Methods in Molecular Biology 1697

Springer Protocols

Alice Pébay
Kursad Turksen
Editors

Sphingosine-1-Phosphate

Methods and Protocols

Second Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:
http://www.springer.com/series/7651

Sphingosine-1-Phosphate

Methods and Protocols

Second Edition

Edited by

Alice Pébay

Centre for Eye Research Australia, The University of Melbourne, East Melbourne, VIC, Australia

Kursad Turksen

Ottawa Hospital Research Institute, Ottawa, ON, Canada

Humana Press

Editors
Alice Pébay
Centre for Eye Research Australia
The University of Melbourne
East Melbourne, VIC, Australia

Kursad Turksen
Ottawa Hospital Research Institute
Ottawa, ON, Canada

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-7412-2 ISBN 978-1-4939-7413-9 (eBook)
https://doi.org/10.1007/978-1-4939-7413-9

Library of Congress Control Number: 2017959561

Printed on acid-free paper

This Humana Press imprint is published by Springer Nature
The registered company is Springer Science+Business Media, LLC
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

Since the first edition of this volume, the many new and important advances in this fast-moving field prompted us to solicit updates for protocols that we thought would be valuable for both experts and novices alike.

We are thus grateful to all of the contributors who took time to present their protocols in the useful format for which the *Methods in Molecular Biology* series is known. We thank them for this and hope that this volume will be as valuable as the first edition was found to be.

We acknowledge the support and guidance of Dr. John Walker, Editor-in-Chief of the *Methods in Molecular Biology* series, for giving us the opportunity to put this volume together. Patrick Marton, Executive Editor of the *Springer Protocols* series, provided continuous support and encouragement from the start to completion of this project - Thank you.

David Casey provided tremendous help and hands-on support by helping us to eliminate any missing parts and details in chapters. A special thank you for this outstanding effort goes to him.

Melbourne, VIC, Australia — ***Alice Pébay***
Ottawa, ON, Canada — ***Kursad Turksen***

Contents

Contributors

Mohammed Alqinyah • *Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA, USA*

Sergio E. Alvarez • *Instituto Multidisciplinario de Investigaciones Biológicas San Luis (IMIBIO-SL) CONICET and Universidad Nacional de San Luis, San Luis, Argentina*

Annie Bang • *Flow Cytometry Facilities, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada*

Kayla J. Bayless • *Department of Molecular and Cellular Medicine, Texas A&M University Health Science Center, College Station, TX, USA*

Erhard Bieberich • *Department of Neuroscience and Regenerative Medicine, Medical College of Georgia, Augusta University, Augusta, GA, USA*

Phillip Callihan • *Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA, USA*

Ludmila E. Campos • *Instituto Multidisciplinario de Investigaciones Biológicas San Luis (IMIBIO-SL) CONICET and Universidad Nacional de San Luis, San Luis, Argentina*

Melina G. Castro • *Instituto Multidisciplinario de Investigaciones Biológicas San Luis (IMIBIO-SL) CONICET and Universidad Nacional de San Luis, San Luis, Argentina*

Alena P. Chumanevich • *Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC, USA*

Lorena T. Davies • *Molecular Signalling Laboratory, Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, SA, Australia*

Camille L. Duran • *Department of Molecular and Cellular Medicine, Texas A&M University Health Science Center, College Station, TX, USA*

Jonathan K. Fleming • *Lpath Incorporated, San Diego, CA, USA*

Suchismita Halder • *Gill Heart and Vascular Institute, University of Kentucky College of Medicine, Lexington Veterans Affairs Medical Center, Lexington, KY, USA*

Shelley B. Hooks • *Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA, USA*

Dimitrios Karamichos • *Department of Ophthalmology/Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*

Roland Kaunas • *Department of Biomedical Engineering, Texas A&M University, College Station, TX, USA*

Satouchi Kiyoshi • *Department of Applied Biological Science, Fukuyama University, Fukuyama, Japan*

Naoki Kobayashi • *Department of Biochemistry, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka, Japan*

Maria P. Kraemer • *Gill Heart and Vascular Institute, University of Kentucky College of Medicine, Lexington Veterans Affairs Medical Center, Lexington, KY, USA*

Stephen J. Lye • *Research Centre for Women's and Infants' Health, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada; Department of Obstetrics and Gynaecology, University of Toronto, Toronto, ON, Canada; Department of Physiology, University of Toronto, Toronto, ON, Canada*

Jun-ichi Morishige • *Department of Cellular and Molecular Function Analysis, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan*

Andrew J. Morris • *Gill Heart and Vascular Institute, University of Kentucky College of Medicine, Lexington Veterans Affairs Medical Center, Lexington, KY, USA*
Sarah E. Nicholas • *Department of Ophthalmology/Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
Tsuyoshi Nishi • *Department of Biomolecular Science and Regulation, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka, Japan; Faculty of Pharmaceutical Science, Osaka University, Suita, Osaka, Japan*
Carole A. Oskeritzian • *Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC, USA*
Alice Pébay • *Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, The University of Melbourne, Melbourne, VIC, Australia; Ophthalmology, Department of Surgery, The University of Melbourne, Melbourne, VIC, Australia*
Martin F. Pera • *Department of Anatomy and Neurosciences, Florey Neuroscience and Mental Health Institute, Walter and Eliza Hall Institute of Medical Research, The University of Melbourne, Melbourne, VIC, Australia*
Melissa R. Pitman • *Molecular Signalling Laboratory, Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, SA, Australia*
Stuart M. Pitson • *Molecular Signalling Laboratory, Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, SA, Australia*
Shrestha Priyadarsini • *Department of Ophthalmology/Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
Gary M. Reynolds • *Centre for Liver Research and NIHR Biomedical Research Unit, University of Birmingham and Queen Elizabeth Hospital Birmingham, Birmingham, UK*
Yamila I. Rodriguez • *Instituto Multidisciplinario de Investigaciones Biológicas San Luis (IMIBIO-SL) CONICET and Universidad Nacional de San Luis, San Luis, Argentina*
Roger Sabbadini • *Stanford University School of Medicine, Stanford, CA, USA*
Susan S. Smyth • *Gill Heart and Vascular Institute, University of Kentucky College of Medicine, Lexington Veterans Affairs Medical Center, Lexington, KY, USA*
Stefka D. Spassieva • *Department of Molecular and Cellular Medicine, Texas A&M Medical Health Sciences Center, Bryan, TX, USA*
Tamotsu Tanaka • *Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan*
Barbara Visentin • *La Jolla Biologics Inc, San Diego, CA, USA*
Guanghu Wang • *Department of Neuroscience and Regenerative Medicine, Medical College of Georgia, Augusta University, Augusta, GA, USA*
Piper A. Wedman • *Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC, USA*
Jonathan M. Wojciak • *Lpath Incorporated, San Diego, CA, USA*
Raymond C.B. Wong • *Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, The University of Melbourne, Melbourne, VIC, Australia; Ophthalmology, Department of Surgery, The University of Melbourne, Melbourne, VIC, Australia*
Ryouhei Yamashita • *Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan*
Jianhong Zhang • *Research Centre for Women's and Infants' Health, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada*

Methods in Molecular Biology (2017) 1697: 1–8
DOI 10.1007/7651_2017_5

Published online: 28 March 2017

Measuring Sphingosine-1-Phosphate/Protein Interactions with the Kinetic Exclusion Assay

Jonathan K. Fleming and Jonathan M. Wojciak

Abstract

By directly detecting the ligand-free binding sites in a sample, the kinetic exclusion assay (KinExA®) provides a compelling alternative to SPR-based techniques for determining equilibrium dissociation constants of protein-ligand interactions. It is especially useful for observing protein-lipid interactions, as binding of native lipids occurs entirely in solution, and monoclonal antibodies can be used to directly compete with a protein of interest for lipid binding. By measuring the antigen-free binding sites on the antibody and using competition affinity analysis, the K_d for the lipid binding the protein and the antibody can be determined simultaneously. Herein, we describe this label-free approach for determining the K_d for S1P-binding serum albumin, which chaperones ~30% of the S1P in human plasma.

Keywords: Anti-lipid antibody, Bovine serum albumin, Competitive affinity analysis, Human serum albumin, Kinetic exclusion assay, Physical biochemistry, Sphingosine-1-phosphate

1 Introduction

The ability to determine accurate, reliable equilibrium dissociation constants for proteins binding lysophospholipids, such as S1P, is challenging due to its lack of inherent traceable characteristics (e.g., fluorescence or UV/Vis absorption). Modifying S1P either by covalently attaching bulky tags or derivatization potentially alters the solubility properties and/or natural mode of protein recognition [1]. In addition, binding studies that rely on physical separation of free and bound S1P species can be difficult because S1P demonstrates poor solubility properties in aqueous media. The aqueous media is, however, necessary to support the structure and function of the protein. To overcome these issues, typically S1P is delivered in complex with fatty acid-free bovine serum albumin (FAF-BSA) [2]. However, the intrinsic affinity of FAF-BSA for S1P (or other lysophospholipids) may confound the binding event being investigated and is seldom considered while interpreting the data.

The KinExA approach described here both overcomes the challenges described above for studying protein-lipid interactions and elucidates the effect of using carrier proteins to deliver

lysophospholipids. The equilibrium binding constant of S1P binding to serum albumin is determined by measuring the unbound fraction of an antibody, LT1009, which competes with serum albumin for S1P binding. This method can also be applied to other S1P-binding proteins and chaperones.

Three equilibrium affinity experiments are used to determine the K_d of serum albumin and the anti-S1P LT1009 antibody (Fig. 1). Two experiments employ a constant serum albumin concentration, which is below the K_d for the S1P interaction, while the third experiment contains a much higher serum albumin concentration, which alters the position and shape of the isotherm relative to the corresponding low serum albumin concentration curve. Fitting all three curves using a direct competition model generates K_d values (K_{d1}, S1P-LT1009; K_{d2}, S1P-serum albumin), the percent of nominal lipid, and residual error between the data points and the theoretical curves (Fig. 1). Although all three experimentally derived curves are necessary to determine these parameters, each curve weighs disproportionately toward the resolution of a particular parameter: the low antibody, low serum albumin experiment yields K_{d1} information; the high antibody, low serum albumin experiment provides titrant concentration information; and the

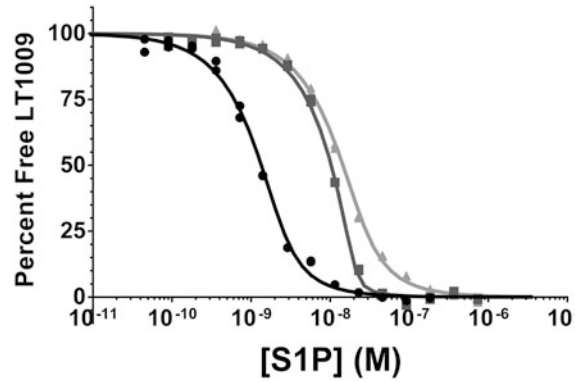

Fig. 1 Competition affinity experiments with FAF-BSA. Global curve fitting of the three affinity experiments used to determine the equilibrium dissociation constants for the S1P binding the antibody and FAF-BSA in solution. The percentage of antigen-free binding sites on the antibody (in duplicate) is plotted as a function of the S1P concentration in each sample. The LT1009 and FAF-BSA concentrations are as follows: *black circles*, 1 nM LT1009 and 1 μM FAF-BSA; *gray squares*, 10 nM LT1009 and 1 μM FAF-BSA; *light gray triangles*, 10 nM LT1009 and 500 μM FAF-BSA [1]. Note: The curves shown here are specific for the reagents and conditions described herein. The use of alternative conditions or chaperones may change the curve shape and spacing due to differences in binding properties

high antibody, high serum albumin experiment affords K_{d2} information.

During this analysis, the concentration of S1P-binding sites offered by serum albumin was set equal to its molar concentration (i.e., 1:1 S1P/albumin), due to the lack of published information on S1P albumin stoichiometry. The antibody concentration is used as a reference, which allows for a parameter that represents the amount of S1P present to float and be determined as a percent of the nominal lipid concentration. This parameter is particularly useful and informative, since a loss of S1P is presumed while pipetting this hydrophobic lipid in aqueous buffer. Together these data provide insight into the interaction between S1P, the commonly used carrier protein serum albumin, and the LT1009 anti-S1P antibody.

2 Materials

Ultrapure water (18 MΩ cm at 25 °C) and analytical grade reagents should be used. The experiments described herein use sodium azide and waste must be disposed of properly.

2.1 Equipment and Software

Pipettes, vortex mixer, scale, water bath sonicator, rocking platform, tabletop centrifuge, spectrophotometer, glass syringes with stainless steel needles, KinExA 3200 with autosampler, and competition n-curve software (Sapidyne Instruments Inc.).

2.2 Reagents and Buffers

Maleimide-activated lyophilized bovine serum albumin, omega-1 thiolated S1P, dimethyl sulfoxide, phosphate-buffered saline, fraction V fatty acid-free BSA, S1P, methanol, dry argon, running buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.005% polysorbate 20, 0.02% NaN_3, pH 7.4), LT1009 antibody, 5% bleach, and fatty acid-free human serum albumin.

2.3 Consumables

Pipette tips, 2 mL amber glass vials, spin desalting columns, PMMA beads (Sapidyne Instruments Inc.), 2 mL microcentrifuge tubes, 12 × 75 mm silanized glass tubes, 50 mL polypropylene conical tubes, 0.2 μm polyethersulfone filters, and KinExA bead vials (Sapidyne Instruments Inc.).

3 Methods

All procedures are to be carried out at room temperature unless noted.

3.1 S1P-BSA Coating Material Preparation

1. Prepare 150 μM maleimide-activated lyophilized bovine serum albumin (BSA) by resuspending in water; vortex gently until dissolved.

2. Prepare 25 mM omega-1 thiolated S1P [3] in DMSO by adding a measured mass of the lipid to an amber glass vial, adding DMSO, and vortexing for 1 min, sonicating for 10 min, and vortexing for an additional 1 min.
3. Add the 150 μM bovine serum albumin made in **step 1** to an amber glass vial followed by a 40-fold molar excess of thiolated S1P in DMSO. Rock slowly for 2 h. Volume made depends on experimental requirements.
4. Buffer exchange spin desalting columns into PBS in a tabletop centrifuge (follow manufacturer instructions).
5. To remove excess unconjugated thiolated S1P, pass the S1P-BSA coating material through the PBS exchanged columns by centrifuging at 1500 RPM for 2 min.
6. Determine BSA concentration of the flow through by measuring A280 spectrophotometrically and using a molar extinction coefficient of 0.66 mL/mg.

3.2 S1P-BSA Bead Preparation

1. Prepare aliquots containing 200 mg of PMMA beads in 2 mL microcentrifuge tubes.
2. In a silanized glass tube, dilute S1P-BSA to 30 μg/mL with PBS. Vortex briefly to mix. 1 mL of solution is needed for every 200 mg of beads.
3. Prepare a 1.5 mM fraction V fatty acid-free BSA (FAF-BSA) stock from dry FAF-BSA in PBS by mixing gently in a polypropylene conical and pass through a 0.2 μm PES filter. Verify concentration by measuring A280 spectrophotometrically and using a molar extinction coefficient of 0.66 mL/mg.
4. In another silanized glass tube, dilute FAF-BSA to 150 μM with PBS. Vortex briefly to mix. 1 mL of solution is needed for every 200 mg of beads.
5. To coat beads, add 1 mL of S1P-BSA in PBS from **step 2** to 200 mg of beads in microcentrifuge tube and then rock for 1 h at 37 °C.
6. Remove the beads from the rocking platform and allow them to settle.
7. To block the beads, aspirate the liquid leaving the beads undisturbed and replace with 1 mL of 150 μM FAF-BSA in PBS.
8. Transfer the resulting slurry containing blocked beads via a 1 mL pipette tip that has been cut at a 45° angle to a glass KinExA bead vial containing 13.5 mL PBS per 200 mg of S1P-BSA coated and blocked beads.

3.3 Label Preparation

1. Prepare goat anti-human heavy and light chain IgG secondary by diluting it in running buffer (*see* **Note 1**).

3.4 S1P Preparation

1. Resuspend powdered S1P of a known mass in methanol to 0.5 mM by vortexing for 1 min, sonicating for 10 min, and vortexing for another minute. S1P concentration can be verified using a colorimetric phosphorus assay [1]. Quantity used is dependent on experimental needs.
2. Dry down enough S1P in methanol in an amber glass vial under a stream of dry argon in order to carry out experiments (*see* **Note 2**). 150 nmol was used for the experiments described herein.
3. Resuspend dry S1P to 0.1 mM in running buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$, 0.005% polysorbate 20, 0.02% NaN_3, pH 7.4) supplemented with 100 μM FAF-BSA by vortexing for 1 min, sonicating for 10 min, and vortexing again for 1 min (*see* **Note 3**).
4. Dilute S1P twofold using glass vials with a glass syringe into running buffer supplemented with 100 μM FAF-BSA (*see* **Note 4**). These aliquots serve as the lipid stocks for subsequent experiments.

3.5 Determination of LT1009 Kinetic Exclusion

1. Prepare 15 mL of 10 nM LT1009 IgG in running buffer in a polypropylene conical.
2. Add 2 mL of running buffer to a silanized glass tube and add FAF-BSA to 100 μM. The volume added should be consistent in the subsequent two steps in order to prevent signal differences due to antibody concentration differences introduced via varying volumes. This sample will provide nonspecific binding (NSB) data.
3. Transfer 2 mL of 10 nM LT1009 IgG to a silanized glass tube and add FAF-BSA to 100 μM. This sample will provide the 100% signal data.
4. Transfer 2 mL of 10 nM LT1009 IgG to a silanized glass tube and add S1P in running buffer supplemented with FAF-BSA to 100 μM. Final S1P concentration: 14 nM. This sample will provide the ~50% signal data.
5. Gently vortex mix all samples and incubate for 6 h.
6. Measure each of the samples using the KinExA starting with the NSB sample, the 100% signal, and the ~50% signal sample at flow rates starting at 0.25 mL/min to 2.25 mL/min incrementally by 0.25 mL/min.
7. Determine the percentage of free antibody by dividing the signal (voltage) of the antibody in the presence of S1P (~50% signal) by the signal of the antibody in the absence of S1P (100% signal) after subtracting NSB from both values (*see* **Note 5**).

8. Clean KinExA after this and all other experiments by passing 5% bleach in water through the autosampler needle as if it were sample until signal reaches baseline. Rinse thoroughly with running buffer.

3.6 Equilibrium Affinity Experiments with BSA, LT1009, and S1P

1. Prepare stock solutions of either 1 or 10 nM LT1009 and 1 or 500 μM FAF-BSA (dependent on experiment) in running buffer and distribute to 15 silanized glass tubes (*see* **Note 6**).
2. Deliver S1P from the twofold S1P stocks made previously in running buffer with 100 μM FAF-BSA to all but one of the 15 tubes containing LT1009 and FAF-BSA (*see* **Note 7**).
3. Vortex samples gently and equilibrate for at least 6 h.
4. Measure samples using the KinExA from low S1P concentration to high at a flow rate of 0.25 mL/min. Repeat measurements to obtain duplicate measurements for each sample.
5. Analyze data in competition n-curve software with drift correction.

3.7 Equilibrium Affinity Experiments with HSA, LT1009, and S1P

1. Prepare a 1.5 mM stock of fatty acid-free HSA (FAF-HSA) as in Section 3.2, **step 3**.
2. Prepare a stock solution of 10 nM LT1009 and 500 μM FAF-HSA and distribute to 15 silanized glass tubes.
3. Deliver S1P from the twofold S1P stocks made previously in running buffer with 100 μM FAF-BSA to all but one of the 15 tubes containing LT1009 and FAF-HSA (*see* **Note 7**).
4. Vortex samples gently and equilibrate for at least 6 h.
5. Measure samples using the KinExA from low S1P concentration to high at a flow rate of 0.25 mL/min. Repeat measurements to obtain duplicate measurements for each sample.
6. Analyze data in competition n-curve software with drift correction (*see* **Note 8**).

4 Notes

1. The concentration of label required to give an acceptable signal (voltage) to noise ratio varies and needs to be determined experimentally per manufacturer recommendations. Factors affecting signal include bead load volume, efficiency of bead coating, label fluorophore, lamp brightness, sample load volume, etc. The experiments described herein used a concentration of 250–500 ng/mL.
2. Dry S1P can be stored under dry nitrogen or argon at -20 °C. Periodic testing should be carried out to verify integrity.
3. Calcium serves as a bridge between the LT1009 and S1P and is thus required for strong affinity binding.

4. In order to minimize lipid carryover during serial dilution of lipid stocks, the following precautions are taken. When using the glass syringe to move lipid from one vial to the next vial, minimal contact with the solution should be maintained. This is carried out by inserting the stainless steel needle just beyond the meniscus of the air-liquid interface. After each transfer, the needle syringe should be washed in methanol, running buffer, and then running buffer supplemented with 100 μM FAF-BSA. The needle should then be wiped with a tissue wipe before continuing with the serial dilution.
5. The percentage of free LT1009 at the tested flow rates should not increase significantly as the flow rate increases. A lack of percent-free LT1009 increase confirms that the fraction of LT1009 in complex with S1P does not dissociate during detection and contribute to the free LT1009 measurements. It is important that this is determined before carrying out equilibrium affinity experiments. It is recommended that each measurement be made in duplicate.
6. The experiments required to obtain equilibrium dissociation constants for both FAF-BSA and LT1009 are as follows: 1 nM LT1009, 1 μM FAF-BSA; 10 nM LT1009, 1 μM FAF-BSA; and 10 nM LT1009, 500 μM FAF-BSA. These concentrations are fixed throughout the sample set.
7. In another effort to minimize lipid carryover, S1P is taken via glass syringe (*see* **Note 4**) from the twofold titrated S1P stock with the lowest concentration first and added to the FAF-BSA or FAF-HSA and LT1009 containing tubes. Subsequently the S1P stock with the next highest concentration of S1P is delivered until all but one of the test tubes contain FAF-BSA or FAF-HSA, LT1009, and S1P. The tube without S1P but with FAF-BSA or FAF-HSA and LT1009 serves as a signal reference point between experiments.
8. Data collected in this step are paired with two of the datasets collected in Section 3.6 and processed together. Datasets: 1 nM LT1009, 1 μM FAF-BSA, and 10 nM LT1009, 1 μM FAF-BSA.

Acknowledgment

The authors thank Tom Glass and Steve Lackie for their extensive advice and technical support.

Conflicts of Interest

KinExA is a registered trademark of Sapidyne Instruments Inc.

LT1009 is a proprietary agent of Lpath Inc. but may be provided upon request.

References

1. Fleming JK, Glass TR, Lackie SJ, Wojciak JM (2016) A novel approach for measuring sphingosine-1-phosphate and lysophosphatidic acid binding to carrier proteins using monoclonal antibodies and the kinetic exclusion assay. J Lipid Res 57(9):1737–1747. doi:10.1194/jlr.D068866
2. Rosen H, Gonzalez-Cabrera PJ, Sanna MG, Brown S (2009) Sphingosine 1-phosphate receptor signaling. Annu Rev Biochem 78:743–768. doi:10.1146/annurev.biochem.78.072407.103733
3. O'Brien N, Jones ST, Williams DG, Cunningham HB, Moreno K, Visentin B, Gentile A, Vekich J, Shestowsky W, Hiraiwa M, Matteo R, Cavalli A, Grotjahn D, Grant M, Hansen G, Campbell MA, Sabbadini R (2009) Production and characterization of monoclonal anti-sphingosine-1-phosphate antibodies. J Lipid Res 50(11):2245–2257. doi:10.1194/jlr.M900048-JLR200

Methods in Molecular Biology (2017) 1697: 9–20
DOI 10.1007/7651_2017_41

Published online: 25 May 2017

An Improved Isoform-Selective Assay for Sphingosine Kinase 1 Activity

Melissa R. Pitman, Lorena T. Davies, and Stuart M. Pitson

Abstract

Sphingosine kinases (SK) are the sole enzymes responsible for the production of sphingosine 1-phosphate (S1P). S1P is a signaling molecule with a plethora of targets, acting as both a second messenger intracellularly and extracellularly via a family of cell surface G-protein-coupled S1P receptors. The two sphingosine kinases, SK1 and SK2, arise from different genes and have some distinct and overlapping cellular functions that are regulated in part by differential cellular localization, developmental expression, and catalytic properties. Here, we describe an improved method for selectively detecting SK1 activity in vitro and cell lysates via the use of the zwitterionic detergent CHAPS, which effectively inhibits SK2 activity and thus allows selective analysis of SK1 activity in a range of cell samples. The assay measures the production of ^{32}P-labeled S1P following the addition of exogenous sphingosine and [γ^{32}P]ATP. The S1P product can be purified by Bligh–Dyer solvent extraction, separated by thin layer chromatography (TLC), and the radiolabeled S1P quantified by exposing the TLC plate to a storage phosphor screen. This sensitive, reproducible assay can be used to selectively detect SK1 activity in tissue, cell, and recombinant protein samples.

Keywords: Sphingosine kinase, D-*erythro*-sphingosine, Sphingosine 1-phosphate, Thin layer chromatography, Bligh–Dyer extraction

1 Introduction

Sphingosine 1-phosphate (S1P) is a bioactive signaling molecule that can modulate a range of cellular responses. S1P can be exported from the cell to act as a ligand for a family of S1P-specific G-protein-coupled receptors or bind to and act on a range of intracellular targets [1, 2]. The production of S1P is catalyzed by two sphingosine kinase (SK) enzymes, SK1 and SK2, that phosphorylate sphingosine at the primary hydroxyl (Fig. 1). SK1 and SK2 are distinct proteins that arise from different genes. Knockout of both SK isoforms is embryonic lethal in mice; however, loss of only one isoform produces no obvious defects, suggesting some level of functional redundancy between these two enzymes [3–5]. Both SK1 and SK2 promote cell survival and proliferation [6, 7], although, in some cases SK2 can exert different physiological functions. As opposed to SK1, SK2 can have proapoptotic effects [8–10], inducing epigenetic and transcriptional regulation by acting on histone deacetylases

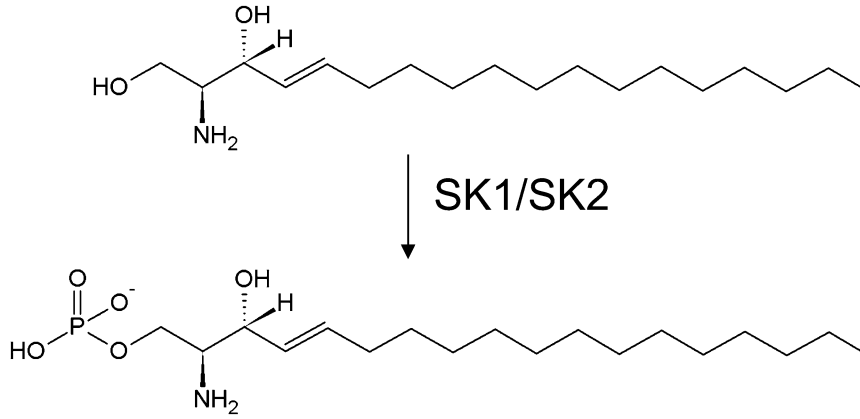

Fig. 1 Sphingosine kinases catalyze the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P)

[11]. In a number of conditions, SK1 and SK2 appear to play distinct roles, including in inflammatory arthritis [12], ischemia-reperfusion injury [13], lipopolysaccharide-induced lung injury [14], in human mast cell functions [15], and S1P distribution in blood and lymphoid tissue [16].

The two SK enzymes share significant sequence similarity; however, they differ in size, post-translational modifications, cellular localization, and expression profile [17]. In particular, SK1 and SK2 differ in catalytic properties, a factor that can be exploited to selectively measure activity of individual isoforms [18, 19]. The most commonly employed and cost-effective SK assay measures the production of ^{32}P-labeled S1P from sphingosine and [γ^{32}P]ATP [20, 21]. Conditions in the assay can be tailored to selectively measure the activity of SK1, SK2, or both. Previously, we and others have reported conditions for the selective assay of SK1 activity using the addition of 0.25 % Triton X-100 to assays, with this detergent largely inhibiting SK2 activity but sustaining SK1 activity when assessed with purified recombinant SK proteins [18, 19]. Unexpectedly, however, we recently found that these previously described SK1-selective assay conditions were not very selective for SK1 when employed with cell lysates. Indeed, using these conditions, cell lysates from embryonic fibroblasts (MEFs) from SK1 knockout mice (which only have the SK2 isoform) showed significant residual SK2 activity (Fig. 2a). Higher concentrations of Triton X-100 also failed to improve SK2 inhibition in lysates (Fig. 2b). Therefore, we tested the ability of other detergents to inhibit purified recombinant SK2 and found that at a concentration of 1 % the zwitterionic detergent CHAPS effectively eliminated SK2 activity (Fig. 3a), albeit with a substantial (3.5-fold) increase in the activity of purified recombinant SK1. At 3 % CHAPS, the activity of SK2 was negligible and the increase in SK1 activity was less than twofold (Fig. 3b). Importantly, when SK2 activity was determined in lysates from SK1 knockout MEFs (which only have the SK2 isoform), the addition of 3 % CHAPS completely inhibited SK2

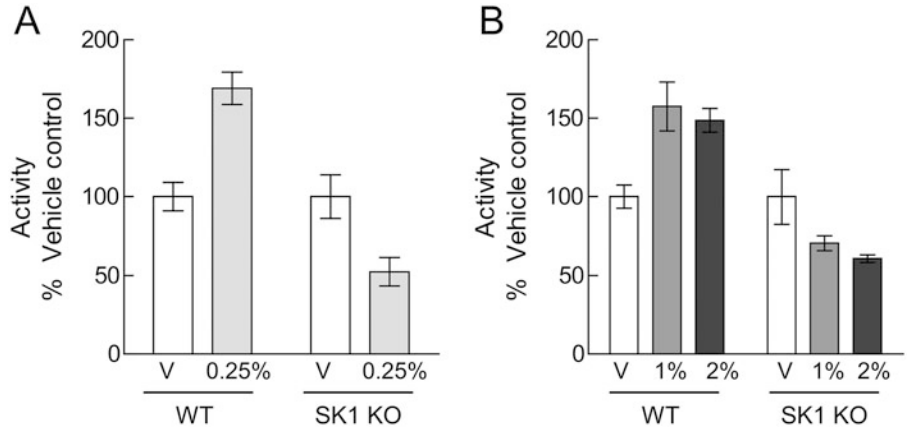

Fig. 2 Triton X-100 does not adequately inhibit SK2 activity in lysates. (**a**) Lysates from wild-type (WT) mouse embryonic fibroblasts (MEFs) (*open bars*) and SK1 knockout (KO) MEFs (*grey bars*) were assayed using non-selective SK assay conditions with either H_2O vehicle (V), as well as 0.25 % Triton X-100 to test SK2 inhibition. (**b**) Triton X-100 was tested at both 1 % (*grey*) and 2 % (*dark grey*) in WT and SK1 KO MEF lysates. Activities are standardized to 100 % of vehicle control for the WT and SK1 KO MEFs and correspond to 618 and 158 pmol/mg/min specific activities, respectively. Data represent the mean ± S.D. of four independent experiments

activity, and modestly elevated SK1 activity (1.3-fold) in lysates from MEFs of wild-type mice (Fig. 4a). This 1.3-fold increase in SK1 activity was consistent with SK1 activity measured in lysates from MEFs of SK2 knockout mice treated with 3 % CHAPS versus vehicle (data not shown). Hence, inclusion of CHAPS in the assay significantly improves the stringency of the SK1 selectivity. Assays employing BSA-solubilized sphingosine without addition of CHAPS can be used in parallel to measure the total SK activity (from both SK1 and SK2) in cell lysates.

At the completion of the assay incubation, the S1P product is isolated using a Bligh–Dyer solvent extraction, where under acidic conditions around 75 % of the S1P partitions to the organic phase [20]. The chloroform phase is then separated by thin layer chromatography (TLC) where the radiolabeled S1P product migrates with an R_f of 0.7 in butanol/ethanol/H_2O/glacial acetic acid (8:2:1:2) (Fig. 4b). The S1P spot can then be quantified by exposing the TLC plate to a storage phosphor screen.

2 Materials

Prepare all solutions with analytical grade reagents using distilled water, and store at room temperature unless otherwise indicated.

2.1 Sample Preparation

1. Lysis buffer: 50 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl, 10 % glycerol (w/v), 1 mM dithiothreitol, 2 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM EDTA (*see* **Note 1**)

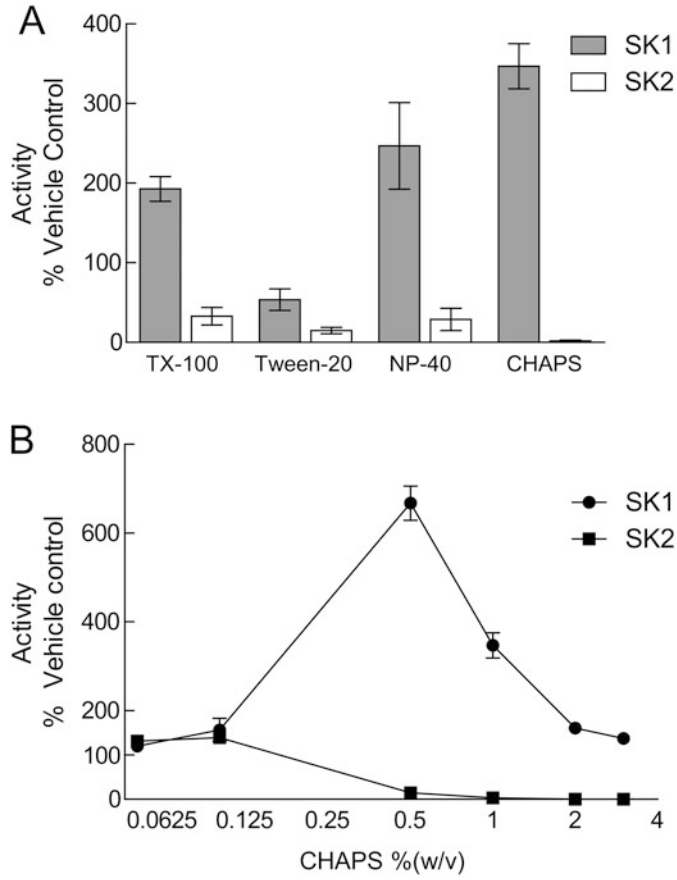

Fig. 3 Effect of other detergents on the activity of SK1 and SK2. (**a**) Purified recombinant human SK1 (*grey bars*) and SK2 (*open bars*) were assayed under nonselective SK assay conditions with the addition of H_2O vehicle or 1 % (w/v, final) detergent (**b**) varying concentrations of CHAPS were tested against recombinant purified SK1 (*circles*) and SK2 (*squares*). Activity of SK1 and SK2 with vehicle was used as the controls, and set as 100 %. Data represent the mean ± S.D. of three independent experiments

2. Protease inhibitor cocktail (Complete™; Roche) prepared as a 50× concentrated stock and stored at −20 °C
3. Bath sonicator with sufficient power to disrupt cells (e.g., Bioruptor™; Diagenode, NY) or 26G needle and syringe
4. Microcentrifuge

2.2 Substrate Preparation

1. Fatty acid-free BSA
2. Sonicator

2.3 Incubation

1. Eppendorf® Safe-Lock® 1.5 mL microcentrifuge tubes (*see* **Note 2**)
2. SK1-selective assay buffer: 100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, and 4.4 % (w/v) CHAPS

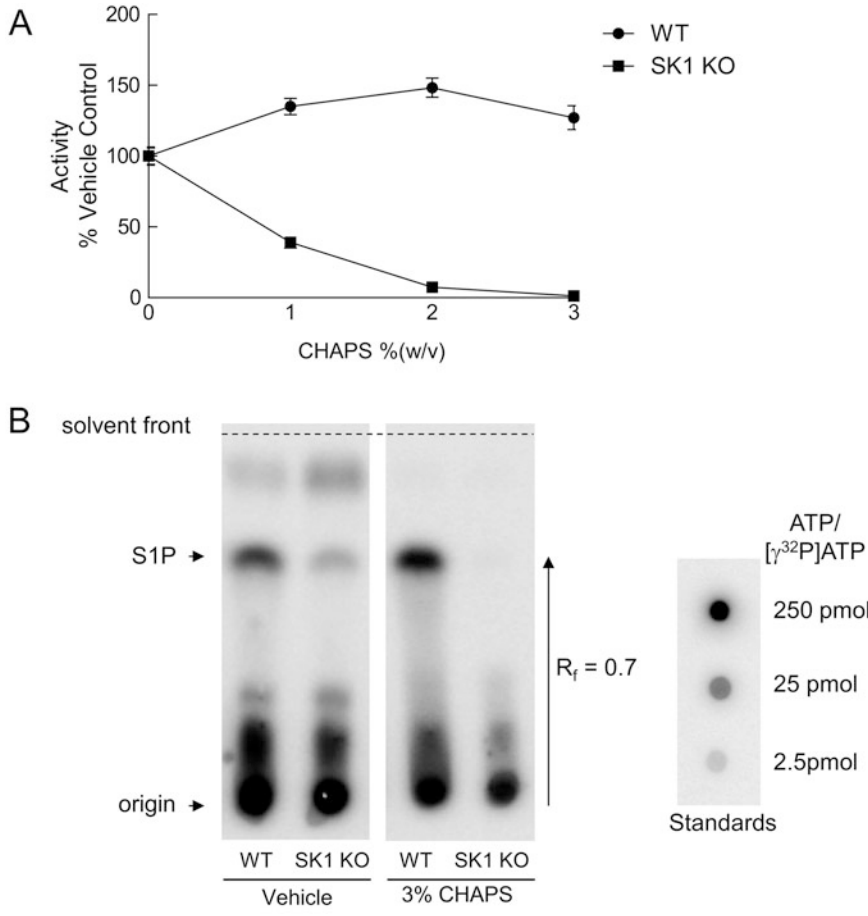

Fig. 4 3 % CHAPS inhibits SK2 activity in cell lysates. (**a**) Lysates from wild-type (WT) MEFs (*circles*) and SK1 knockout (KO) MEFs (*squares*) were assayed using nonselective SK assay conditions with either H_2O vehicle (V), as well as 1–3 % CHAPS to test SK2 inhibition. Activities are standardized to 100 % of vehicle control for the WT and SK1 KO MEFs and correspond to 618 and 158 pmol/mg/min specific activities, respectively. Data represent the mean ± S.D. of four independent experiments. (**b**) Image of TLC plates with S1P spots from nonselective (vehicle) and SK1 selective (3 % CHAPS) assays using lysates from WT or SK1 KO MEFs (using 25 μg of protein, 2 μCi [γ^{32}P]ATP, and 0.5 mM adenosine-5′-triphosphate (ATP)). The chloroform phase was spotted onto the plate at the origin and using the described mobile phase S1P migrates with an (R_f) of 0.7. The S1P can be quantified and converted to pmol S1P generated by comparison to standards made from dilution of the assay reaction mixture 1 in 10, 1 in 100, and 1 in 1,000 to allow application of 250, 25, and 2.5 pmol of [γ^{32}P]ATP, respectively (for standard assay conditions with 1 μCi [γ^{32}P]ATP outlined in Table 1, with 2 μCi [γ^{32}P]ATP values are 500, 50, and 5 pmol)

3. Total SK assay buffer: 100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, and 10 mM NaF
4. D-*erythro*-sphingosine (Biomol)
5. 20 mM Adenosine-5′-triphosphate (ATP) prepared in 1 M Tris/HCl buffer (pH 7.4) containing 200 mM $MgCl_2$ and stored at −20 °C (*see* **Note 3**)

6. [γ^{32}P]ATP (Perkin Elmer, NEG502A)
7. 50 mM 4-deoxypyridoxine (Sigma, DO501) stock solution stored at −20 °C (*see* **Note 4**)
8. Perspex screens for use with radionuclides
9. Geiger counter
10. Water bath or incubator set at 37 °C
11. Whatman paper (3 M)

2.4 Extraction

1. Chloroform/methanol/conc. HCl (100:200:1)
2. Chloroform
3. 5 M KCl
4. Microcentrifuge
5. Aspiration apparatus
6. Silica gel TLC plates (Sigma, Z193291, pore size 60 Å, aluminum backing)

2.5 Resolution and Quantitation of Sphingosine 1-Phosphate

1. Source of compressed air or hair dryer (on cool setting)
2. 1-Butanol/ethanol/glacial acetic acid/H_2O (8:2:1:2)
3. Glass TLC developing tank
4. Plastic ziplock bags or cling wrap
5. Storage phosphor screen (GE Healthcare)
6. Phosphorimaging system (e.g., Typhoon; GE Healthcare)
7. ImageQuant™ (GE Healthcare) software or equivalent

3 Methods

Ensure all procedures using [γ^{32}P]ATP are carried out with protective perspex shielding using standard radiation safety techniques. Radiation should be monitored with a Geiger counter. Dispose of all waste strictly in accordance with local radioactive waste disposal regulations.

3.1 Sample Preparation

1. Resuspend cell pellets in lysis buffer.
2. Lyse by sonication in a bath sonicator (e.g., 4 × 25 s pulses with 25 s breaks in a 200 W Bioruptor™ apparatus) or by five passages through a 26G needle.
3. Samples can be assayed as whole cell lysates, or clarified lysate can be prepared by centrifuging the whole cell lysate at 13,000 × *g* for 15 min at 4 °C.
4. Assess protein concentration in the samples by standard protein assay methods.

3.2 Preparing the Sphingosine Substrate

1. Weigh out the desired quantity of sphingosine to make up 2 mM sphingosine in the required volume.
2. Add 2 % fatty acid-free BSA in 50 mM Tris/HCl (pH 7.4).
3. Sonicate on ice until the solution becomes clear.
4. Aliquot and store at −20 °C (*see* **Note 5**).

3.3 Incubation

1. Add 20 μl of enzyme sample to an Eppendorf® Safe-Lock® microcentrifuge tube (*see* **Note 6**).
2. Add 80 μl of reaction mixture, the composition of which differs depending on the SK isoform to be analyzed (*see* Table 1 and **Notes 7** and **8**).
3. Incubate at 37 °C for 30 min.
4. For later conversion of radioactive signal to phosphate concentration, perform a tenfold serial dilution of the leftover reaction mixture into water and spot 2 μl of the 1 in 10, 1 in 100, and 1 in 1,000 dilutions onto pre-marked Whatman paper (*see* **Note 9**).

3.4 Extraction

1. To the 100 μl assay mixture, add 270 μl of chloroform/methanol/conc. HCl (100:200:1).
2. Add 20 μl of 5 M KCl (*see* **Note 10**).
3. Add 70 μl chloroform to create a phase separation.
4. Vortex to mix well.

Table 1
Example of assay setup

	Volume per assay (μl)	
Reagent	**SK1**	**SK1 and SK2**
20 mM Mg-ATP	5	5
10 μCi/μl [γ^{32}P]ATP	0.1	0.1
2 mM sphingosine in 2 % fatty acid-free BSA	5	5
50 mM 4-deoxypyridoxine	1	1
Assay buffer with 4.4 % (w/v) CHAPS	69	0
Assay buffer	0	69
Cell lysate or recombinant SK protein	20	20
Total volume	**100**	**100**

For selective detection of SK1 activity, use 3 % CHAPS to inhibit SK2. To nonselectively assay total SK activity, use BSA-solubilized sphingosine and omit CHAPs from the assay. It is recommended to prepare a batch of reaction mixture of sufficient volume to assay all of the required samples. To do this, we recommend multiplying each of the volumes shown by $n + 1$, where n is the number of samples to be assayed

5. Centrifuge for 5 min at 13,000 × *g* to fully separate the phases (*see* **Note 11**).
6. Remove the upper aqueous/methanol phase by aspiration.

3.5 Thin Layer Chromatography

1. Cut a 20 cm × 20 cm Silica TLC plate into half (*see* **Note 12**).
2. Measure 2 cm from the bottom of the TLC plate and draw a line with pencil along the long edge.
3. Mark on this line with pencil where the samples will be applied (the origin), ensuring that the spots are no closer than 1.5 cm from the edge of the plate and no closer than 1.3 cm to each other (*see* **Note 13**).
4. Apply 50 μl of the remaining lower chloroform phase onto the TLC plate by slowly and repeatedly spotting a few microliters of liquid onto the plate with the pipette tip so that no more than a 5 mm diameter circle of the TLC plate is wet (*see* **Note 14**). Between each spot, allow the liquid to absorb into the plate and then carefully dry the spot with a stream of air.

3.6 Resolution and Quantitation of Sphingosine 1-Phosphate (See Note 15)

1. Develop the TLC plate with 1-butanol/ethanol/glacial acetic acid/H_2O (8:2:1:2) in a glass TLC developing tank until the mobile phase is within 1 cm of the top of the TLC plate (*see* **Note 16**).
2. Remove the TLC plate from the developing tank with tweezers and allow to air dry in a fume hood for 15 min.
3. Cover the dried TLC plate in cling wrap or a place in a ziplock plastic bag.
4. Expose to a storage phosphor screen overnight (*see* **Note 17**). Include the Whatman paper with the assay mixture dilutions to allow quantification of the phosphor signal.
5. Read the storage phosphor screen on a phosphorimager.
6. Quantify the S1P spot which with the mobile phase employed will have a relative migration (R_f) of approximately 0.7 (see Fig. 4).
7. Using the included [γ^{32}P]ATP standard curve, the sample protein concentrations, and a multiplication coefficient of 4.27 to account for incomplete S1P extraction and spotting of only part of the chloroform phase onto the TLC plate (*see* **Note 18**), the S1P spot intensity can be converted to amount of phosphate transferred/min/mg protein.

4 Notes

1. Since both SK1 and SK2 can be activated by phosphorylation [22, 23], inclusion of phosphatase inhibitors NaF, Na_3VO_4, and β-glycerophosphate in the lysis buffer is important.

Activation of Na_3VO_4 is required prior to its use to enable phosphatase inhibition [24].

2. The Eppendorf® Safe-Lock® microcentrifuge tubes have a better sealing cap that contains the radioactive aerosols during centrifugation steps more effectively than regular microcentrifuge tubes. If other microcentrifuge tubes are employed, the tops of the closed tubes should be covered with Parafilm™ to minimize potential microcentrifuge contamination by radionuclides.
3. ATP must be in complex with Mg to be a substrate for phosphotransferases. The ATP solution must be prepared in a buffer to prevent the solution becoming too acidic which will result in ATP hydrolysis.
4. 4-Deoxypyridoxine is an inhibitor of S1P lyase and is only required for use when assaying SK activity in cell lysates to prevent dephosphorylation of the S1P product.
5. This solution should be aliquoted but can tolerate several freeze-thaw cycles.
6. **Cell lysates**: 20–50 μg of cell lysates are generally used for the assay. SK1 selectivity with >50 μg lysate would need to be tested empirically. **Recombinant Protein**: For purified recombinant SK proteins it is recommended to use between 0.1 and 10 ng of recombinant protein per assay. Too much enzyme will cause the substrates to become limiting, causing a nonlinear reaction rate. It is advisable to initially do a time course, stopping the assay at 10, 20, and 30 min timepoints to check that the substrates are not limiting.
7. The CHAPS concentration in the reaction mixture is 1.47× to account for the 1.47-fold dilution by the 20 μl sample and substrates/additives. It is recommended to prepare a batch of reaction mixture of sufficient volume to assay all of the required samples (see Table 1). The following gives the assay components and their final concentrations in each of the assays:

   SK1-selective assay: 1 mM Mg-ATP, 1 μCi [γ^{32}P]ATP, 100 μM sphingosine in 0.1 % fatty acid-free BSA (prepared from 2 mM sphingosine stock in 2 % fatty acid-free BSA), 0.5 mM 4-deoxypyridoxine, and assay buffer with 3 % (w/v, final) CHAPS

   Nonselective SK assay: 1 mM Mg-ATP, 1 μCi [γ^{32}P]ATP, 100 μM sphingosine in 0.1 % fatty acid-free BSA (prepared from 2 mM sphingosine stock in 2 % fatty acid-free BSA), 0.5 mM 4-deoxypyridoxine, and CHAPS-free assay buffer
8. If enhanced assay sensitivity is required the amount of [γ^{32}P] ATP added to the assay can be increased twofold, and the concentration of unlabeled ATP can be decreased to 0.5 mM. Lower ATP concentrations than this should not be routinely

used to ensure that ATP does not limit the reaction velocity since the K_M value for ATP of SK1 is approximately 80 μM [25].

9. Using standard assay conditions (Table 1), 2 μl of reaction mixture diluted 10, 100, and 1,000-fold will contain 250, 25, and 2.5 pmol of [γ^{32}P]ATP, respectively (Fig. 4).
10. Addition of KCl enhances S1P extraction into the organic (chloroform) phase [26].
11. In cell lysates, this step often results in the appearance of a "skin" between the two phases that consists mostly of precipitated proteins.
12. The solvent system described here effectively separates S1P from other radiolabeled contaminants in the chloroform phase within 9 cm migration of the mobile phase from the origin. Thus, TLC plates of 10 cm height are sufficient.
13. It is recommended that a maximum of 14 samples be spotted per 20 cm TLC plate for optimum results. Plates can be cut down further for smaller assays.
14. Rinse pipette tips with chloroform prior to transfer of the chloroform phase to minimize dripping of the sample from the pipette tip.
15. Solvent preparation and development of the TLC plates should be carried out in a fume hood.
16. This mobile phase will take approximately 1.5 h to migrate up the TLC plate. We find this mobile phase to be the most effective at resolving the S1P spot, but a number of other mobile phases have also been employed in this assay. These include: 1-butanol/glacial acetic acid/H_2O (3:1:1) [26], methanol/chloroform/glacial acetic acid/H_2O (10:4:3:2:1) [27], and chloroform/methanol/ammonium hydroxide (4:1:0.1, v/v) [20].
17. Storage phosphor screens capture images produced by ionizing radiation, such as ^{32}P. During reading, the phosphorimager stimulates the screen using lasers to convert the latent signal to light. This light is proportional to the amount of radioactivity in the sample. The image can then be quantified using standard quantification software such as ImageQuant™. The screens are reusable and can be cleared by exposure to extra-bright light using a light box. If a phosphorimager is not available, X-ray film can be employed, but this can be ten- to 100-fold less sensitive than the storage phosphor screen/phosphorimager approach.
18. Values calculated from the standard curve must be adjusted by a factor of 4.27 to account for the incomplete extraction of only 75 % of the generated S1P into the chloroform phase [21], and application of only 31.5 % of the chloroform phase onto the TLC plate.

Acknowledgements

This work was supported by the Fay Fuller Foundation, the Royal Adelaide Hospital Research Fund through an Early Career Research Fellowship to M.R.P, and the National Health and Medical Research Council of Australia through a Senior Research Fellowship to S.M.P. (1042589).

References

1. Strub GM, Maceyka M, Hait NC et al (2010) Extracellular and intracellular actions of sphingosine-1-phosphate. Adv Exp Med Biol 688:141–155
2. Pitson SM (2011) Regulation of sphingosine kinase and sphingolipid signaling. Trends Biochem Sci 36:97–107
3. Allende ML, Sasaki T, Kawai H et al (2004) Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. J Biol Chem 279:52487–52492
4. Kharel Y, Lee S, Snyder AH et al (2005) Sphingosine kinase 2 is required for modulation of lymphocyte traffic by FTY720. J Biol Chem 280:36865–36872
5. Mizugishi K, Yamashita T, Olivera A et al (2005) Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol 25:11113–11121
6. Xia P, Gamble JR, Wang L et al (2000) An oncogenic role of sphingosine kinase. Curr Biol 10:1527–1530
7. Neubauer HA, Pham DH, Zebol JR et al (2016) An oncogenic role for sphingosine kinase 2. Oncotarget 7:64886–64899
8. Maceyka M, Sankala H, Hait NC et al (2005) SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. J Biol Chem 280:37118–37129
9. Hofmann LP, Ren S, Schwalm S et al (2008) Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. Biol Chem 389:1399–1407
10. Okada T, Ding G, Sonoda H et al (2005) Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. J Biol Chem 280:36318–36325
11. Hait NC, Allegood J, Maceyka M et al (2009) Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science 325:1254–1257
12. Lai WQ, Irwan AW, Goh HH et al (2009) Distinct roles of sphingosine kinase 1 and 2 in murine collagen-induced arthritis. J Immunol 183:2097–2103
13. Jo SK, Bajwa A, Ye H et al (2009) Divergent roles of sphingosine kinases in kidney ischemia-reperfusion injury. Kidney Int 75:167–175
14. Wadgaonkar R, Patel V, Grinkina N et al (2009) Differential regulation of sphingosine kinases 1 and 2 in lung injury. Am J Physiol Lung Cell Mol Physiol 296:L603–L613
15. Oskeritzian CA, Alvarez SE, Hait NC et al (2008) Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. Blood 111:4193–4200
16. Sensken SC, Bode C, Nagarajan M et al (2010) Redistribution of sphingosine 1-phosphate by sphingosine kinase 2 contributes to lymphopenia. J Immunol 184:4133–4142
17. Neubauer HA, Pitson SM (2013) Roles, regulation and inhibitors of sphingosine kinase 2. FEBS J 280:5317–5336
18. Liu H, Sugiura M, Nava VE et al (2000) Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. J Biol Chem 275:19513–19520
19. Pitman MR, Pham DH, Pitson SM (2012) Isoform-selective assays for sphingosine kinase activity. Methods Mol Biol 874:21–31
20. Olivera A, Barlow KD, Spiegel S (2000) Assaying sphingosine kinase activity. Methods Enzymol 311:215–223
21. Olivera A, Spiegel S (1998) Sphingosine kinase. Assay and product analysis. Methods Mol Biol 105:233–242
22. Hait NC, Bellamy A, Milstien S et al (2007) Sphingosine kinase type 2 activation by ERK-mediated phosphorylation. J Biol Chem 282:12058–12065
23. Pitson SM, Moretti PA, Zebol JR et al (2003) Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J 22:5491–5500

24. Gordon JA (1991) Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods Enzymol 201:477–482
25. Pitson SM, D'Andrea RJ, Vandeleur L et al (2000) Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes. Biochem J 350(Pt 2):429–441
26. Kralik SF, Du X, Patel C et al (2001) A method for quantitative extraction of sphingosine 1-phosphate into organic solvent. Anal Biochem 294:190–193
27. Vessey DA, Kelley M, Karliner JS (2005) A rapid radioassay for sphingosine kinase. Anal Biochem 337:136–142

Methods in Molecular Biology (2017) 1697: 21–30
DOI 10.1007/7651_2017_42

Published online: 12 May 2017

Methods for Analyzing Sphingosine-1-Phosphate Signaling in Human and Mouse Primary Mast Cells

Alena P. Chumanevich, Piper A. Wedman, and Carole A. Oskeritzian

Abstract

Mast cells produce a potently bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) constitutively and upon activation. The ligation of S1P to its type 2 receptor on mast cells triggers a novel downstream signaling pathway that we discovered links activation of transcription factor signal transducer and activator of transcription 3 to mast cell-derived chemokine release in both humans and mice. In this chapter, we describe the methods used to study S1P signaling in human and mouse primary mast cells.

Keywords: Primary mast cells, Sphingosine-1-phosphate, Signaling, Protein phosphorylation, Signal transducer and activator of transcription 3, Activation, Chemokines, Inflammation, Western blot, Quantitative PCR

Abbreviations

BMMC	Mouse bone marrow-derived mast cell
BSA	Bovine serum albumin
C_t	Threshold cycle
DI	Deionized
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
MC	Mast cell
PBS	Phosphate-buffered saline
p-Stat3	Phosphorylated Stat3
QPCR	Real-time quantitative PCR
rhSCF	Recombinant human stem cell factor
rm	Recombinant murine
S1P	Sphingosine-1-phosphate
S1PR	S1P receptor
SkMC	Human skin-derived mast cell
Stat3 or STAT3	Signal transducer and activator of transcription 3
WB	Western blot

1 Introduction

Mast cells (MC) are uniquely able to produce and release a multifunctional sphingolipid metabolite, sphingosine-1-phosphate (S1P), not only constitutively but also upon activation in the context of inflammation [1–4]. Moreover, MC express two out of the five receptors for S1P, S1PR1 conveying cell movement [2, 4] and S1PR2 which ligation with S1P triggers MC activation accompanied with cytokine and chemokine secretion [1, 2, 5]. Of relevance to inflammation but also cancer, S1P may stimulate tissue-resident MC in an autocrine and/or paracrine manner also acting on MC phenotypic plasticity and responsiveness as well as modulate tissue microenvironment [6–8]. We have previously established the importance of the MC/S1P/S1PR2 axis in endowing the acutely allergic host with an early phase perivascular T-cell recruitment that is dependent on MC-derived chemokines and necessitates activation of signal transducer and activator of transcription 3 (Stat3) signaling in MC [2]. We discovered a new signaling pathway in MC, linking S1P ligation of S1PR2 to chemokine release through Stat3 activation [2]. This was confirmed using genetic, molecular, and pharmacological approaches. Importantly, this novel mechanism of action of S1P is relevant to both human and mouse primary MC [2]. Consistent with our findings, its significance to human atopic disease was recently substantiated by another group who reported compromised allergic reactions and MC degranulation in patients carrying STAT3 mutations, in human MC transduced with small hairpin RNA against Stat3 and in a corresponding mouse model of Stat3 mutation [9].

In this chapter, we describe in great detail the experimental conditions to activate primary human and mouse pure MC with S1P for subsequent molecular analysis of signaling by Real-time quantitative PCR (QPCR) and Western blot (WB). These highly reproducible protocols can be adapted to monitor many signaling molecules, in addition to transcription factors, expressed in mouse bone marrow-derived mast cell (BMMC) and in human MC derived from skin.

2 Materials

2.1 Mast Cells

1. Human skin mast cells (SkMC) expanded ex vivo for at least 4 weeks after fresh human skin processing and up to 3–4 months post-processing [6, 10]
2. Lonza BioWhittaker X-VIVO 15 Hematopoietic Serum-Free Culture Medium. Recombinant human stem cell factor (rh SCF, 200 ng/ml)

3. Mouse mast cells (BMMC) obtained from bone marrow progenitors cultured for at least 3 weeks and up to 3–4 months of culture [6]
4. Culture medium for BMMC: RPMI-1640 medium supplemented with 10 %-heat inactivated Fetal Bovine Serum (FBS), 1 mM HEPES, 2 mM L-glutamine, 1 mM Sodium Pyruvate, 1 % Antibiotic Antimycotic Solution (penicillin 10,000 I.U./ml, streptomycin 10,000 μg/ml, and amphotericin B 25 μg/ml), recombinant (r) murine (m) IL-3 (2 ng/ml), rm SCF (20 ng/ml), and 50 μM 2-mercaptoethanol

2.2 Sphingosine-1-Phosphate

(a) Prepare S1P (Biomol) as a 1 mM stock solution in methanol (see **Note 1**), divide into aliquots of ~240 μl, and store in clean glass bottle with screw tops wrapped with saran wrap at −20 °C (see **Note 2**).

(b) Pipet the desired amount of S1P with a positive displacement pipette equipped with appropriate size capillaries and pistons. Transfer in siliconized 5-ml round bottom glass tube (see **Note 3**). Evaporate methanol under N_2 stream (see **Note 4**).

(c) Dissolve the S1P film in PBS supplemented with 4 mg/ml fatty acid-free Bovine Serum Albumin (BSA) (**Note 5**) to yield concentration of 125 μM (**Note 6**).

(d) Cover glass tube with parafilm. To bring S1P into solution, use a water bath sonicator (1 min) and vortex regularly during this time.

(e) Keep tube in water bath (37 °C) for 30 min, vortexing every 5 min (see **Note 7**). Keep the tube containing resolubilized S1P in a water bath maintained at 37 °C for the entire length of the experiment (see **Note 8**).

2.3 Cell Lysis

1. QIAzol Lysis Reagent
2. Lysis buffer: RIPA buffer (1×) (Thermo Scientific™ Pierce) supplemented with 0.2 mM sodium orthovanadate (Na_3VO_4) and Protease Inhibitor Cocktail (Sigma) (at 1:500 dilution)
3. Protein Assay Dye (Bio-Rad)

2.4 Cytokine/Chemokine Expression by Real-Time Quantitative PCR

1. RNeasy Mini Kit (Qiagen)
2. Nuclease-free water (Ultrapure grade)
3. Chloroform/isoamyl alcohol 24:1, for molecular biology, DNAse, RNAse, and protease free
4. Ethanol for molecular biology, 200 proof
5. RNase-Free DNase Set (50) (Qiagen)
6. SensiFAST cDNA Synthesis Kit (Bioline)
7. SensiFAST SYBR No-ROX Kit (Bioline)
8. MicroAmpFast 96-well Reaction Plate (Applied Biosystems)

2.5 Measurement of Proteins

1. Mini-PROTEAN Electrophoresis System (Bio-Rad), Mini Trans-Blot cell (Bio-Rad), and Mini-PROTEAN TGX Gels (Bio-Rad)
2. Running buffer: Tris/Glycine/SDS Buffer (Bio-Rad)
3. Transfer buffer (25 mM Tris base, 192 mM glycine, pH 8.3, 20 % methanol)
4. Blocking buffer (wash buffer supplemented with 5 % milk (Blotting-Grade Blocker, Bio-Rad))
5. Nitrocellulose membrane (Bio-Rad)
6. Precision Plus Protein Dual Color Standards (Bio-Rad)
7. Primary antibodies: pStat3 (Santa Cruz Biotechnology, cat# sc-81523); Stat3 (Cell Signaling, cat# 4904) and GAPDH (Cell Signaling, cat# 2188)
8. Secondary antibodies: for p-Stat3 (Santa Cruz Biotechnology, cat# sc-2055); for Stat3 (Cell Signaling, cat# 7074)
9. SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific)

3 Methods

3.1 Activation of Human and Mouse Mast Cells with Sphingosine-1-Phosphate

1. Primary human SkMC were cultured in X-VIVO 15 medium supplemented with human SCF (see **Note 9**). Collect pellets and culture medium supernatants of SkMC by centrifugation at 1,200× rpm for 10 min at RT.
2. Resuspend pellets in medium saved after centrifugation at a concentration of 1×10^6 cells/ml and 5×10^6 SkMC/sample. Transfer in 50-ml conical tubes.
3. Primary mouse BMMC were cultured in BMMC culture medium. Collect pellets and culture medium supernatant of BMMC by centrifugation at 1,200× rpm for 10 min at RT.
4. Resuspend pellets in medium saved after centrifugation at a concentration of 1×10^6 cells/ml and between 5 and 10×10^6 BMMC/sample (see **Note 10**). Transfer in 50-ml conical tubes.
5. Add S1P to a final concentration of 100 nM to each sample. Make an additional tube for S1P vehicle control (cells in PBS with 4 mg/ml BSA buffer).
6. Incubate cells at 37 °C in an incubator shaker.
7. Stop the reaction by transferring tubes on ice and adding ice-cold PBS to the cells up to 50 ml at different time points (see **Note 11**). Keep your samples on ice unless otherwise indicated.

8. Remove supernatant by centrifugation (1,200× rpm for 10 min, 4 °C).
9. Resuspend cell pellets in 1 ml or less of ice-cold PBS and transfer cells into Eppendorf tubes.
10. Centrifuge at 1,200× rpm for 5 min at 4 °C.
11. Discard supernatant. Pellets may be stored at −80 °C until use.

3.2 Cell Lysis

Keep samples on ice at all time.

For **QPCR** analysis:

Add 700 µl of QIAzol Lysis Reagent to each cell pellet. Vortex or pipet up and down several times to homogenize well. Lysed cells can be stored in QIAzol reagent at −80 °C until RNA extraction.

For **WB** analysis:

1. 5–10 × 10^6 cell pellets can be lysed with 120–180 µl of lysis buffer.
2. Sonicate (Qsonica 4422 Q55 Sonicator microprobe 1/8″) each sample 3 times on ice for 10 s with 1 min interval at the amplitude intensity level 10.
3. Keep samples on ice for 15 min to yield a homogenous suspension (see **Note 12**).
4. Measure protein concentration of lysates using Bradford technique (Bio-Rad Protein Assay Dye, VWR Cuvettes PS Semi-Micro and Beckman Coulter DU 800 spectrophotometer). Three to four milligrams of protein per milliliter are expected per sample.

3.3 Determination of Gene Expression

1. Total RNA Extraction
   (a) Place samples on the bench at RT for 5 min to defrost.
   (b) Add 140 µl of chloroform to each tube and cap securely. Shake each tube vigorously for 15 s.
   (c) Let stand at RT for 5 min.
   (d) Centrifuge for 15 min at 12,000 × g (15,000 rpm) at 4 °C (see **Note 13**).
   (e) Use Qiagen miRNAeasy Mini Kit essentially following manufacturer's recommendations. Carefully transfer the upper colorless aqueous phase to a new tube (supplied) using a 200-µl pipette tip.
   (f) Measure the exact volume of each sample and add 1.5 volumes of 100 % molecular grade ethanol accordingly. Mix thoroughly by pipetting up and down several times. Continue without delay with step g.

(g) Pipet up to 700 μl of the sample into an RNeasy Mini spin column in 2-ml collection tube (supplied). Centrifuge at 8,000 × g (10,000 rpm) for 15 s at RT. Discard the flow-through.

(h) Repeat step g using the remainder of the sample. Discard the flow-through.

(i) Add to the sample 350 μl of RWT buffer (supplied) and centrifuge for 15 s at 8,000 × g (10,000 rpm), RT. Discard the flow-through.

(j) In advance, prepare DNase mix: 10 μl of DNase (Qiagen RNase-Free DNase) per 70 μl of RDD buffer (supplied). Add DNase mix directly onto the mesh of the RNeasy Mini spin column. Let sit for 15 min at RT.

(k) On top of the mesh of each RNeasy Mini spin column, add 350 μl of RWT buffer (supplied). Centrifuge for 15 s at 8,000 × g (10,000 rpm), RT. Discard the flow-through.

(l) Add to each RNeasy Mini spin column 500 μl of RPE buffer (supplied). Centrifuge for 15 s min at 8,000 × g (10,000 rpm), RT. Discard the old collection tubes with the flow-through.

(m) Add another 500 μl of RPE buffer (supplied) to each RNeasy Mini spin column. Centrifuge for 2 min at 8,000 × g (10,000 rpm), RT.

(n) Carefully without allowing flow-through to touch the bottom of the spin column, transfer RNeasy Mini spin columns into new tubes with no lid. Centrifuge at full speed for 1 min at RT. Discard the old collection tubes with the flow-through.

(o) Transfer RNeasy Mini spin column again into another set of new tubes with no lid. Carefully add 50 μl of RNase-free water (supplied) on top of the mesh. Let sit for 2 min. Centrifuge for 1 min at 8,000 × g (10,000 rpm), RT, to elute the RNA.

(p) Collect the first eluate of RNA and transfer on top of the mesh again. Let sit for 2 min. Centrifuge for 1 min at 8,000 × g (10,000 rpm), RT.

(q) Transfer RNase-free Eppendorf into tubes and keep on ice until concentration determination (see **Note 14**).

(r) Aliquot and store at −80 °C.

2. cDNA Synthesis

1 μg of total RNA was used to synthesize 20 μl of cDNA exactly as indicated in the manufacturer's instructions.

3. QPCR

   (a) Prepare individual QPCR reactions, as follows: 10 μl of SensiFAST SYBR no-ROX mix (2×), 500 nM (final concentration) of each forward and reverse primers, and 200 ng (final concentration) of cDNA template (see **Note 15**). Bring to a final volume of 20 μl with nuclease-free water (see **Note 16)**.

   (b) Seal the plate, centrifuge at 1,500 rpm for 5 min at RT to get rid of air bubbles. Transfer the plate to a real-time PCR cycler machine (Bio-Rad CFX Connect) and perform the following reactions: 95 °C for 10 min (polymerase activation), followed by 40 cycles of: (a) 95 °C for 10 s (denaturation), (b) 55 °C (for BMMC) and 59 °C (SkMC) for 20 s (annealing), and (c) 72 °C for 15 s (extension).

   (c) The relative fold expression of genes of interest was calculated [5] (see **Note 17**).

3.4 Western Blot Analysis

1. For detection of p-Stat3, Stat3 in whole cell lysate, load 9–12 μg of protein per sample per well. For one gel, perform electrophoresis starting at 60 V for the first 20 min then increase to 100 V.
2. Transfer proteins at 100 V, 1 h, on ice onto nitrocellulose membrane.
3. Block the membrane in blocking buffer for 40 min to 1 h on a rocking shaker at RT.
4. Wash the membrane 3 × 10 min with wash buffer, rocking.
5. Cut the membrane at the appropriate molecular weight based on the migration of protein markers and incubate membranes at 4 °C overnight gently shaking with the following primary antibodies: pStat3 diluted 1:200, Stat3 diluted 1:500, and GAPDH diluted 1:3,000.
6. The next day, wash the membrane with wash buffer 3 × 10 min. Incubate the membrane with secondary antibodies: for p-Stat3, use Santa Cruz Biotechnology, cat# sc-2055, diluted 1:2,000, and for Stat3, use Cell Signaling, cat# 7074, diluted 1:2,000.
7. Wash the membrane 3 × 10 min with wash buffer, then rinse with deionized (DI) water 3 times.
8. Soak the membrane for 2–3 min in SuperSignal West Pico Chemiluminescent substrate or SuperSignal West Dura Extended Duration substrate.
9. Expose membrane to film using the automated X-Ray Film Processor.

10. Band quantitation can be carried out using ImageJ (National Institutes of Health). Integrated density numbers (in pixels) of p-Stat3 were normalized to total Stat3 [3].

4 Notes

1. It is recommended to heat the methanol at 65 °C, vortex and sonicate to solubilize S1P.
2. Clean glassware by placing it in hot soapy water (use a non-phosphate detergent); rinse with tap water then with DI water and finally with methanol at least twice to remove excess water and cover.
3. Clean glass tubes as indicated in **Note 2** and dry. Sigmacote® reagent is used to siliconize the glass tubes and prevent surface adsorption of S1P. One milliliter of Sigmacote is transferred from tube to tube as the reaction is instantaneous. Allow the treated glass surface to air dry in a hood. Rinse the siliconized tubes with DI water and reverse tubes to remove excess water. Store siliconized tubes covered.
4. S1P lipid is deposited as a film in the bottom of the siliconized tube.
5. The molar ratio of S1P to its carrier BSA matters. The recommended range is S1P:BSA 2:1 M ratio, therefore 125 μM S1P in 4 mg/ml BSA. However, a concentration of 100 μM will also work if convenient for subsequent dilutions.
6. S1P is not water soluble as it is a lipid. Instead, S1P binds to BSA that acts as a carrier allowing S1P solubilize in aqueous solutions.
7. Sonication and incubation at 37 °C maximize S1P-BSA complex formation, with sonication helping disperse S1P lipid into solution.
8. Never keep S1P solution on ice! This will prevent S1P to stay in solution.
9. Use cells at least 48 h after last medium change.
10. 10×10^6 BMMCs/sample recommended.
11. Recommended time points: 0, 1, 3, 6, and 16 h.
12. For better results, after step 3, perform one to two freeze-and-thaw cycles at −80 °C.
13. After centrifugation, the sample separates into three phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.

14. Quantify final RNA concentration and assess for impurities by determining the ratio of absorbance at 260 versus 230 and 280 nm (NanoDrop). Expect 5–15 μg RNA per sample.
15. Prepare a master mix in a separate RNase-free tube (in the dark) by adding nuclease-free water first, then add primers (the total volume of that mixture is 6 μl per sample). Then add SensiFAST SYBR no-ROX mix (2×) (10 μl per sample). Transfer 16 μl of master mix to each well of the PCR plate. Add 4 μl of cDNA to each well. Avoid generating air bubbles.
16. Primers were synthesized and purchased from Thermo Fisher Scientific, Inc., with melting temperatures ranging from 59.9 to 64.5 °C [5].
17. The threshold cycle (Ct) value is determined by using CFX Manager software (Bio-Rad). The difference in Ct between target and housekeeping gene (GAPDH or beta-actin) (for both unstimulated and S1P-stimulated cells) is found (ΔCt). The ΔCt from unstimulated cells is subtracted from the ΔCt of S1P stimulated cells Ct (ΔΔCt). Due to the logarithmic nature of these calculations, fold change is obtained using 2^-ΔΔCt.

Acknowledgements

This work was supported by grants from the US National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases R01 AI095494 and NIH/National Institute of Arthritis and Musculoskeletal and Skin Diseases R21 AR067996 to C.A.O.

References

1. Mitra P, Oskeritzian CA, Payne SG, Beaven MA, Milstien S, Spiegel S (2006) Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. Proc Natl Acad Sci U S A 103:16394–16399
2. Oskeritzian CA, Price MM, Hait NC, Kapitonov D, Falanga YT, Morales JK et al (2010) Essential roles of sphingosine-1-phosphate receptor 2 in human mast cell activation, anaphylaxis, and pulmonary edema. J Exp Med 207:465–474
3. Oskeritzian CA, Hait NC, Wedman P, Chumanevich A, Kolawole EM, Price MM et al (2015) The sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 axis regulates early airway T-cell infiltration in murine mast cell-dependent acute allergic responses. J Allergy Clin Immunol 135:1008–1018.e1001
4. Oskeritzian CA, Alvarez SE, Hait NC, Price MM, Milstien S, Spiegel S (2008) Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. Blood 111:4193–4200
5. Chumanevich A, Wedman P, Oskeritzian CA (2016) Sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 axis can promote mouse and human primary mast cell angiogenic potential through upregulation of vascular endothelial growth factor-A and matrix metalloproteinase-2. Mediators Inflam 2016:1503206
6. Price MM, Oskeritzian CA, Falanga YT, Harikumar KB, Allegood JC, Alvarez SE et al (2013) A specific sphingosine kinase 1 inhibitor attenuates airway hyperresponsiveness and inflammation in a mast cell-dependent murine model of allergic asthma. J Allergy Clin Immunol 131:501–511.e501
7. Oskeritzian CA (2015) Mast cell plasticity and sphingosine-1-phosphate in immunity, inflammation and cancer. Mol Immunol 63:104–112
8. Rodriguez YI, Campos LE, Castro MG, Aladhami A, Oskeritzian CA, Alvarez SE (2016)

Sphingosine-1-phosphate: a new modulator of immune plasticity in the tumor microenvironment. Front Oncol 6:218

9. Siegel AM, Stone KD, Cruse G, Lawrence MG, Olivera A, Jung MY et al (2013) Diminished allergic disease in patients with STAT3 mutations reveals a role for STAT3 signaling in mast cell degranulation. J Allergy Clin Immunol 132:1388–1396
10. Kambe N, Kambe M, Kochan JP, Schwartz LB (2001) Human skin-derived mast cells can proliferate while retaining their characteristic functional and protease phenotypes. Blood 97:2045–2052

Methods in Molecular Biology (2017) 1697: 31–42
DOI 10.1007/7651_2017_55

Published online: 03 August 2017

Measurement of Lysophosphatidic Acid and Sphingosine-1-Phosphate by Liquid Chromatography-Coupled Electrospray Ionization Tandem Mass Spectrometry

Maria P. Kraemer, Suchismita Halder, Susan S. Smyth, and Andrew J. Morris

Abstract

Lysophosphatidic acids and sphingosine-1-phosphate are bioactive lipids that regulate diverse cellular and physiological processes through actions that are largely mediated by cell surface receptors. The roles played by these lipids in multiple disease processes make the enzymes and receptors involved in their synthesis, inactivation, and signaling attractive targets for pharmacological therapies. In this chapter we describe methods for sensitive accurate quantitation of LPA and S1P levels in biological fluids using liquid chromatography-coupled electrospray ionization tandem mass spectrometry.

Keywords: Liquid chromatography, Lysophospholipids, Mass spectrometry

1 Introduction

LPA and S1P are bioactive lipid mediators that exert largely or wholly cell surface receptor mediated effects on a wide variety of cells. Studies using genetic and pharmacological approaches in animal models implicate LPA and S1P in a broad range of physiological and pathophysiological processes including inflammation, cancer, cardiovascular disease, pain, and neurological injury [1–8]. While both of these lipids can be generated intracellularly as intermediates in pathways of lipid synthesis and degradation they are also both present in extracellular biological fluids including cerebrospinal fluid, lymph, and blood plasma [9]. Although other mechanisms are possible, the most parsimonious theory holds that these extracellular pools of LPA and S1P are the source of biologically active "signaling" lipids. LPA can be generated extracellularly by the secreted lysophospholipase D autotaxin and possibly also by pathways that involve A-type phospholipases [10, 11]. S1P has to be formed intracellularly by ATP dependent kinases and mechanisms for secretion of intracellular S1P involving various plasma membrane transporters have been reported [7]. Because LPA and S1P are not water soluble they are necessarily bound to

"chaperone" molecules in biological fluids. This has been best studied in plasma where both lipids show associations with serum albumin and with circulating lipoproteins. In the case of S1P a specific association with apolipoprotein M mediates association with high density lipoprotein. There is evidence that HDL/apoM associated S1P has distinct signaling functions from albumin bound S1P and indeed the concept that binding of these lipids to plasma proteins and protein lipid complexes protects them from degradation and may influence their signaling properties is particularly interesting [12]. LPA and S1P are involved in multiple pathological processes including tumor growth and metastasis, fibrosis, vascular inflammation and atherosclerosis, neuropathic pain and brain injury and immune system function. Consequently the enzymes and receptors involved in synthesis and signaling by these bioactive lipids are attractive targets for development of small molecule and perhaps also antibody therapeutics [13]. Studies of LPA and S1P metabolism and signaling in preclinical and clinical models are critically dependent on the availability of analytical methods to enable their precise detection and quantitation at low (sub pmol) levels in complex matrices that often contain other lipids at far higher (micro to millimolar) levels. Mass spectrometry methods primarily using reverse phase HPLC coupled with electrospray ionization and selected ion monitoring approaches using triple quadrupole instruments have been widely used to measure LPA and S1P [14–24]. Literature methods for measurement of these lipids are largely very similar and primarily differ in the choice of column and associated solvent system. We have used these approaches to measure the activity of LPA and S1P generating and degrading enzymes, to study LPA and S1P metabolism in isolated cells and to examine the impact of genetic and pharmacological approaches targeting LPA and S1P metabolism and signaling in preclinical models and clinical settings. Although the basic approach used in our laboratory for these assays has been consistent we have continued to improve these methods to address a number of issues that have arisen in the literature that have been suggested to confound these measurements. This includes suggestions that LPA can be formed by hydrolysis of phospholipids during acidic extractions [25], that LPA can be formed by ion source fragmentation of anionic lysophospholipids during electrospray ionization [26]. The most persistent limitation of these measurements remains sample to sample carry over which can be significant for these lipids and has a major impact on assay precision and robustness. In this brief chapter we discuss our approaches to these measurements and present standard approaches currently used in our laboratory.

2 Materials

2.1 Mass Spectrometry Instrumentation

We have used ABSciex hybrid triple quadrupole linear ion trap instruments coupled with Shimadzu chromatography equipment for these measurements. Specifically the methods described below have been developed and validated using ABSciex 4000 Q-Trap instruments with an ABSciex Turbo V ion source and a Shimadzu Prominence binary HPLC system with an autosampler and column oven and an ABSciex 6500 plus Q-Trap instrument with an ion drive ion Turbo V ion source and a Shimadzu Nexera X2 UPLC system with a rack changing autosampler and column oven. The 4000 series instruments are configured with a switching valve between the column and ion source that enables diversion of the column flow to waste. The 6500 series instrument has an integral switching valve. Use of the switching valve during sample loading and equilibration reduces contamination of the ion source improving assay sensitivity and robustness. Instruments of this type are expensive to acquire, maintain, and operate so it is likely that investigators seeking to use these methods may not have access to identical instrumentation and may have to work with shared or facility core owned and operated instrumentation. All of the methods described below could be set up on HPLC coupled triple quadrupole mass spectrometer systems with electrospray ionization capabilities. As explained below the accuracy and sensitivity of these measurements depend on the speed and sensitivity of the instrumentation, particularly the rate at which data can be collected in selected ion monitoring mode which is the primary determinant of the accuracy of peak integration. Accordingly assay performance (i.e., limits of detection, quantitation, and accuracy) on other analytical platforms would have to be evaluated on an instrument specific basis.

2.2 Solvents, Tubes, Internal Standards

Extractions are performed in 8 ml borosilicate glass tubes with Teflon lined caps which are available from multiple commercial sources. Solvents and other reagents are HPLC grade and again available from many suppliers. Internal standards can be purchased from Avanti Polar Lipids, Alabaster, AL.

2.3 Other Equipment

Thorough mixing of the samples before and after phase separation is important. We use a Tallboys Multi Tube Vortexer, Troemner Inc., Thorofare, NJ, USA for efficient mixing of batches of tubes. Any low speed centrifuge with a swinging bucket rotor and adapters for the extraction tubes is suitable for centrifugation of the extraction tubes. For evaporation of solvents we use a Zymark/Biotage Turbo Vap concentrator (Biotage AB Charlotte, NC, USA) that has been adapted for use with 4 ml vials although an N-evap (Organomation Inc., Berlin, MA) with Pasteur pipettes is an effective

substitute. We use a Parker/Balston NitroVap membrane nitrogen generator connected to an air compressor to generate nitrogen gas for these evaporators but these methods would also work with tanked/compressed nitrogen gas. Dispensing of samples, internal standards, and resuspension of extracted material for analysis requires calibrated air displacement pipettes.

3 Methods

3.1 Lipid Extraction from Biological Fluids-Blood, Plasma, Cell Culture Media

1. Label 8 ml borosilicate glass tubes with Teflon lined cap.
2. Add 2 ml MeOH, 1 ml $CHCl_3$ to each tube.
3. Add internal standard to tube (generally 50 pmol C17 LPA or C17 S1P, i.e., 50 μl of a 1 μM solution in MeOH).
4. Add the sample to the tube (for blood or plasma we extract 50 μl, for other biological fluids the volume can be larger up to the total aqueous volume limit of 0.5 ml).
5. Add 0.1 M HCl so the total aqueous volume is 0.5 ml (i.e., for 50 μl blood add 0.45 ml 0.1 M HCl).
6. Vortex for 5 min. This will generate a single phase.
7. Add 1 ml $CHCl_3$ and 1.3 ml 0.1 M HCl to separate phases.
8. Vortex for 5 min.
9. Centrifuge at $>3000 \times g$ for 10 min. This will generate two phases with a "cake" of denatured protein and other insoluble materials at the interface.
10. Transfer lower phase to 4 ml screw cap vial using a Pasteur pipette changing the pipette between samples. Take care to avoid the protein interface and upper phase. Leave behind ~50 μl (don't worry about being more accurate than this because there is an internal standard to correct for recovery). It is important not to transfer the protein cake material at the interface into the vial because this impedes effective reconstitution of the sample and can result in insoluble material being introduced into the chromatography system.
11. Evaporate to dryness under N_2.
12. Resuspend/dissolve dried material in 0.1 ml MeOH, vortex and allow to stand for ~10 min.
13. Transfer to autosampler vial for LC MS analysis. We use 8 mm 100 μl polypropylene vials with Teflon septa. The choice of vial needs to be consistent with the LC system autosampler.

3.2 Measurement of S1P

3.2.1 HPLC Method

Column: Agilent Zorbax Eclipse XDB C8 column, 5 μm, 4.6 × 150 mm.

Solvent A: 75/25 of methanol/water with formic acid (0.5%) and 5 mM ammonium formate (0.1%).

Solvent B: 99/1of methanol/water with formic acid (0.5%) and 5 mM ammonium formate (0.1%).

Flow rate: 0.5 ml/min; Injection volume: 10 μl; Oven temperature: 30 °C.

3.2.2 MS Parameters: Positive Mode Electrospray Ionization, AB Sciex 4000 Q-Trap

Instrument Settings

Declustering potential (DP): 61.0 V.

Collision Energy (CE): 23.0 V.

Collision Cell Exit Potential (CXP): 16.0 V.

Ion spray voltage: 5500 V; Curtain gas: 20 Psi; CAD: Medium.

Temperature: 550.0 °C; Gas 1: 40.0 psi; Gas 2: 40.0 psi.

1. Analysis of S1P is conducted using a Shimadzu UFLC coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode.
2. C17 S1P is used as an internal standard.
3. Samples are analyzed using an Agilent Zorbax Eclipse XDB C8 column, 5 μm, 4.6 × 150 mm column. The mobile phase consists of 75/25 of methanol/water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent A and 99/1of methanol/water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent B. For the analysis of S1P and DH-S1P the separation is achieved using a gradient of 0% B for 1 min, 0% B to 100% B in the next minute, maintained at 100% B for the next 10 min, and equilibrated to the starting conditions in 3 min (Table 1).
4. The flow rate is 0.5 ml/min and the column temperature is 30 °C.
5. The sample injection volume is 10 μl.
6. The mass spectrometer is operated in the positive electrospray ionization mode with optimal ion source settings determined by synthetic standards of S1P, DH-S1P, and C17 S1P with a declustering potential of 61 V, entrance potential of 10 V, collision energy of 23 V, collision cell exit potential of 16 V, curtain gas of 20 psi, ion spray voltage of 5500 V, ion source gas1/gas2 of 40 psi, and temperature of 550 °C. MRM transitions are shown in Table 2.
7. Quantitation of the indicated S1P species is achieved by normalization to the internal standard and reference to external calibration curves generated using authentic standards.

Table 1
Gradient elution for S1P measurements

Time (min)	%B
0	0
1	0
2	100
11	100
12	0

Table 2
Precursor product ion pairs for S1P measurements

	Q1 *m/z*	Q3 *m/z*
C17-S1P	366.1	250.2
	366.1	82.0
	366.1	79.0
DH-S1P	382.1	284.2
	382.1	266.1
	382.1	95.0
S1P	380.1	264.1
	380.1	82.1

3.3 Measurement of LPA

3.3.1 HPLC Method

Column: Machery-Nagel Nucleodur C8 Gravity column; particle size: 5 μm; length: 125 mm; internal diameter: 2.0 mm.

Solvent A: 75/25 of methanol/water with 0.5% formic acid and 0.1% 5 mM ammonium formate.

Solvent B: 80% (99/1of methanol/water with 0.5% formic acid and 0.1% 5 mM ammonium formate) + 20% (Chloroform).

Flow rate: 0.5 ml/min; Injection volume: 10 μl.

Oven temperature: 30 °C.

3.3.2 Instrument Settings

Declustering potential (DP): −90.0 V.

Collision Energy (CE): −26.0 V.

Collision Cell Exit Potential (CXP): −11.0 V.

Curtain gas: 20 psi.

Collision Gas: Medium.

Ion spray voltage: −4500 V.

Temperature: 550.0 °C.

Gas 1: 40.0 psi; Gas 2: 40.0 psi.

1. Analysis of LPA is conducted using a Shimadzu UFLC coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode.
2. C17 LPA is used as an internal standard.
3. Samples are analyzed using a Machery-Nagel Nucleodur C8 Gravity column; particle size: 5 μm; length: 125 mm; internal diameter: 2.0 mm. The mobile phase consists of 75/25 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent A and 80% (99/1of methanol/water with 0.5% formic acid and 0.1% 5 mM ammonium formate) + 20% (Chloroform) as solvent B.
4. The separation is achieved using a gradient of 0% B for 1 min, 0% B to 100% B in the next minute, maintained at 100% B for the next 6 min, and equilibrated to the starting conditions in 3 min (Table 3).
5. The flow rate is 0.5 ml/min and the column temperature is 30 °C.

   The sample injection volume is 10 μl.
6. The mass spectrometer is operated in the negative electrospray ionization mode with optimal ion source settings determined by tuning with synthetic standards of LPA species with a declustering potential of −90 V, entrance potential of −10 V, collision energy of −26 V, collision cell exit potential of −11 V, curtain gas of 20 psi, ion spray voltage of −4500 V, ion source gas1/ gas2 of 40 psi, and temperature of 550 °C. LPA species specific instrument settings can be refined by tuning with authentic synthetic standards. LPA species specific MRM transitions are shown in Table 4.
7. Quantitation of LPA species is achieved by using the internal standard and reference to calibration curves generated using authentic standards.

Table 3
Gradient elution for LPA measurements

Time (min)	%B
0	0
1	0
2	100
6.0	100
7.0	0

Table 4
Precursor product ion pairs for LPA measurements

	Q1 *m/z*	Q3 *m/z*
LPA 12:0	353.2	153.0
LPA 14:0	381.2	153.0
LPA 16:0	409.2	153.0
LPA 16:1	407.2	153.0
LPA 17:0	423.3	153.0
LPA 18:0	437.3	153.0
LPA 18:1	435.3	153.0
LPA 18:2	433.2	153.0
LPA 18:3	431.2	153.0
LPA 19:0	451.3	153.0
LPA 20:0	465.3	153.0
LPA 20:2	461.3	153.0
LPA 20:3	459.3	153.0
LPA 20:4	457.2	153.0
LPA 20:5	455.2	153.0
LPA 22:0	493.3	153.0
LPA 22:2	489.3	153.0
LPA 22:4	485.3	153.0
LPA 22:5	483.3	153.0
LPA 22:6	481.2	153.0

3.4 Concluding Comments

Standard operating procedures (SOPs) are critical to quality management in any analytical laboratory. The information described in this chapter can be used to develop SOPs that are specific to the laboratory adopting the methods. The SOP needs to separate process based quality assurance components, for example, validation of instrument/equipment performance (i.e., instrument tuning and calibration) from assay specific quality control (e.g., use of internal standards). The major complication with these methods is carry over between samples. This can be minimized by washing the autosampler needle and port and monitored by inclusion of solvent blanks in the sample batches. We have observed batch to batch variation in carry over between HPLC columns which may relate to differences in end capping of the silica column matrix. In ongoing work we have found that this carry over issue is further mitigated by

use of columns with a polymeric silica matrix, for example, Waters bridged ethylene silica columns. We hope to publish revised methods using this type of column and a UPLC system that provide greater sensitivity that the methods reported here in the coming year. Finally, the sample preparation method described here is robust and efficient but cannot be simply adapted for automated sample processing because of the phase separation step and the need to recover the lower phase. In our hands, supported liquid extraction methods that absorb the aqueous and insoluble materials using diatom based supports do not result in efficient recovery of lipids although as newer reagents of this type become available this approach is certainly worth considering. Alternate sample preparation methods using methanol or butanol based extractions in which the lipids partition into the upper phase of the system offer a strategy to automate the sample preparation using standard liquid handling approaches with adaptation of these methods to use microplate formats an obvious strategy to increase sample throughput [27, 28].

4 Notes

1. Phospholipids are sensitive to acid hydrolysis and there have been literature reports that LPA can be generated by acid hydrolysis of lysophospholipids during extraction methods using acidified organic solvents. In our experience, this is only seen with much higher concentrations of HCl in the extraction solvent system than the 0.1 M concentration used in our method. To investigate this issue we have added a series of odd carbon chain phospholipids to plasma and attempted to detect the corresponding odd carbon chain LPA species using the LC MS methods described below with triple quadrupole or multistage high resolution instruments. In these studies levels of these LPA species were consistently below the detection limit of the instrument.
2. The assay procedure described above is optimized for analysis of biological fluids. It can be modified for extraction of lipids from small quantities (up to 100 mg) of tissue by homogenizing the tissue in 0.5 ml of 0.1 M HCl and then following the procedure from 2 omitting steps 4 and 5. We use a Tekmar tissumizer homogenizer for this purpose with a disposable micro tip.
3. In general measurements of these lipids in biological fluids are normalized to the starting volume of the sample. For measurements in tissues lipid levels can be normalized to lipid phosphorous measured after wet digestion in perchloric acid or by

measuring levels of more abundant lipids, for example, phosphatidylcholines [27, 28].

4. Because the mass spectrometry method for S1P measurement is monitoring no more than eight separate MRM transitions a 150 ms dwell time results in at least nine data points across each peak which provides accurate and sensitive quantitation. On the 4000 Q-Trap instrument the limit of quantitation of this assay is ~10 fmol of S1P on the column. The limit of detection is ~1 fmol of S1P. The assay is linear up to ~10 pmol of S1P on the column. These assay parameters would have to be determined empirically on the particular instrument system.
5. Avanti Polar Lipids sell a deuterated S1P standard that can be substituted for C17 S1P in the above method and used for quantitation of S1P using standard stable isotope dilution methods.
6. We have validated these methods for use with human and mouse plasma and some tissues and other biological fluids. As with all LC MS methods validation using different matrices would be needed before the methods could be used with confidence to analyze other samples.
7. Our standard LPA method on the 4000 Q-Trap uses 100 ms dwell times which results in at least seven data points across a chromatographic peak even when measuring 15 different LPA species. The limit of quantitation for C17 LPA is ~10 fmol LPA on the column with a limit of detection of ~1 fmol. The method is linear up to 10 pmol LPA on the column.
8. The sensitivity of this method for different LPA species is highly dependent on the chain length and degree of saturation, presumably because of the differential efficiency with which these species are ionized, are stable during ionization and transmission through the instrument. Specifically, the instrument response to short chain saturated LPA species can be two- to threefold higher than to longer chain saturated species. Generation of LPA species specific calibration curves is therefore important if absolute quantitation of LPA species is desired.
9. These methods have been validated for measurement of LPA in mouse and human plasma and would need to be independently validated if used with other samples and matrices.
10. It has been suggested that LPA can be formed by source fragmentation of some anionic lysophospholipids, predominantly lysophosphatidylserine and lysophosphatidylinositol and that this phenomenon would lead to overestimation of the true levels of LPA in the sample. Using the instrument settings described in this method with a modified chromatographic method that separates different classes of lysophospholipids we found that ~1% of lysophosphatidylinositol

and up to 5% of lysophosphatidylserine are fragmented to generate LPA consequently in our hands because plasma levels of these other lysophospholipids are lower than levels of LPA we do not consider this to be a significant issue. Separation of these different lysophospholipid classes can be achieved by modifying the chromatographic method to employ a 10 min linear gradient from solvent A to solvent B. This would be important if analyzing samples that contain higher levels of other anionic lysophospholipids.

11. It is well established that lysophosphatidic acid can be formed in biological fluids ex vivo through the actions of autotaxin on lysophospholipids which are abundant in these systems [11]. Although not directly relevant to the analytical methods discussed here rapid extraction of plasma or storage on ice before extraction is important to minimize this process.

References

1. Abdel-Latif A, Heron PM, Morris AJ, Smyth SS (2015) Lysophospholipids in coronary artery and chronic ischemic heart disease. Curr Opin Lipidol 26(5):432–437. doi:10.1097/mol.0000000000000226
2. Binder BY, Williams PA, Silva EA, Leach JK (2015) Lysophosphatidic acid and Sphingosine-1-phosphate: a concise review of biological function and applications for tissue engineering. Tissue Eng Part B Rev 21 (6):531–542. doi:10.1089/ten.TEB.2015.0107
3. Sheng X, Yung YC, Chen A, Chun J (2015) Lysophosphatidic acid signalling in development. Development 142(8):1390–1395. doi:10.1242/dev.121723
4. Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic acid signaling in the nervous system. Neuron 85(4):669–682. doi:10.1016/j.neuron.2015.01.009
5. Aikawa S, Hashimoto T, Kano K, Aoki J (2015) Lysophosphatidic acid as a lipid mediator with multiple biological actions. J Biochem 157 (2):81–89. doi:10.1093/jb/mvu077
6. Chew WS, Wang W, Herr DR (2016) To fingolimod and beyond: the rich pipeline of drug candidates that target S1P signaling. Pharmacol Res 113(Pt A):521–532. doi:10.1016/j.phrs.2016.09.025
7. Pyne S, Adams DR, Pyne NJ (2016) Sphingosine 1-phosphate and sphingosine kinases in health and disease: recent advances. Prog Lipid Res 62:93–106. doi:10.1016/j.plipres.2016.03.001
8. Pyne NJ, Pyne S (2017) Sphingosine 1-phosphate receptor 1 signaling in mammalian cells. Molecules (Basel, Switzerland) 22(3). doi:10.3390/molecules22030344
9. Morris AJ, Selim S, Salous A, Smyth SS (2009) Blood relatives: dynamic regulation of bioactive lysophosphatidic acid and sphingosine-1-phosphate metabolism in the circulation. Trends Cardiovasc Med 19(4):135–140. doi:10.1016/j.tcm.2009.07.005
10. Aoki J, Inoue A, Okudaira S (2008) Two pathways for lysophosphatidic acid production. Biochim Biophys Acta 1781(9):513–518. doi:10.1016/j.bbalip.2008.06.005
11. Nakanaga K, Hama K, Aoki J (2010) Autotaxin—an LPA producing enzyme with diverse functions. J Biochem 148(1):13–24. doi:10.1093/jb/mvq052
12. Proia RL, Hla T (2015) Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. J Clin Invest 125 (4):1379–1387. doi:10.1172/jci76369
13. Kihara Y, Mizuno H, Chun J (2015) Lysophospholipid receptors in drug discovery. Exp Cell Res 333(2):171–177. doi:10.1016/j.yexcr.2014.11.020
14. Baker DL, Desiderio DM, Miller DD, Tolley B, Tigyi GJ (2001) Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatography-mass spectrometry. Anal Biochem 292(2):287–295. doi:10.1006/abio.2001.5063

15. Fulkerson Z, Wu T, Sunkara M, Kooi CV, Morris AJ, Smyth SS (2011) Binding of autotaxin to integrins localizes lysophosphatidic acid production to platelets and mammalian cells. J Biol Chem 286(40):34654–34663. doi:10.1074/jbc.M111.276725
16. Salous AK, Panchatcharam M, Sunkara M, Mueller P, Dong A, Wang Y, Graf GA, Smyth SS, Morris AJ (2013) Mechanism of rapid elimination of lysophosphatidic acid and related lipids from the circulation of mice. J Lipid Res 54(10):2775–2784. doi:10.1194/jlr.M039685
17. Keune WJ, Potjewyd F, Heidebrecht T, Salgado-Polo F, Macdonald SJ, Chelvarajan L, Abdel Latif A, Soman S, Morris AJ, Watson AJ, Jamieson C, Perrakis A (2017) Rational design of autotaxin inhibitors by structural evolution of endogenous modulators. J Med Chem 60 (5):2006–2017. doi:10.1021/acs.jmedchem.6b01743
18. Georas SN, Berdyshev E, Hubbard W, Gorshkova IA, Usatyuk PV, Saatian B, Myers AC, Williams MA, Xiao HQ, Liu M, Natarajan V (2007) Lysophosphatidic acid is detectable in human bronchoalveolar lavage fluids at baseline and increased after segmental allergen challenge. Clin Exp Allergy 37(3):311–322. doi:10.1111/j.1365-2222.2006.02626.x
19. Ackerman SJ, Park GY, Christman JW, Nyenhuis S, Berdyshev E, Natarajan V (2016) Polyunsaturated lysophosphatidic acid as a potential asthma biomarker. Biomark Med 10 (2):123–135. doi:10.2217/bmm.15.93
20. Sonoda H, Aoki J, Hiramatsu T, Ishida M, Bandoh K, Nagai Y, Taguchi R, Inoue K, Arai H (2002) A novel phosphatidic acid-selective phospholipase A1 that produces lysophosphatidic acid. J Biol Chem 277 (37):34254–34263. doi:10.1074/jbc.M201659200
21. Kurano M, Suzuki A, Inoue A, Tokuhara Y, Kano K, Matsumoto H, Igarashi K, Ohkawa R, Nakamura K, Dohi T, Miyauchi K, Daida H, Tsukamoto K, Ikeda H, Aoki J, Yatomi Y (2015) Possible involvement of minor lysophospholipids in the increase in plasma lysophosphatidic acid in acute coronary syndrome. Arterioscler Thromb Vasc Biol 35 (2):463–470. doi:10.1161/atvbaha.114.304748
22. Le Stunff H, Giussani P, Maceyka M, Lepine S, Milstien S, Spiegel S (2007) Recycling of sphingosine is regulated by the concerted actions of sphingosine-1-phosphate phosphohydrolase 1 and sphingosine kinase 2. J Biol Chem 282(47):34372–34380. doi:10.1074/jbc.M703329200
23. Nagahashi M, Yamada A, Aoyagi T, Allegood J, Wakai T, Spiegel S, Takabe K (2016) Sphingosine-1-phosphate in the lymphatic fluid determined by novel methods. Heliyon 2(12): e00219. doi:10.1016/j.heliyon.2016.e00219
24. Nagahashi M, Yamada A, Miyazaki H, Allegood JC, Tsuchida J, Aoyagi T, Huang WC, Terracina KP, Adams BJ, Rashid OM, Milstien S, Wakai T, Spiegel S, Takabe K (2016) Interstitial fluid Sphingosine-1-phosphate in murine mammary gland and cancer and human breast tissue and cancer determined by novel methods. J Mammary Gland Biol Neoplasia 21(1-2):9–17. doi:10.1007/s10911-016-9354-7
25. Liebisch G, Scherer M (2012) Quantification of bioactive sphingo- and glycerophospholipid species by electrospray ionization tandem mass spectrometry in blood. J Chromatogr B Analyt Technol Biomed Life Sci 883-884:141–146. doi:10.1016/j.jchromb.2011.10.037
26. Onorato JM, Shipkova P, Minnich A, Aubry AF, Easter J, Tymiak A (2014) Challenges in accurate quantitation of lysophosphatidic acids in human biofluids. J Lipid Res 55 (8):1784–1796. doi:10.1194/jlr.D050070
27. Zhao Z, Xu Y (2010) An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples. J Lipid Res 51(3):652–659. doi:10.1194/jlr.D001503
28. Alshehry ZH, Barlow CK, Weir JM, Zhou Y, McConville MJ, Meikle PJ (2015) An efficient single phase method for the extraction of plasma lipids. Metabolites 5(2):389–403. doi:10.3390/metabo5020389

Methods in Molecular Biology (2017) 1697: 43–56
DOI 10.1007/7651_2017_44

Published online: 31 May 2017

Immunohistochemical Detection of Sphingosine-1-Phosphate and Sphingosine Kinase-1 in Human Tissue Samples and Cell Lines

Gary M. Reynolds, Barbara Visentin, and Roger Sabbadini

Abstract

Sphingosine-1-phosphate (S1P) and the enzyme primarily responsible for its production, sphingosine kinase-1 (SphK-1), are dysregulated in multiple human diseases including cancer, multiple sclerosis (MS), diabetes, neurological diseases, fibrosis, and certain pathologies associated with impaired angiogenesis such as age-related macular degeneration (AMD). Antibody-based techniques to identify and localize S1P and SphK-1 within cells and tissue specimens represent a powerful tool, not only to understand biological role of these molecules but also to validate these unique in-class targets in multiple state diseases. Consequently, the potential applications of these molecules for therapy and diagnostic purposes are currently under investigation. Here, we describe a new improved technique, Agitated Low Temperature Epitope Retrieval (ALTER) for staining procedures, to identify expression of S1P and SphK-1 in human frozen tissue samples. The challenges encountered in the process of localization in tissue samples of lipid molecules such as S1P are discussed.

Keywords: Bioactive lipids, S1P, SPHK1, Anti-S1P antibody, LT002, ALTER technique, Immunohistochemistry, Liver cancer

1 Introduction

Sphingolipids are primary structural components of cell membranes that also serve as cellular signaling and regulatory molecules [1, 2]. The sphingolipid signaling cascade includes the bioactive lipid mediators, ceramide (CER), sphingosine (SPH), and sphingosine-1-phosphate (S1P). These mediators are derived from sphingomyelin which is present in the plasma membranes of all mammalian cells. The bioactive lipid, S1P, is now appreciated for its roles in cardiovascular diseases, neurological diseases, inflammation, angiogenesis, and tumor biology [3].

S1P is produced from SPH through the action of sphingosine kinase (SphK). Two isoforms of the kinase have been identified, SphK-1 and SphK-2 [4, 5]. While CER and SPH are commonly associated with apoptosis, S1P is typically viewed as a mediator of cell proliferation and activation of survival pathways and SphK-1 is thought to control this sphingolipid rheostat [6]. S1P has recently

been identified as an extracellular mediator that can act as a ligand for a set of G-Protein Coupled Receptors (GPCRs) belonging to the S1P receptor family, formerly known as Edg receptors [7]; however, intracellular actions of S1P have also been described [8].

Even though S1P is involved as a bioactive lipid signaling mediator in several pathophysiological processes, the most recognized role for S1P is in cancer biology [9–11]. It has been proposed that S1P is upregulated and thereby, responsible for promoting tumor growth [12, 13]. It has been suggested that SphK-1 may be the product of an oncogene [12, 13], a concept supported by the finding that RNA for SphK-1 was overexpressed in many solid tumors, such as those of the breast, colon, lung, ovary, stomach, uterus, kidney, and rectum [14–17]. Increased expression of the SphK-1 in tumor samples has been correlated with significant decreases in survival rate in patients with several types of cancer [18–23]. Importantly, SphK-1 enzymatic activity has been correlated with enhanced SphK-1 immunohistochemical (IHC) staining in tumor specimens obtained from, for example, prostate cancer patients [24]. SphK-1 specific inhibitor reduced tumor growth in several animal models of human cancers [25–27].

Recently, the literature has suggested that S1P is released from tumor cells, and that S1P may be a novel biomarker for early stage cancer detection [28–32]. For example, Sutphen et al. have shown that serum S1P levels are elevated in early stage ovarian cancer patients [29]. Beyond thinking of S1P as a cancer biomarker, S1P levels have been shown to be increased in the bronchiolar lavage of human asthma patients [33], in CSF fluid of multiple sclerosis patients [34] and in serum of patients with coronary artery disease [35].

As a result, academic scientists and industry need simple and accurate means to make measurements of S1P in biological samples, including blood, animal tissues, and biopsy material, in order to validate the putative role of S1P in a particular physiological or pathological process. IHC localization of S1P and SphK-1 may be useful in that regard. In the first edition, we reported an IHC technique that for the first time allowed the demonstration of S1P in tissue sections. Here, we describe an improved methodology, applicable to both S1P and SphK-1 staining.

Since the early 1990s, heat induced epitope retrieval (HIER) techniques have been employed for the reversal of formalin fixation effects and subsequent IHC staining of tissue antigens [36]. Some antigens and tissue constituents however can still not be demonstrated, possibly due to loss during tissue fixation and/or processing or, subsequent loss or denaturation with the high temperatures utilized in subsequent HIER techniques [37]. In 2002, Reynolds published his novel technique, agitated low temperature epitope retrieval, ALTER to address the problems associated with HIER [38]. Using a lower temperature over an extended period, with agitation to

enhance the formalin fixation reversal effects on antigens ensured that many more epitopes could be visualized. We demonstrate the application of the technique here, for the detection of S1P in tissues and improved IHC for SphK-1, providing a simple single methodology.

2 Materials

2.1 Basic Equipment

1. Hotplate stirrer with temperature probe for the ALTER technique
2. Moisture chamber for antibody incubation steps or an automated immunostainer
3. A hydrophobic barrier pen to circle tissue samples, providing a water repellent barrier, which keeps reagents such as antibody solutions localized on the tissue sections
4. Microscope equipment with camera for image acquisition

2.2 Human Frozen Tissue Samples

In the method described below, S1P is localized in human frozen tissue samples. The choice of frozen material is mandatory for S1P staining (*see* **Notes 1** and **3**). Frozen tissue samples were also utilized for the localization of SphK-1; however, staining can be also performed on formalin-fixed and paraffin embedded (FFPE) sections with few modifications for this antibody (*see* **Note 4**). Liver cancer samples stained for S1P and SphK-1 were from The Centre for Liver Research (University of Birmingham, UK). Where possible, cancer patient cases were compared to age-matched healthy control liver samples and distal "normal" (data not shown).

2.3 Primary Antibodies

The S1P staining was performed using the murine monoclonal anti-S1P antibody, or LT1002, generated by Lpath, Incorporated [39]. This antibody represents a high affinity monoclonal $IgG_{1\kappa}$ that recognizes S1P with a high degree of specificity. A commercially available isotype-matched antibody control (NS $IgG_{1\kappa}$) obtained from Southern Research (cat#0102-01) was used as an isotype-matched control mAb for these experiments (*see* **Note 6**).

The SphK-1 staining as described was with the commercially available rabbit polyclonal antibody raised against human SphK-1 (ABCAM; cat#ab16491). To demonstrate specificity of the anti-SphK-1 Ab, a synthetic peptide derived from within residue 350 to the C-terminus of human SphK-1 enzyme was also purchased from ABCAM (cat#ab16634). This peptide was the immunogen used to obtain the primary antibody. The peptide can be utilized to saturate the antibody binding sites when premixed with the antibody and then added to the tissue (*see* **Note 6**).

2.4 Buffers and Solutions

1. 10 % neutral-buffered formalin (NBF) solution to use as a fixative
2. 1 mM EDTA pH 8 with Tween-20 (or similar high pH retrieval solution)
3. Tris-based wash solution pH 7.6 with Tween-20
4. Endogenous peroxidase blocking solution (H_2O_2)
5. 2 % Casein solution for nonspecific binding site blocking

3 Methods

3.1 Pretreatment of Tissue Samples for Immunohistochemical Staining Using Agitated Low Temperature Epitope Retrieval (Figs. 1 and 2)

1. Frozen liver tissues (*see* **Note 1**) cryo-sectioned at 4 μm thick are placed onto charged microscope slides (*see* **Note 2**) and fixed in NBF (CellPath) prior to staining procedure (*see* **Note 3**).
2. Briefly wash sections in tap water.
3. Slides are placed into a metal rack and then incubated in 1 L of preheated EDTA buffer solution (Binding Site Ltd) in a 2 L beaker, on a hotplate stirrer (Fig. 1) overnight (16 h). Agitation is achieved using a 30 × 5 mm magnetic bar, with the stirrer set to 500 rpm (*see* **Note 4**).
4. Removing the beaker from the hotplate, tap water is run into the beaker of buffer until room temperature (RT) is reached.

3.2 Staining of Spingosine-1-Phosphate and Spingosine Kinase-1 in Liver Tissue Samples and Cell Lines (Figs. 3 and 4)

1. Remove the slides from the beaker and rack, drain off excess water, wipe carefully around the tissue section, and with the hydrophobic barrier pen (Vector Labs) circle around each section. This step is not required if an automated immunostainer is being used.
2. Place slides into the incubating chamber (or onto the automated immunostaining machine).
3. Slides are washed in TBS wash buffer solution (Dako) for 5 min.
4. Excess buffer is drained off the sections and the endogenous peroxidase blocking step is performed (Dako) for 10 min.
5. Tipping off the hydrogen solution, wash the slides in TBS wash buffer solution for 5 min (twice within this period).
6. Drain off excess buffer and apply the nonspecific binding site blocking, by incubating the slides in a 2× casein solution (Vector) for 10 min (*see* **Note 5**).
7. Prepare the primary antibodies solutions: anti-S1P antibody (45 μg/mL) and/or anti-human SphK-1 antibody (0.5 μg/mL) in TBS wash buffer solution. Appropriate negative controls should also be run (*see* **Note 6**). After draining off the blocking solution from each slide, replace with the primary antibody solutions, incubating the slides for 1 h at RT.

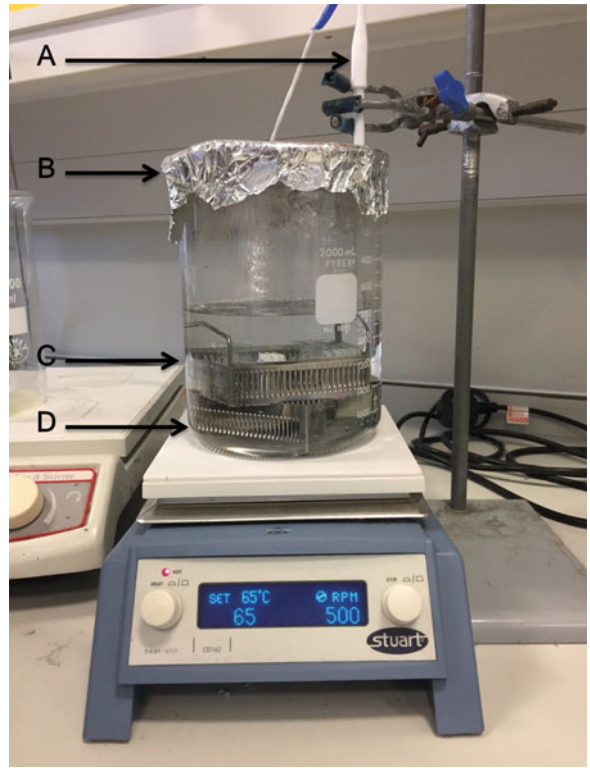

Fig. 1 Hotplate Stirrer with temperature probe used for the Agitated Low Temperature Epitope Retrieval (ALTER) technique. Essential equipment required for the ALTER technique. (**a**) The temperature probe to ensure minimal fluctuation in temperature. (**b**) Tin foil placed over the beaker to prevent evaporation of fluid overnight. (**c**) A metal rack for the slides. (**d**) An inverted metal rack or similar platform in the bottom of the beaker to raise the slide rack with slides above the magnetic bar used in the agitation

8. Draining off the primary antibody, wash the slides in TBS wash buffer solution for 5 min (twice within this period).
9. Incubate sections in the secondary ImmPRESS universal HRP reagent solution (Vector Labs) for 30 min at RT.
10. Draining off the secondary antibody, wash the slides in TBS wash buffer solution for 10 min (four times within this period).
11. To develop the antibody-labeled sections, the ImmPACT Nova-RED peroxidase substrate (Vector Labs) solution is prepared as per manufacturers instructions. Incubate the tissue samples with the substrate solution for 5 min. Other substrates for HRP detection (e.g., DAB solution) can alternatively be used.
12. Wash with tap water.
13. Counterstain with Mayers Hematoxylin (pfm Medical) 30 s.

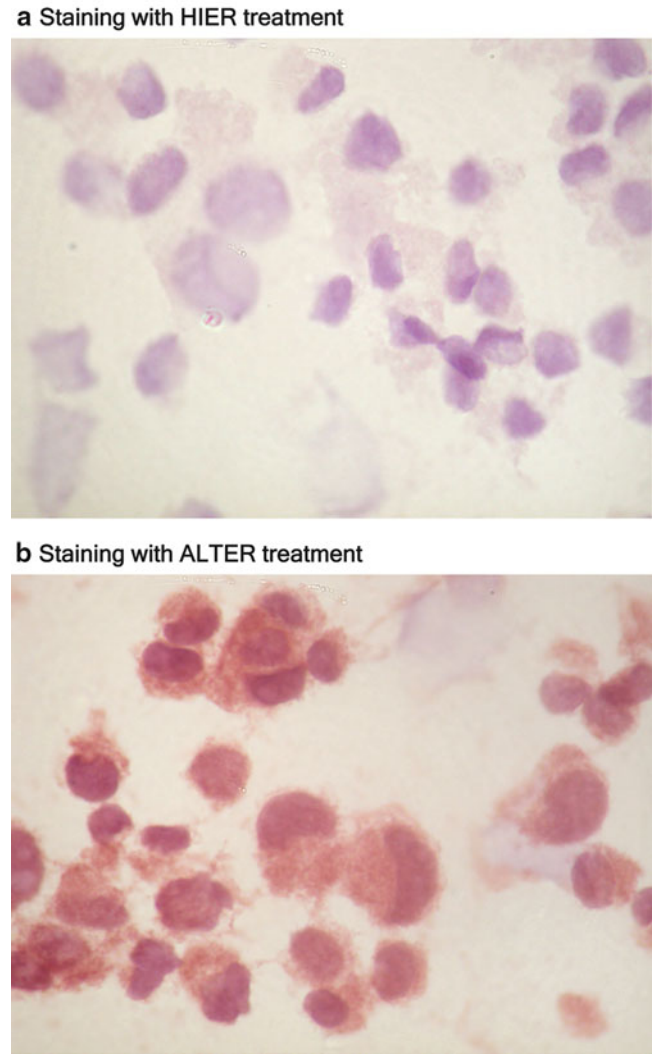

Fig. 2 Sphingosine-1-phosphate (S1P) staining is only achievable with standard fixation procedures in neutral-buffered formalin if the ALTER technique is employed. Using standard heat induced epitope retrieval (HIER) techniques such as microwaving removes the bioactive lipid from tissues or cells. HepG2 cell lines stained for LT1002 (**a**) after HIER showing the absence of S1P and (**b**) using the ALTER technique, with positive cytoplasmic staining for S1P in *red*

14. Wash in tap water again.
15. Dehydrate slides rapidly in two changes of absolute alcohol, 1 min each.
16. Place the slides in Clearene or similar xylene substitute solvent and mount using Expert XTF medium (CellPath) or similar xylene free mountant.

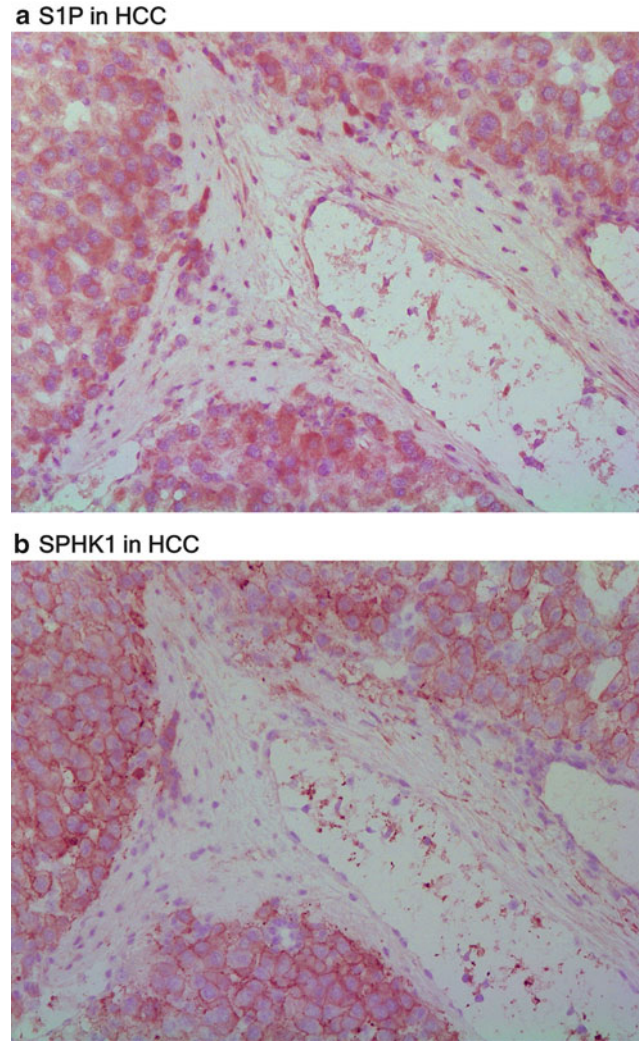

Fig. 3 Frozen tissue sections of hepatocellular carcinoma (HCC) obtained from The Centre for Liver Research (University of Birmingham). Using serial sections, the same tumor cells can be seen to express both (**a**) S1P, using the LT1002 antibody and (**b**) sphingosine kinase-1 (SPHK1), positive staining in *red*

3.3 Microscopy (Fig. 5)

After the staining procedures, let the slides dry in a chemical hood. Slides can then be observed using a conventional light microscope. A digital camera is useful to acquire tissue sample images in tiff or jpg format.

4 Notes

1. While FFPE is generally used and preferred for clinical evaluation of biopsy specimens due to superior morphological preservation, some tissue constituents are lost during tissue processing. With the evolution of epitope retrieval techniques, most proteins can

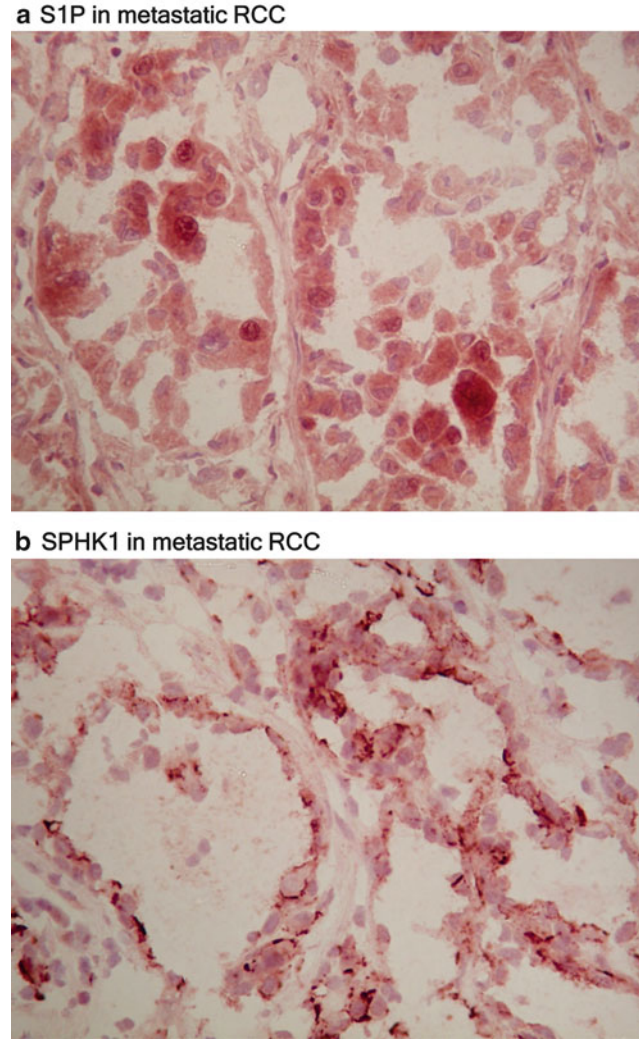

Fig. 4 Serial frozen tissue sections, from The Centre for Liver Research (University of Birmingham), stained for (**a**) S1P and (**b**) SPHK1, in metastatic renal cell carcinoma

now be demonstrated in FFPE tissues, but some are still not detectable, for example, due to becoming denatured in subsequent retrieval techniques due to the high temperatures used (refer to Note 4). Bioactive lipids such as S1P are an example of cellular products that are not fixed and can be lost in the FFPE process, therefore frozen tissue is the only method of choice at present.

2. Highly adhesive glass slides help to reduce the risk of tissue loss and architectural damage during staining procedure. Highly adhesive slides which are slightly positive charges are commercially available. Other adhesive slides can be prepared by

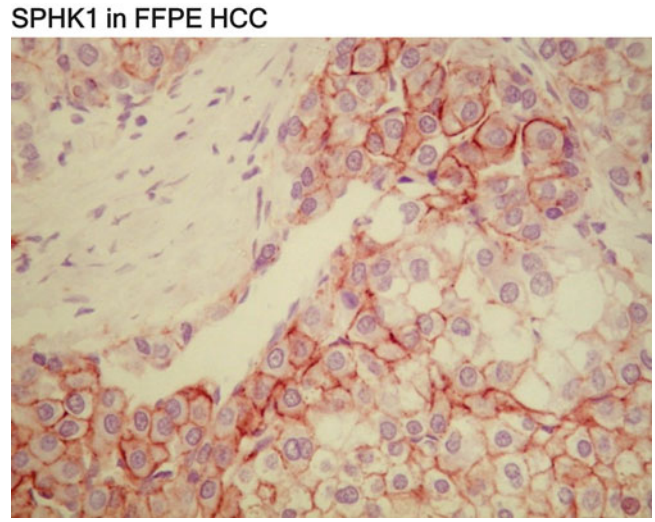

Fig. 5 SPHK1 can also be demonstrated in formalin-fixed paraffin embedded (FFPE) tissue sections. From The Centre for Liver Research (University of Birmingham), an FFPE case of hepatocellular carcinoma (HCC), with tumor cells demonstrating upregulation of SPHK1 (*red*)

pretreating with chemically agents (i.e., Vectabond from Vector Laboratories) or coating with poly-L lysine (note that increased background may be experienced using poly-L lysine coated slides).

3. Setting up an IHC protocol for lipid staining is a challenging task mostly because the effect of fixation on lipids remains largely unknown. Hydrophobic long chain lipids are removed by acetone in most of the tissue and polar lipids can be lost in aqueous formalin solutions. Hydrophobic lipids can be retained by osmium tetroxide and potassium dichromate but these agents obviously cause coloration (e.g., osmium tetroxide stains lipid in black) which interferes with immunostaining interpretation. Baker's fixative (a solution of formalin with the addition of $CaCl_2$) may be recommended for some types of lipids. S1P is a molecule which possesses both hydrophilic and hydrophobic properties (i.e., amphipathic). S1P is soluble in organic solvents such as methanol, ethanol, and xylenes and is generally insoluble in aqueous solutions unless bound with carrier proteins such as fatty acid free BSA. Due to its lipid nature, a 10 % neutral-buffered formalin solution was determined experimentally as the optimal fixative for preservation of S1P. Be aware that formalin contains methanol (~10 %), added by the manufactured because it slows down the polymerization process leading to the precipitation of paraformaldehyde. Furthermore, unbuffered formalin contains a small amount of formate ions derived from the reaction of two formaldehyde molecules, one of which can be reduced to

methanol and the other oxidized to formic acid (Cannizzaro's reaction). Because this is a slow reaction, the concentrations of methanol and formate in any formaldehyde solution can increase slowly with prolonged storage. Thus, we recommend the use of fresh solutions and to avoid the build of formic acid and methanol with long storages of the fixative.

4. In the first edition of these methodologies, the time of fixation for S1P was experimentally determined to be shorter (30 s for cells and 2 min for tissue samples) than that normally used for the localization of antigens (e.g., 10 min for SphK-1). A brief fixation of the tissue is designed to retain all the tissue constituents, but not long enough to cause exaggerated cross-linking events which ultimately may make S1P undetectable. In addition, sections should not be left in prolonged water washings to avoid reversing the cross linking of antigens and consequent loss of them during subsequent immunostaining procedures. In this second edition, we demonstrate how time in fixative is now an irrelevance providing that the ALTER technique is applied for antigen retrieval. Reynolds first described this novel method in 2002 (37) whereby he was able to demonstrate IL-6 in tissue sections by IHC (previously only detectable by in situ hybridization). The theory revolves around the fact that some antigens are less well preserved through fixation than others and thereby, with only minimal reversal of fixation, they become exposed and then denatured or lost by the traditional high temperatures employed. Using a lower temperature over a longer time period, with agitation to enhance the chemical reversal means that loss or denaturation does not occur as demonstrated in Fig. 2.
5. In the S1P staining protocol, a 2× casein solution was utilized for the blocking step instead of serum. Serum is known to contain micromolar concentrations of this bioactive lipid.
6. Controls: The use of controls is crucial in performing any immunochemical procedure. Negative, positive, and experimental controls are essential to guarantee quality of both staining procedures and antibodies used. Negative controls are generally used to assess specificity of the staining. For monoclonal primary antibodies, the negative controls include nonspecific antibodies which are preferably isotype-matched. For example, in the S1P staining, a nonspecific $IgG_{1\kappa}$ was used as negative control for LT1002 which is also $IgG_{1\kappa}$. For polyclonal antibodies such as the rabbit anti-SphK-1 antibody used here, pre-immune serum from the animal the antibody was raised in can be used if available. In the case of cells, siRNA techniques may be utilized to knockdown the gene of interest and hence the protein. Alternatively experimental controls are obtained by pre-absorbing the primary antibody with the specific antigen used during its generation as described in the Sphk-1 staining. As result of this

preincubation performed for a time (generally half an hour) prior the addition of the primary solutions to the slides, the signal obtained with the primary antibody is totally quenched. For the majority of antibodies raised against peptides, the antigen used for the antibody generation may be commercially available. The SphK-1 peptide is premixed with its rabbit polyclonal anti-SphK-1 antibody for 30 min at RT under agitation, prior the primary antibody incubation step. The concentration of SphK-1 peptide utilized to quench the reactivity of the anti-SphK-1 antibody was determined experimentally. Primary antibody was premixed with increasing concentrations of antigen/peptide (1:1, 1:2, 1:10 and 1:100, antibody:peptide ratio). Then normal protocol was followed. In presence of an opportune amount of SphK-1 peptide (in this case 2 μg/mL), the SphK-1 staining is totally quenched as observed. The use of an experimental control with antibodies generated against lipids such as the anti-S1P antibody is more challenging. This is mainly due to nature of the antigen, a lipid, which has evident solubility issues. S1P is a "sphingolipid" characterized by a hydrophilic head (phosphate group) and a long hydrophobic tail. S1P is in general a sticky molecule which binds nonspecifically to plastic surfaces if not properly conjugated with a carrier protein. Fatty acid free BSA can be used to solubilize S1P and deliver S1P in cells and tissues. Unfortunately S1P conjugated BSA cannot be used to deliver S1P to the anti-S1P antibody and pre-absorb the antibody binding sites. The use of excess of S1P/BSA conjugate can cause an increase of non-specific binding within the tissue and even the glass slide. This is expected considering that BSA is sometime preferred to serum as blocking agent in IHC and also other types of assay (e.g., ELISA). To avoid any nonspecific binding, we tested a commercially available omega (ω)-biotinyl-S1P conjugate (Avanti Polar, cat#860552) as antigen to pre-absorb to LT1002. We premixed an excess of biotinyl-S1P conjugate with LT1002 (e.g., 1:1; 2:1, 10:1, 100:1, 200:1, ω-biotinyl-S1P:LT1002, moles ratio), 30 min prior primary antibody incubation step and then we incubate the tissue samples with either the biotinyl-S1P/LT1002 complex or LT1002 solution. We obtained a gradual reduction of signal in tissue sections incubated with increasing concentration of the biotinyl-S1P/LT1002 complex compared to the ones incubated with LT1002 alone. As negative control, the 12:0 biotinyl-LPA (Avanti Polar Lipids, cat# 860552) was premixed with LT1002 as the same ratio than the ω-biotinyl-S1P. No reduction of signal was observed in the case of biotinyl-LPA/LT1002 complex. The ability of the biotinyl-S1P (but not of the 12:0 biotinyl-LPA) to bind specifically to LT1002 was also confirmed in a competition antibody-based ELISA assay using LT1002 (data not shown). Specificity of the

secondary antibodies can be evaluated by simply omitting the primary antibody; as a consequence, no staining should be visible.

7. LT1002 is prepared and delivered to the tissue in a 5 μM $CaCl_2$ solution. The addition of micromolar concentrations of calcium guarantees a favorable environment for the binding of LT1002 to S1P. As reported by Wojciak et al. [40] on studying the crystal structure of the humanized variant generated from LT1002, two metal ions (Ca^{2+}) form bridges in complementary regions from the antibody light chain and S1P. The addition of metal chelators or the introduction of mutations in key positions of the antibody amino acid sequence in the light chain which coordinate the metal atoms interactions decreases S1P binding to LT1002.
8. In this chapter, we reported a protocol to how detect SphK-1 in human frozen tissue samples. Although we preferred to set up and optimize our IHC protocols for frozen tissue in order to localize both S1P and SphK-1 in sequential tissue sections, SphK-1 detection may be achieved in FFPE samples (Fig. 5).

References

1. Hannun YA, Bell RM (1993) The sphingomyelin cycle: a prototypic sphingolipid signaling pathway. Adv Lipid Res 25:27–41
2. Igarashi Y (1997) Functional roles of sphingosine, sphingosine-1-phosphate, and methylsphingosines: in regard to membrane sphingolipid signaling pathways. J Biochem 122:1080–1087
3. Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S (2006) Sphingosine kinases, sphingosine-1-phosphate, apoptosis and diseases. Biochim Biophys Acta 1758:2016–2026
4. Liu H, Sugiura M, Nava VE, Edsall LC, Kono K, Poulton S, Milstein S, Kohama S, Spiegel S (2000) Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. J Biol Chem 275:19513–19520
5. Nava VE, Hobson JP, Murthy S, Milstien S, Speigel S (2002) Sphingosine kinase type 1 promotes estrogen-dependent tumorgenesis of breast cancer MCF-7 cells. Exp Cell Res 281:115–127
6. Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S, Spiegel S (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature 381:800–803
7. Chun J, Rosen H (2006) Lysophospholipid receptors as potential drug targets in tissue transplantation and autoimmune diseases. Curr Pharm Des 12:161–171
8. Van Brocklyn JR, Lee M, Menzeleev R, Olivera A, Edsall L, Cuvillier O, Thomas DM, Coopman PJP, Thangada S, Liu CH, Hla T, Speigel S (1998) Dual actions of sphingosine- 1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. J Cell Biol 142:229–240
9. Pyne NJ, Pyne S (2010) Sphingosine-1-phosphate and cancer. Nat Rev Cancer 10:489–503
10. Ader I, Malavaud B, Cuvillier O (2009) When the sphingosine kinase 1/sphingosine-1-phosphate pathway meets hypoxia signaling: new targets for cancer therapy. Cancer Res 69(9):3723–3726
11. Sabbadini RA (2010) Sphingosine-1-phosphate antibodies as potential agents in the treatment of cancer and age-related macular degeneration. Br J Pharmacol 162:1225–1238
12. Xia P, Gamble J, Wang L, Pitson SM, Moretti PA, Wattenberg BW, D'Andrea RJ, Vadas MA (2000) An oncogenic role of sphingosine kinase. Curr Biol 10:1527–1530
13. Vadas MA, Gamble JR (1996) Endothelial adhesion molecules in atherogenesis. Circ Res 79:1216–1217
14. Johnson KR, Johnson KY, Crellin HG, Ogretmen B, Boylan AM, Harley RA, Obeid LM (2005) Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. J Histochem Cytochem 59:1159–1166

15. French KJ, Schrecengost RS, Lee BD, Zhuang Y, Smith SN, Eberly JL, Yun JK, Smith CD (2003) Discovery and evaluation of inhibitors of human sphingosine kinase. Cancer Res 63:5962–5969
16. Kawamori T, Osta W, Johnson KR, Pettus BJ, Bielawski J, Tanaka T, Wargovich MJ, Reddy BS, Hannun YA, Obeid LM, Zhou D (2006) Sphingosine kinase 1 is up-regulated in colon carcinogenesis. FASEB J 20:386–388
17. Shida D, Fang X, Kordula T, Takabe K, Lepine S, Alvarez SE, Milstien S, Spiegel S (2008) Cross-talk between LPA1 and epidermal growth factor receptors mediates up-regulation of sphingosine kinase 1 to promote gastric cancer cell motility and invasion. Cancer Res 68:6569–6577
18. Long JS, Edwards J, Watson C, Tovey S, Mair KM, Schiff R, Natarajan V, Pyne NJ, Pyne S (2010) Sphingosine kinase 1 induces tolerance to human epidermal growth factor receptor 2 and prevents formation of a migratory phenotype in response to sphingosine-1-phosphate in estrogen receptor-positive breast cancer cells. Mol Cell Biol 30:3827–3841
19. Li JY, Wang H, May S, Song X, Fueyo J, Fuller GN (2008) Constitutive activation of c-Jun N-terminal kinase correlates with histologic grade and EGFR expression in diffuse gliomas. J Neurooncol 88:11–17
20. Ruckhaberle E, Rody A, Engels K, Gaetje R, von Minckwitz G, Schiffmann S, Grosch S, Geisslinger G, Holtrich U, Karn T, Kaufmann M (2008) Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. Breast Cancer Res Treat 112:41–52
21. Facchinetti MM, Gandini NA, Fermento ME, Sterin-Speziale NB, Ji Y, Patel V, Gutkind JS, Rivadulla MG, Curino AC (2010) The expression of sphingosine kinase-1 in head and neck carcinoma. Cells Tissues Organs 192 (5):314–324
22. Li W, Yu CP, Xia JT, Zhang L, Weng GX, Zheng HQ, Kong QL, Hu LJ, Zeng MS, Zeng YX, Li M, Li J, Song LB (2009) Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. Clin Cancer Res 15:1393–1399
23. Van Brocklyn JR, Jackson CA, Pearl DK, Kotur MS, Snyder PJ, Prior TW (2005) Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. J Neuropathol Exp Neurol 64:695–705
24. Malavaud B, Pchejetski D, Mazerolles C, de Paiva GR, Calvet C, Doumerc N, Pitson S, Rischmann P, Cuvillier O (2010) Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. Eur J Cancer 46:3417–3424
25. Kapitonov D, Allegood JC, Mitchell C, Hait NC, Almenara JA, Adams JK, Zipkin RE, Dent P, Kordula T, Milstien S, Spiegel S (2009) Targeting sphingosine kinase 1 inhibits Akt signaling, induces apoptosis, and suppresses growth of human glioblastoma cells and xenografts. Cancer Res 69:6915–6923
26. Paugh SW, Paugh BS, Rahmani M, Kapitonov D, Almenara JA, Kordula T, Milstien S, Adams JK, Zipkin RE, Grant S, Spiegel S (2008) A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. Blood 112(4):1382–1391
27. Pchejetski D, Doumerc N, Golzio M, Naymark M, Teissie J, Kohama T, Waxman J, Malavaud B, Cuvillier O (2008) Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. Mol Cancer Ther 7:1836–1845
28. Shen Z, Wu M, Elson P, Kennedy AW, Belinson J, Casey G, Xu Y (2001) Fatty acid composition of LPA and LPI in plasma from patients with ovarian cancer and other gynecological diseases. Gynecol Oncol 83:25–31
29. Sutphen R, Xu Y, Wilbanks GD, Fiorica J, Grendys EC Jr, LaPolla JP, Arango H, Hoffman MS, Martino M, Wakeley K, Griffin D, Blanco RW, Cantor AB, Xiao YJ, Krischer JP (2004) Lysophospholipids are potential biomarkers of ovarian cancer. Cancer Epidemiol Biomarkers Prev 13:1185–1191
30. Xiao Y, Chen Y, Kennedy AW, Belinson J, Xu Y (2000) Evaluation of plasma lysophospholipids for diagnostic significance using electrospray ionization mass spectrometry (ESI-MS) analyses. Ann N Y Acad Sci 905:242–259
31. Xiao YJ, Schwartz B, Washington M, Kennedy A, Webster K, Belinson J, Xu Y (2001) Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. Anal Biochem 290:302–313
32. Xu Y, Shen Z, Wiper DW, Wu M, Morton RE, Elson P, Kennedy AW, Belinson J, Markman M, Casey G (1998) Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. JAMA 280:719–723
33. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S, Panettieri RA Jr (2001) Sphingosine-1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. FASEB J 15:1212–1214

34. Kulakowska A, Zendzian-Piotrowska M, Baranowski M, Kononczuk T, Drozdowski W, Gorski J, Bucki R (2010) Intrathecal increase of sphingosine-1-phosphate at early stage multiple sclerosis. Neurosci Lett 477:149–152
35. Deutschman D, Carstens J, Klepper R, Smith W, Page MT, Young TR, Gleason L, Nakajima N, Sabbadini R (2003) Predicting obstructive coronary artery disease using serum sphingosine-1-phosphate. Am Heart J 146:62–68
36. Reynolds GM, Young FI, Young JA, Williams A, Rowlands DC (1994) Microwave oven antigen retrieval applied to the immunostaining of cytopathology specimens. Cytopathology 5:345–358
37. Reynolds GM, Williams A, Gregory J, Rowlands DC (1995) Abolition of endogenous alkaline phosphatase activity by microwave oven heating. J Pathol 176(2):217
38. Reynolds GM, Billingham LJ, Gray LJ, Flavell JR, Najafipour S, Crocker J, Nelson P, Young LS, Murray PG (2002) IL-6 expression by Hodgkin/Reed-Sternberg cells is associated with the presence of 'B' symptoms and failure to achieve complete remission in patients with advanced Hodgkin's disease. Br J Haematol 118:1–7
39. O'Brien N, Jones ST, Williams DG, Cunningham HB, Moreno K, Visentin B, Gentile A, Vekich J, Shestowsky W, Hiraiwa M, Matteo R, Cavalli A, Grotjahn D, Grant M, Hansen G, Campbell MA, Sabbadini R (2009) Production and characterization of monoclonal anti-sphingosine-1-phosphate antibodies. J Lipid Res 50:2245–2257
40. Wojciak JM, Zhu N, Schuerenberg KT, Moreno K, Shestowsky WS, Hiraiwa M, Sabbadini R, Huxford T (2009) The crystal structure of sphingosine-1-phosphate in complex with a Fab fragment reveals metal bridging of an antibody and its antigen. Proc Natl Acad Sci U S A 106:17717–17722

Methods in Molecular Biology (2017) 1697: 57–71
DOI 10.1007/7651_2017_6

Published online: 31 March 2017

A Cleanup Method for Mass Spectrometric Analysis of Sphingosine- and Ceramide-1-Phosphate in Blood and Solid Tissue Using a Phosphate Capture Molecule

Jun-ichi Morishige, Ryouhei Yamashita, Tamotsu Tanaka, and Kiyoshi Satouchi

Abstract

Cleanup technology and mass spectrometric determination of sphingosine-1-phosphate (S1P) using a phosphate capture molecule are shown. The protocol is rapid, requires neither thin-layer chromatography nor liquid chromatography, and is applicable to both blood and solid tissue samples. The mass spectrometric method is also applicable to ceramide-1-phosphate.

Keywords: Ceramide-1-phosphate, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Phos-tag, Sphingosine-1-phosphate

1 Introduction

Mass spectrometric analysis is a frequently used method for detection of sphingosine-1-phosphate (S1P). Since S1P is a minor component in the lipids of serum or tissues, separation of S1P from other phospholipids is indispensable when matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is applied for the detection. Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI MS) is another mass spectrometric device used for the detection of S1P [1, 2]. Although this device is equipped with a separation system, carry-over contamination becomes a problem when crude lipid extracts of serum or tissues are successively injected. Here, we show a cleanup technology for S1P using 1,3-bis[bis (pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex, Phos-tag (Fig. 1) [3]. Phos-tag is a dinuclear zinc(II) complex acting as a phosphate capture molecule [4]. Not only phosphorylated peptide but also phospholipids such as S1P, lysophosphatidic acid (LPA), phosphatidic acid, and ceramide-1-phosphate (C1P) can be bound to Phos-tag in the ratio of 1:1 [5–9]. Using this property, we developed a method for S1P enrichment from

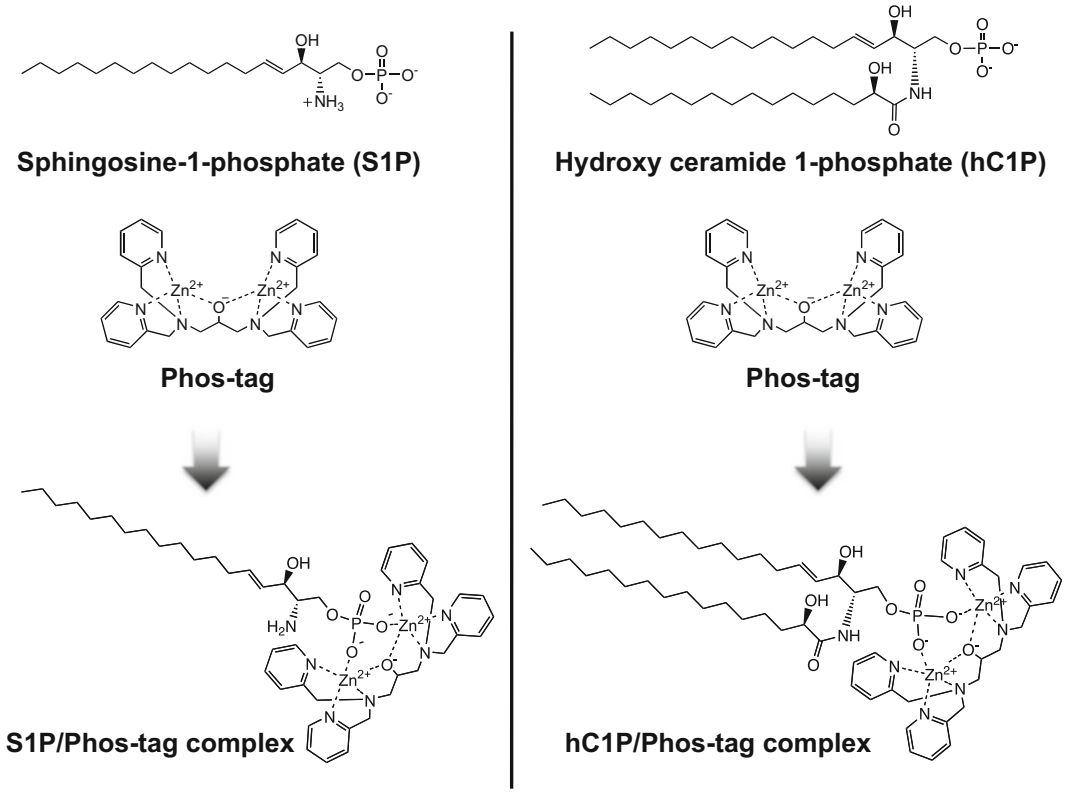

Fig. 1 Structures of S1P, hC1P, Phos-tag, S1P/Phos-tag complex, and hC1P/Phos-tag complex

biological samples by two-step partition without use of LC or TLC. The resulting S1P/Phos-tag complex is detectable by MALDI-TOF MS. By using internal standard, such as C17 analogue of S1P, S1P in biological samples is quantifiable. Here, we show an analytical method for S1P in tissues or assay mixture of sphingosine kinase (SphK). We also showed application of this method to C1P.

2 Materials

^{68}Zn-Phos-tag can be obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) (*see* Ref. [10]). 1,3-Dihydroxy-2-amino-4-octadecene-1-phosphate (C18 S1P) is from Biomol (Hamburg, Germany). 1,3-Dihydroxy-2-amino-4-heptadecene-1-phosphate (C17 S1P) and *D-erythro*-sphingosine from porcine brain are from Avanti Polar Lipids (Birmingham, AL, USA).

2,4,6-Trihydroxyacetophenone (THAP) and 28 % aqueous ammonia are from Aldrich Chemical Co. (Milwaukee, WI, USA) and Nacalai Tesque (Kyoto, Japan), respectively. 4-Deoxypyridoxine hydrochloride and adenosine 5′ triphosphate (ATP) are from Sigma-Aldrich Chemical, CO (St. Louis, MO, USA), respectively. Prepare all solutions using analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Lipid Extraction and Partition

1. Standard S1P solution: Weigh 1 mg C17 S1P (or C18 S1P) and dissolve it in chloroform/methanol (2:1, v/v) mixture, and then add small volume of water (0.1–0.2 mL) to a volume of 5 mL (*see* **Note 1**). Store at −20 °C.
2. One-phase solvent: Mix 1 volume of chloroform, 2 volume of methanol, and 0.8 volume of water (chloroform/methanol/water = 1:2:0.8, v/v).
3. EDTA solution (200 mM): Dissolve 74.4 mg EDTA (2 Na) in 1 mL of 0.24 N NaOH. Store at 4 °C.
4. Homogenizing solution: 100 mM *o*-vanadate and 1 mM EDTA in 0.9 % NaCl. Dissolve 36.8 mg *o*-vanadate in 1.99 mL of 0.9 % NaCl. Add 0.01 mL of 200 mM EDTA. Store at 4 °C.
5. Standard C1P solution: Weigh 1 mg *d*18:1/12:0, *d*18:1/16:0, or *d*18:1/24:0 C1P, and dissolve it in 5 mL chloroform/methanol (2:1, v/v) mixture. Store at −20 °C.

2.2 SphK Assay

1. SphK lysis buffer: 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 15 mM NaF, 0.5 mM deoxypyridoxine, 20 % glycerol, 40 mM β-glycerophosphate, and 1 mM *o*-vanadate. Store at 4 °C.
2. 20 mM ATP: Weigh 1.1 mg ATP, and dissolve in 0.1 mL of 200 mM $MgCl_2$.
3. 1 mM sphingosine: Dissolve 5 mg D-*erythro* sphingosine in 5 mL of chloroform/methanol (2:1, v/v) mixture (store at −20 °C). Take 50 nmol of D-*erythro* sphingosine in 1.5 mL Eppendorf tube, and evaporate the solvent under the stream of nitrogen. Dissolve in 0.05 mL of ethanol, vortex, and then add 0.45 mL of 100 mM Tris-HCl (pH 7.4) (*see* **Note 2**). Sonicate for 5 min in bath-type sonicator.

2.3 MALDI-TOF MS

1. 0.1 % ammonia in methanol: Mix 0.02 mL of 28 % aqueous ammonia with 0.08 mL water (5.6 % aqueous ammonia). Add 0.089 mL of 5.6 % aqueous ammonia and 4.91 mL of methanol (0.1 % ammonia in methanol).
2. ^{68}Zn-Phos-tag solution (0.1 mM) (*see* **Note 3**): Dissolve 0.86 mg ^{68}Zn-Phos-tag (diperchloric acid, monohydrate, formula weight 865.4) in 1 mL water (1 mM ^{68}Zn-Phos-tag solution). Dilute 0.1 mL of 1 mM ^{68}Zn-Phos-tag solution to 1 mL with water. Store at 4 °C.
3. THAP solution: Dissolve 10 mg THAP in 1 mL acetonitrile (*see* **Note 4**). Store it with protection from light.
4. Silica suspension (*see* **Note 5**): Suspend 100 mg silica gel in 1 mL methanol.

3 Methods

Carry out all procedures at room temperature.

3.1 Lipid Extraction from Liquid Sample

1. Mix 0.4 mL of serum with equal volume of 5 % KCl in a glass tube (*see* **Note 6**).
2. Add 3 mL of chloroform/methanol (1:2, v/v) mixture containing 0.32 nmol of C17 S1P.
3. Vortex for 15 s.
4. Sonicate for 30 s in a bath-type sonicator and leave for 20 min on ice.
5. Add 1 mL of chloroform, 1 mL of water, and 0.005 mL of 28 % aqueous ammonia. Vortex for 15 s, and stand for 1 min for phase separation. Adjust pH of the upper phase to 9–10 with 28 % aqueous ammonia (*see* **Note 7**).
6. Centrifuge at 1300 × *g* for 10 min at 4 °C, and remove the lower chloroform phase by a Pasteur pipet (*see* **Note 8**).
7. Add 2 mL of chloroform/methanol (17:3, v/v) mixture to the remaining upper water/methanol phase (*see* **Note 9**). Vortex 15 s, centrifuge at 1300 × *g* for 10 min at 4 °C, and remove the lower phase with a Pasteur pipet.
8. Repeat the **step 7**.
9. Add 2 mL of chloroform/methanol (17:3, v/v) mixture and 0.005 mL of 1 mM ^{68}Zn-Phos-tag to the remaining upper water/methanol phase.
10. Vortex for 15 s, centrifuge at 1300 × *g* for 10 min at 4 °C, and collect the lower phase in another new glass tube with new a Pasteur pipet (*see* **Note 10**).
11. Evaporation of the solvent under stream of nitrogen gas in a water bath at 37 °C. Dissolve the residue in 1 mL of chloroform/methanol (2:1, v/v) mixture. Store at −20 °C until use. Scheme of these procedures is shown in Fig. 2.

3.2 Lipid Extraction from Solid Sample

1. Put 0.1 g (wet weight) of fresh animal tissue in a glass tube, and add 1 mL of ice-cold homogenizing solution (*see* **Note 11**).
2. Homogenize the animal tissue with a Polytron or Potter-Elvehjem homogenizer on ice (*see* **Note 12**).
3. Add 5 mL of ice-cold acetone and 0.65 nmol of C17 S1P (*see* **Note 13**).
4. Vortex 30 s and leave 20 min on ice. Centrifuge at 1300 × *g* for 5 min at 4 °C, and remove supernatant with a Pasteur pipet.
5. Add 2.5 mL of ice-cold acetone, and vortex 30 s. Centrifuge at 1300 × *g* for 5 min at 4 °C, remove supernatant, and add fresh ice-cold acetone.

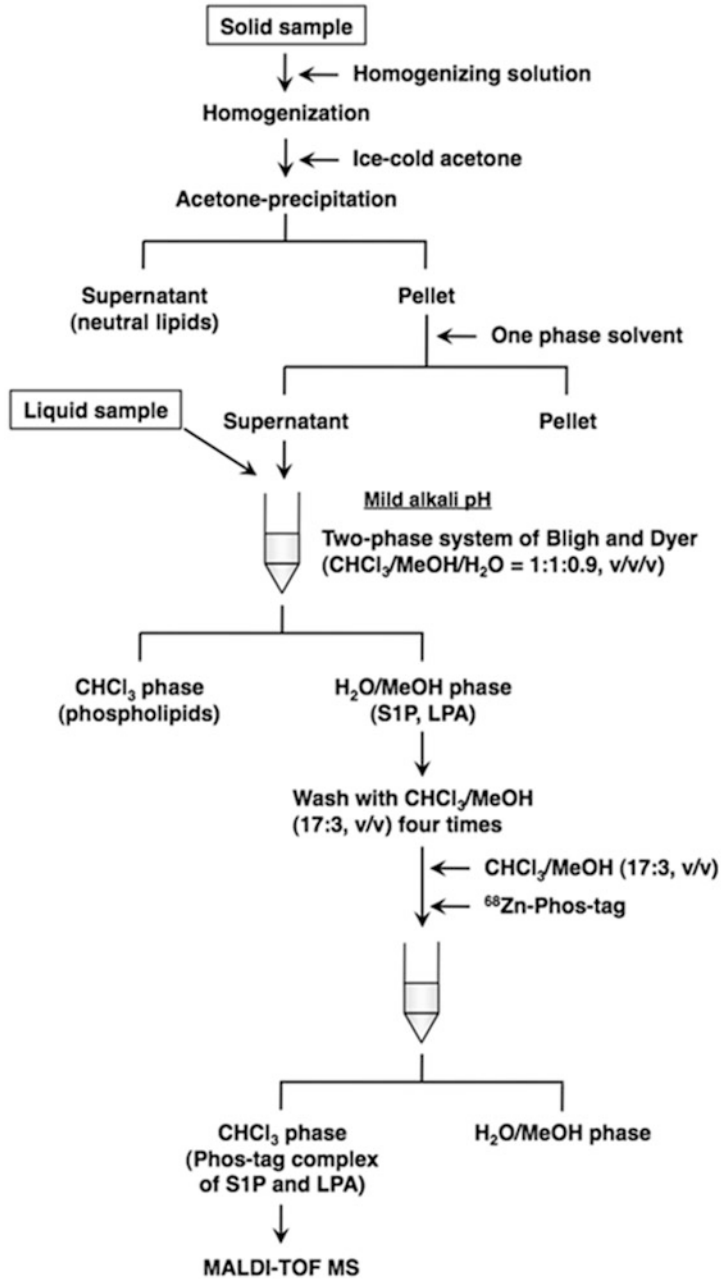

Fig. 2 Schematic presentation of the two-step partition using Phos-tag for the enrichment of S1P

6. Repeat **step 4**.
7. Dry the pellet under stream of nitrogen gas in a water bath at 37 °C.
8. Crush the dried pellet by spatula (*see* **Note 14**).

9. Add 1.9 mL of one-phase solvent, and vortex for 15 s. Sonicate for 30 s in a bath-type sonicator and leave for 20 min on ice.
10. Centrifuge at 1300 × *g* for 10 min at 4 °C, and collect supernatant in a new glass tube with a Pasteur pipet.
11. Add 1.9 mL of one-phase solvent to the remaining pellet, and vortex for 15 s. Centrifuge at 1300 × *g* for 10 min at 4 °C.
12. Combine the supernatant with the former supernatant.
13. Add 1 mL of chloroform, 1 mL of 5 % KCl, and 0.005 mL of 28 % aqueous ammonia to the combined supernatant.
14. Vortex for 15 s, and adjust pH of the upper water/methanol phase to 9–10 with 28 % aqueous ammonia (*see* **Note 7**).
15. Centrifuge at 1300 × *g* for 10 min at 4 °C, and remove the lower phase with a Pasteur pipet (*see* **Note 8**).
16. Add 2 mL of chloroform/methanol (17:3, v/v) mixture. Vortex 15 s, centrifuge at 1300 × *g* for 10 min at 4 °C, and remove the lower phase with a Pasteur pipet.
17. Repeat **step 16** three more times (*see* **Note 15**).
18. Add 2 mL of chloroform/methanol (17:3, v/v) mixture and 0.02 mL of 1 mM ^{68}Zn-Phos-tag solution to the remaining upper phase.
19. Vortex for 15 s, centrifuge at 1300 × *g* for 10 min at 4 °C, and collect the lower phase in another new glass tube with a Pasteur pipet (*see* **Note 10**).
20. Evaporate the solvent under stream of nitrogen gas in a water bath at 37 °C. Dissolve the residue in 1 mL of chloroform/methanol (2:1, v/v) mixture. Store at −20 °C until use. Outline of these procedures is shown in Fig. 2.

3.3 MALDI-TOF MS for S1P

1. Evaporate the organic solvent of S1P/Phos-tag fraction under stream of nitrogen gas. Dissolve the residue in 0.1 mL of 0.1 % ammonia in methanol.
2. Transfer 10 μL of the solution into a 0.5 mL microtube. Add 5 μL of water and 2 μL of silica suspension.
3. Vortex for 10 s. Spot 0.5 μL of the sample suspension on a sample plate for MALDI-TOF MS (*see* **Note 16**).
4. Immediately layer 0.5 μL of THAP solution over the sample suspension on the plate (*see* **Note 17**).
5. Air-dry at room temperature, and subject to MALDI-TOF MS (*see* **Note 18**, Figs. 3, 4, 5).

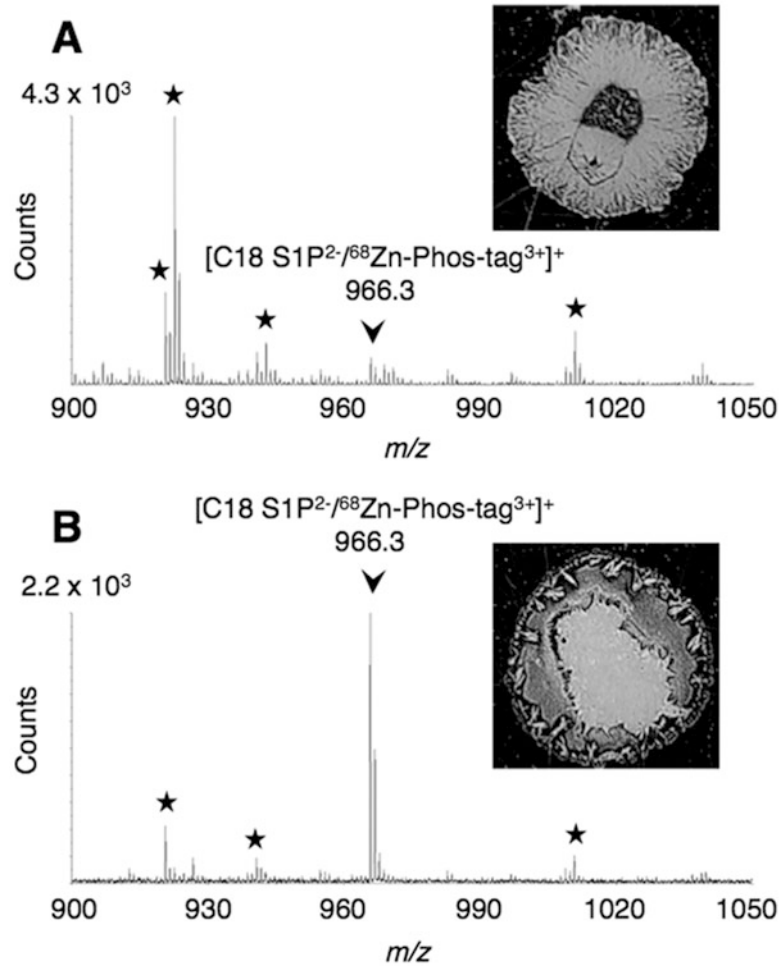

Fig. 3 MALDI-TOF mass spectra of Phos-tag complexes of C18 S1P in the absence (**a**) or presence (**b**) of silica gel. C18 S1P (0.32 nmol) was subjected to MALDI-TOF MS as shown in Section 3.4. *Asterisks* indicate unidentified adducts of ^{68}Zn-Phos-tag. Stains of analyte/matrix on targeting plates are shown in insets

3.4 MALDI-TOF MS for C1P

1. Add standard C1P or TLC-purified C1P in a glass tube, and evaporate the solvent under a stream of nitrogen in a water bath (*see* **Note 20**).
2. Dissolve the residue in 100 μL of 0.3 % ammonia in methanol.
3. Transfer 10 μL of the C1P solution into a 0.5 mL microtube.
4. Add 5 μL of 0.1 mM ^{68}Zn-Phos-tag solution.
5. Vortex for 10 s. Spot 0.5 μL of the sample suspension on a plate for MALDI-TOF MS. Immediately layer 0.5 μL of THAP solution on the sample suspension on the plate.
6. Air-dry at room temperature, and subject to MALDI-TOF MS (Fig. 6).

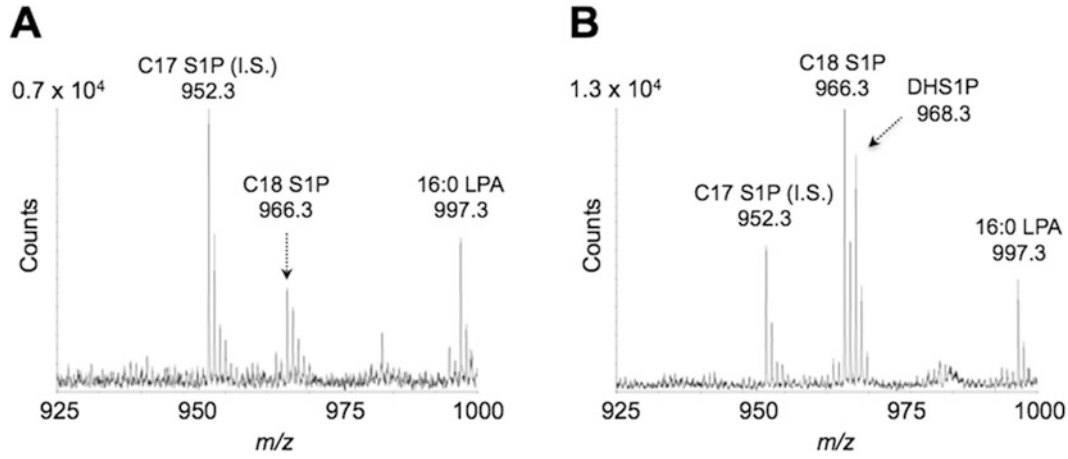

Fig. 4 MALDI-TOF mass spectra of Phos-tag complexes of S1P homologues obtained from calf serum (**a**) and rat lung (**b**). Calf serum (0.4 mL) and rat lung (0.1 g) were mixed with 0.32 and 0.65 nmol of C17 S1P, respectively, as internal standard. S1P-enriched fractions were prepared through the two-step partition using ^{68}Zn-Phos-tag as shown in Sections 3.2 and 3.3 and subjected to MALDI-TOF MS

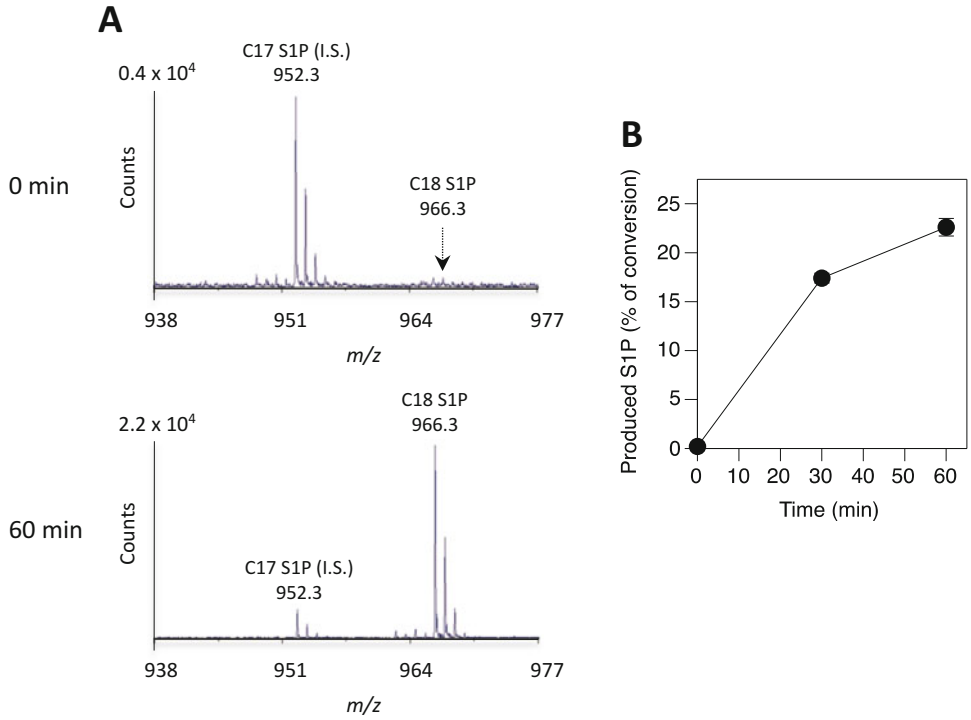

Fig. 5 SphK assay. (**a**) MALDI-TOF mass spectra of Phos-tag complexes of S1P homologues obtained from SphK assay. After incubation, 0.32 nmol of C17 S1P was added into assay mixture. S1P-enriched fractions were prepared as shown in Section 3.8, and subjected to MALDI-TOF MS. (**b**) Conversion rate of S1P form sphingosine during incubation

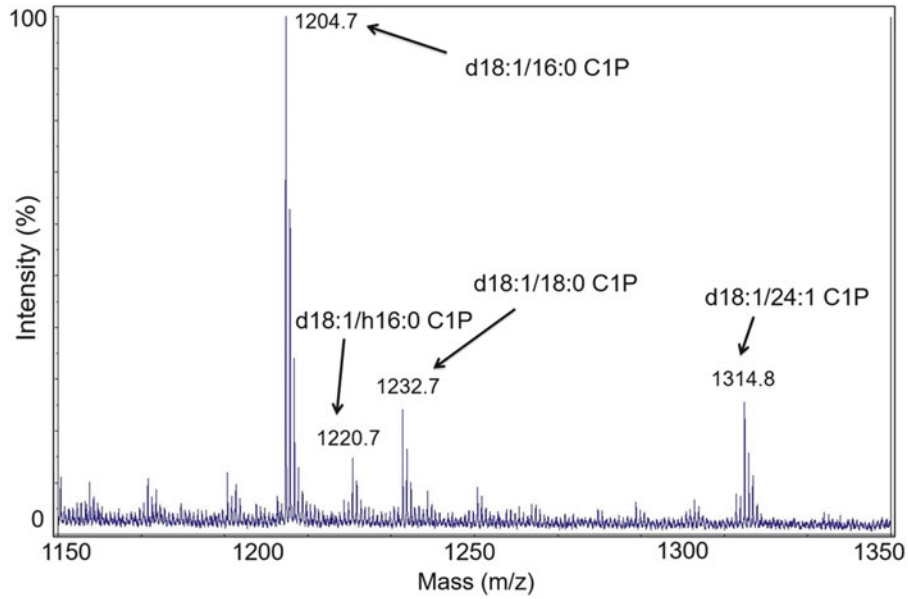

Fig. 6 MALDI-TOF mass spectrum of Phos-tag complexes of C1P prepared from mouse skin. TLC-purified C1P fraction was subjected to MALDI-TOF MS as shown in Section 3.4

3.5 MALDI-TOF MS for Calibration Curve for S1P

1. Mix 0.32 nmol of C17 S1P and various amounts of C18 S1P in a glass tube.
2. Evaporate the solvent under a stream of nitrogen in a water bath at 37 °C.
3. Dissolve the residue in 100 μL of 0.1 % ammonia in methanol.
4. Transfer 10 μL of the mixed S1P solution into a 0.5 mL microtube.
5. Add 5 μL of 0.1 mM ^{68}Zn-Phos-tag solution.
6. Add 2 μL of silica suspension.
7. Vortex for 10 s. Spot 0.5 μL of the sample suspension on a plate for MALDI-TOF MS. Immediately layer 0.5 μL of THAP solution on the sample suspension on the plate.
8. Air-dry at room temperature, and subject to MALDI-TOF MS (*see* **Note 19**).

3.6 MALDI-TOF MS for Calibration Curve for C1P

1. Mix 1 nmol of *d*18:1/12:0 C1P and various amounts of *d*18:1/16:0 or *d*18:1/24:0 C1P in a glass tube.
2. Evaporate the solvent under a stream of nitrogen in a water bath.
3. Dissolve the residue in 100 μL of 0.3 % ammonia in methanol.
4. Transfer 10 μL of the mixed C1P solution into a 0.5 mL microtube.
5. Add 5 μL of 0.1 mM ^{68}Zn-Phos-tag solution.

6. Vortex for 10 s. Spot 0.5 μL of the sample suspension on a plate for MALDI-TOF MS. Immediately layer 0.5 μL of THAP solution on the sample suspension on the plate.
7. Air-dry at room temperature, and subject to MALDI-TOF MS (*see* **Note 21**).

3.7 Preparation of Cytosol for SphK Assay

1. Let the Cos-7 cells (vector control cells and SphK-overexpressing cells) grow until confluent.
2. Aspirate media and wash the cells with PBS.
3. Collect and precipitate the cells by centrifugation at 180 × *g* for 5 min.
4. Remove the supernatant, add 1 mL of SphK lysis buffer to the cell pellet, and make cell suspension.
5. Disrupt the suspended cells completely with probe-type sonicator for 1 min, and centrifuge at 14,000 × *g* for 20 min at 4 °C.
6. Withdraw the supernatant as a cytosol fraction to a new plastic tube. Keep it on ice.
7. Determine protein concentration of the cytosol fraction by standard protein assay methods.
8. Stored it at −80 °C until use.

3.8 SphK Activity Assay

1. Add 25 μg protein of the cytosol fraction, 0.01 mL of 1 mM C18 sphingosine, and SphK lysis buffer to make up volume to 0.18 mL in conical glass tube.
2. Start SphK reaction by adding 0.01 mL of 20 mM ATP to the conical tube and vortex the tubes.
3. Incubate for 30 and 60 min at 37 °C.
4. Add 1.5 mL of chloroform/methanol (1:2, v/v) mixture containing 0.32 nmol of C17 S1P to stop the enzyme reaction, and add 0.2 mL of saline.
5. Vortex for 15 s.
6. Sonicate the reaction mixture for 30 s in a bath-type sonicator, and leave it for 20 min on ice.
7. Add 0.5 mL each of chloroform and saline to the reaction mixture. Vortex for 15 s and stand it for 1 min for phase separation. Adjust pH of the upper phase to 2–3 with 6 N HCl (*see* **Note 22**).
8. Centrifuge at 1300 × *g* for 10 min at 4 °C, and collect the lower chloroform phase in new glass tube with a Pasteur pipet.
9. Evaporate the solvent under stream of nitrogen gas in a water bath at 37 °C.

10. Dissolve the residue in 2 mL of chloroform/methanol (1:1, v/v) mixture. Then, add 0.9 mL of saline for phase separation.
11. Vortex for 15 s, and adjust pH of the upper water/methanol phase to 9–10 with 28 % aqueous ammonia.
12. Centrifuge at 1300 × *g* for 10 min at 4 °C, and remove the lower phase with a Pasteur pipet.
13. Add 2 mL of chloroform/methanol (17:3, v/v) mixture and 0.01 mL of 1 mM ^{68}Zn-Phos-tag to the remaining upper water/methanol phase.
14. Vortex for 15 s, centrifuge at 1300 × *g* for 10 min at 4 °C, and collect the lower phase in a new glass tube with a Pasteur pipet.
15. Evaporate the solvent under stream of nitrogen gas in a water bath at 37 °C.
16. Dissolve the residue in 1 mL of chloroform/methanol (2:1, v/v) mixture. Store at −20 °C until use.

4 Notes

1. A small volume of water is required for complete solubilization. Exact weighing of lipid powder is difficult. So, concentration of the standard S1P solution should be determined by specific methods. Bartlett method (*see* Ref. [11]) and Chalvardjian-Rudnicki method (*see* Ref. [12]) are frequently used methods that are based on a measurement of inorganic phosphorus. Alternatively, add premixed solvent directly to a reagent bottle. Then, vortex and sonicate for complete solubilization.
2. Triton X-100 is commonly employed to solubilize sphingosine for SphK1 assay, because Triton X-100 activates SphK1, but not SphK2 (*see* Ref. [13]). However, Triton X-100 should not be used here, because that it cannot be removed in our protocol and that it interferes ionization of objective molecules in MALDI-TOF MS.
3. Zinc is composed of five stable isotopes. Thus, multiple peaks due to zinc isotopes are detected when natural zinc-containing Phos-tag is used. Use monoisotopic zinc-containing Phos-tag (^{64}Zn- or ^{68}Zn-Phos-tag) for mass spectrometry.
4. THAP gave the best result among matrices tested (THAP, 3,5-dihydroxybenzoic acid, a-cyano-4-hydroxycinnamic acid, sinapinic acid, and harmane) in our analytical condition.
5. We use silica gel scraped off from the TLC plate (Merck 5721, Darmstadt, Germany). The peak intensity of S1P/Phos-tag complex is increased when a small amount of silica gel is mixed at the step of matrix/analyte cocrystallization (*see* Fig. 3). Although the exact reason of the increment of

detection efficiency is unknown, silica gel might condense analyte/matrix on its surface (*see* Ref. [3]). At present, detection limit of C18 S1P on a sample plate is 0.1 pmol under our analytical condition.

6. When protein-rich samples are used, a phase of fluffy material appeared between the water/methanol phase and the chloroform phase. The KCl was added to diminish the fluffy phase. When protein content in a sample is relatively low, such as aqueous humor and saliva, the addition of KCl is not necessary.
7. A sheet of pH test paper can be used for adjustment. Majority of lipids are partitioned into the chloroform phase in the two-phase system of the Bligh and Dyer (*see* Ref. [14]), while S1P goes to the water/methanol phase in a weak alkali condition. The water/methanol phase contains acidic lysophospholipids such as LPA, lysophosphatidylserine, and lysophosphatidylinositol in addition to S1P.
8. Do not remove the upper phase and interfacial fluffy phase.
9. To keep the original solvent composition of chloroform/methanol/water in two-phase system of the Bligh and Dyer method, premixed solvent (chloroform/methanol = 17:3, v/v) should be added. If one uses chloroform instead of premixed solvent for the second separation, S1P might be lost.
10. S1P exists in this chloroform phase as a Phos-tag complex (*see* Fig. 7). LPA, another lysophospholipid having a phosphate group, also exists as a Phos-tag complex in this fraction. These complexes in this fraction can be detected by MALDI-TOF MS (*see* Fig. 4).
11. *o*-Vanadate (a general inhibitor of phosphatase) and EDTA are used to avoid production and/or degradation of S1P by endogenous enzymes in the process of homogenization.
12. When a cultured cell is used as a sample, probe-type and bath-type sonicators can be used for homogenization.
13. Phospholipids including S1P sediment in the presence of acetone. Compounds binding to Phos-tag other than S1P, which is used in the homogenization, are removed in this step. When the ratio of water increases over 20 %, S1P is excluded from tissue pellet together with neutral lipids.
14. To increase the recovery, the dried pellet that contains phospholipids should be crushed into small pieces.
15. Exclusion of phosphatidylcholine, which is the most abundant phospholipid in blood and animal tissues, is necessary for successful detection of a S1P/Phos-tag complex in MALDI-TOF MS. Washing with premixed solvent should be repeatedly done to avoid the matrix effect due to phosphatidylcholine.

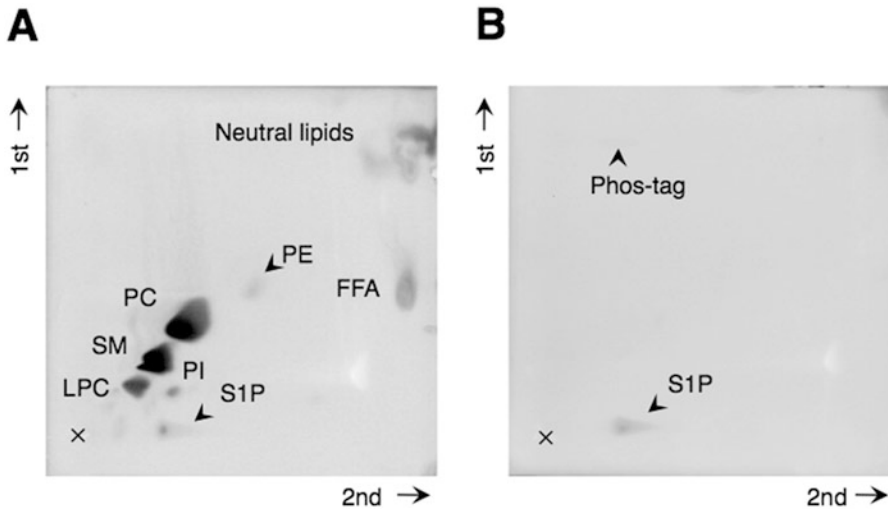

Fig. 7 A model experiment for the enrichment of S1P from calf serum. A serum lipid extract was mixed with 16.1 nmol of C18 S1P. The mixed lipids were subjected to the two-step partition using Phos-tag as shown in Section 3.2. Aliquot of the serum lipid extract (**a**) and resulting S1P-enriched fraction (**b**) are subjected to two-dimensional TLC. First and second solvents are chloroform/methanol/28 % aqueous ammonia (60:35:8, v/v) and chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, v/v), respectively. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *SM* sphingomyelin, *LPC* lysophosphatidylcholine, *FFA* free fatty acid

16. Silica gel in the sample suspension precipitates easily. Withdraw the suspension immediately after vortex.
17. Premix method is better than post-mix method (*see* Section 3.3) in the process of analyte/matrix mixing. We found that intensity of ion of S1P/Phos-tag complex is higher in the sample prepared by the post-mixed method.
18. Typical conditions used in our laboratory are the following: MALDI-TOF mass spectrum is acquired on a Voyager-DE STR (Applied Biosystems, Framingham, MA) in the positive mode. The wavelength of the nitrogen-emitting laser, the pressure in the ion chamber, and the accelerating voltage are 337 nm, 3.7×10^7 Torr, and 20 kV, respectively. The detection is conducted in the reflector mode. The low mass gate is set at 400 Da. To enhance the reproducibility, 256 single shots from the laser are averaged for each mass spectrum. The actual intensity of an objective ion is calculated by subtracting the intensity of an average noise level. Based on the calibration curve of C18 S1P described in **Note 19** and the intensity ratio of $[\text{C18 S1P/Phos-tag}]^+$ against $[\text{C17 S1P/Phos-tag}]^+$, the amount of C18 S1P is determined.
19. A calibration curve is constructed by plotting the intensity ratio of $[\text{C18 S1P/Phos-tag}]^+$ against $[\text{C17 S1P/Phos-tag}]^+$ on *y*-axis versus the molar ratio of $[\text{C18 S1P/Phos-tag}]^+$ against $[\text{C17 S1P/Phos-tag}]^+$ on *x*-axis.

20. C1P cannot be extracted by the above-described method.
21. A calibration curve for C1Ps is constructed by plotting the intensity ratio of $[d18:1/16:0\ C1P/Phos\text{-}tag]^+$, $[d18:1/24:0\ C1P/Phos\text{-}tag]^+$, and $[d18:1/h16:0\ C1P/Phos\text{-}tag]^+$ against $[d18:1/12:0\ C1P/Phos\text{-}tag]^+$ on *y*-axis versus the molar ratio of $[d18:1/16:0\ C1P/Phos\text{-}tag]^+$, $[d18:1/24:0\ C1P/Phos\text{-}tag]^+$, and $[d18:1/h16:0\ C1P/Phos\text{-}tag]^+$ against $[d18:1/12:0\ C1P/Phos\text{-}tag]^+$ on *x*-axis. Quantification is done using d18:1/12:0 C1P as internal standard (*see* Ref. [15]).
22. To avoid removal of zinc in Phos-tag by EDTA contained in SphK lysis buffer, lipids are extracted under acidic condition.

Acknowledgments

We thank Professor Yoh Takuwa for kind donation of SphK-overexpressed cells. This work was supported by the Strategic Support Project of Research Infrastructure Formation for Private Universities from the MEXT, Japan.

References

1. Murph M, Tanaka T, Pang J et al (2007) Liquid chromatography mass spectrometry for quantifying plasma lysophospholipids: potential biomarkers for cancer diagnosis. Methods Enzymol 433:1–25
2. Tokumura A, Carbone LD, Yoshioka Y et al (2009) Elevated serum levels of arachidonoyl-lysophosphatidic acid and sphingosine 1-phosphate in systemic sclerosis. J Med Sci 6:168–176
3. Morishige J, Urikura M, Takagi H et al (2010) A clean-up technology for the simultaneous determination of lysophosphatidic acid and sphingosine-1-phosphate by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a phosphate-capture molecule, Phos-tag. Rapid Commun Mass Spectrom 24:1075–1084
4. Kinoshita E, Takahashi M, Takeda H et al (2004) Recognition of phosphate monoester dianon by an alkoxide-bridged dinuclear zinc (II) complex. Dalton Trans 1189–1193
5. Takeda H, Kawasaki A, Takahashi M et al (2003) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of phosphorylated compound using a novel phosphate capture molecule. Rapid Commun Mass Spectrom 17:2075–2081
6. Tanaka T, Tsutsui K, Hirano K et al (2004) Quantitative analysis of lysophosphatidic acid by time-of-flight mass spectrometry using a phosphate capture molecule. J Lipid Res 45:2145–2150
7. Hirano K, Matsui T, Tanaka T et al (2004) Production of 1,2-didocosahexaenoyl phosphatidylcholine by bonito muscle lysophosphatidylcholine/transacylase. J Biochem 136:477–483
8. Morishige J, Touchika K, Tanaka T et al (2007) Production of bioactive lysophosphatidic acid by lysophospholipase D in hen egg white. Biochim Biophys Acta 1771:491–499
9. Tanaka T, Horiuchi G, Matsuoka M et al (2009) Formation of lysophosphatidic acid, a wound-healing lipid, during digestion of cabbage leaves. Biosci Biotechnol Biochem 73:1293–1300
10. http://www.phos-tag.com/english/index.html
11. Bartlett GR (1959) Phosphorus assay in column chromatography. J Biol Chem 234:466–468
12. Chalvardjian A, Rudnicki E (1970) Determination of lipid phosphorus in the nanomolar range. Anal Biochem 36:225–230

13. Liu H, Sugiura M, Nava VE et al (2000) Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. J Biol Chem 275:19513–19520
14. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
15. Yamashita R, Tabata Y, Iga E et al (2016) Analysis of molecular species profiles of ceramide-1-phosphate and sphingomyelin using MALDI-TOF mass spectrometry. Lipids 51:263–270

Methods in Molecular Biology (2017) 1697: 73–82
DOI 10.1007/7651_2017_25

Published online: 30 April 2017

A Rapid Fluorescence Assay for Measuring Sphingosine-1-Phosphate Transporter Activity in Erythrocytes

Naoki Kobayashi and Tsuyoshi Nishi

Abstract

Sphingosine-1-phosphate (S1P) is an intercellular signaling molecule that is present in the plasma and plays an important role in recruiting lymphocytes from the thymus and secondary lymphoid organs. Erythrocytes are the most abundant cells in the blood and substantially contribute to the S1P supply in the plasma by releasing intracellularly synthesized S1P via an S1P transporter. Thus, the S1P transporter in erythrocytes is a potential target for immuno-suppressing drugs.

In this chapter, we describe a rapid method for measuring the activity of the erythrocyte S1P transporter by using the fluorescent S1P analog, 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD)-labeled S1P. This method does not require chromatography performed with high-performance liquid chromatography, liquid chromatography-tandem mass spectrometry, or thin-layer chromatography methods. Furthermore, S1P transporter activity can be detected by measuring the increase in fluorescence intensity in the extracellular buffer without performing lipid extraction.

Keywords: Sphingosine-1-phosphate, Transporter, Erythrocyte, NBD, Fluorescence, 96-Well plate reader

1 Introduction

Sphingosine-1-phosphate (S1P) is a crucial signal transmitter in the immune system that recruits lymphocytes from the thymus and secondary lymphoid organs. Blood plasma contains higher concentrations (~1 μM) of S1P than are found in marginal areas of the thymus and secondary lymphoid organs, thus resulting in an S1P concentration gradient. Lymphocytes sense S1P with a receptor expressed on the cell surface and move toward higher concentrations of S1P. Plasma S1P is essential for lymphocyte migration, because depletion of plasma S1P markedly decreases the number of lymphocytes present in the blood. Erythrocytes supply the majority of plasma S1P. Erythrocytes constitutively synthesize and release S1P into the blood. We have previously shown that S1P release from erythrocytes is mediated by an S1P transporter [1], but the specific gene has not been identified to date. The S1P transporter in erythrocytes may be a favorable drug target for immunosuppression. To screen agents that might inhibit S1P transporter

activity, a rapid and high-throughput method for measuring activity of the S1P transporter is needed. To date, established methods to measure S1P transport activity require thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. These methods are highly sensitive for detecting S1P but also are time consuming because separation of lipids by chromatography is required. Thus, we established a rapid method for measuring S1P transporter activity by using a fluorescently labeled S1P analog, 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD)-labeled S1P [2].

In erythrocytes, S1P is intracellularly synthesized from internalized extracellular sphingosine by sphingosine kinase 1 and is released from cells via the S1P transporter (Fig. 1). Similarly to sphingosine, NBD-labeled sphingosine is internalized into erythrocytes and is phosphorylated to form NBD-S1P (Fig. 1) [2]. The synthesized intracellular NBD-S1P, similarly to S1P, is released from erythrocytes in a time-dependent manner (Fig. 1) [2]. Release of both S1P and NBD-S1P from erythrocytes is inhibited by glyburide. Furthermore, S1P and NBD-S1P release from erythrocytes is competitively inhibited by intracellular NBD-S1P and S1P, respectively. Thus, NBD-S1P is transported via the S1P transporter in erythrocytes [2]. An important characteristic of S1P metabolism in erythrocytes is that internalized sphingosines are metabolized only to S1P. NBD-sphingosine, similarly to sphingosine, is metabolized only to NBD-S1P in erythrocytes. Thus, changes in NBD-S1P fluorescence in either extracellular buffer or cells are attributed to changes in the activities of sphingosine kinase 1 and/or the S1P transporter.

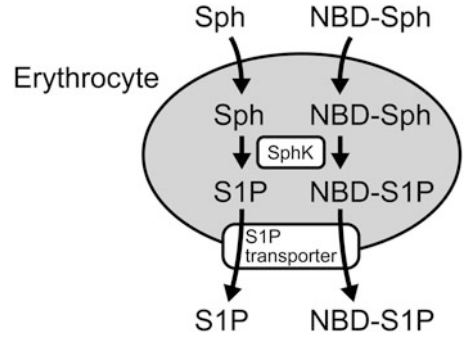

Fig. 1 Schematic illustration of S1P and NBD-S1P synthesis and release in erythrocytes. Erythrocytes incorporate extracellular sphingosine (Sph) and NBD-sphingosine (NBD-Sph), which are phosphorylated by Sphingosine kinase (SphK) to S1P and NBD-S1P, respectively. An erythrocyte S1P transporter exports S1P and NBD-S1P in a time-dependent manner without any stimuli

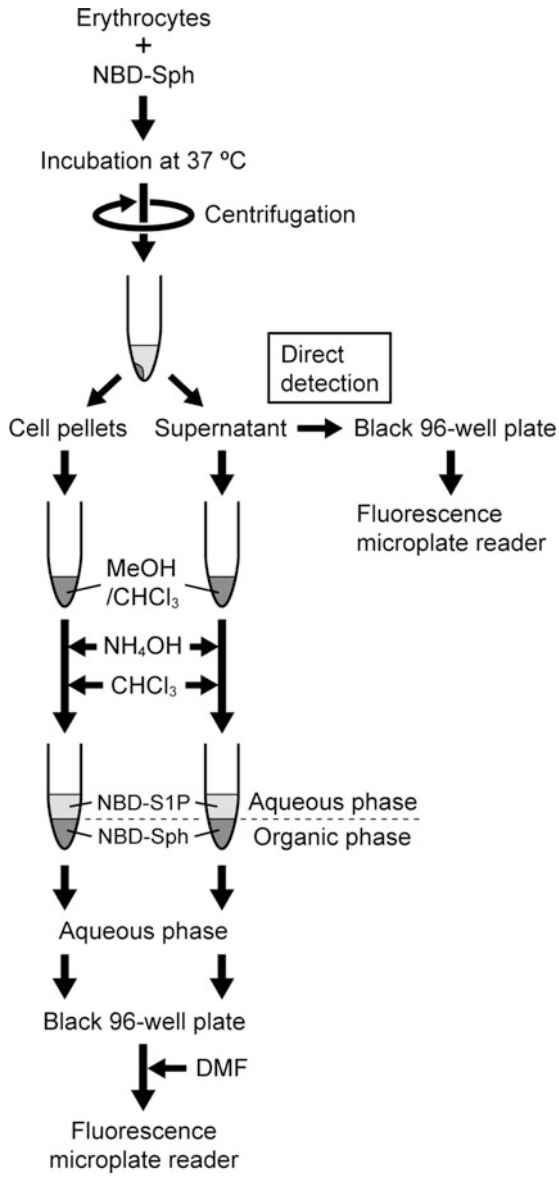

Fig. 2 Outline of the method for measuring NBD-S1P release from erythrocytes. Erythrocytes were mixed with NBD-sphingosine (NBD-Sph) and incubated for the required time. Then the mixture was centrifuged and separated into supernatant (extracellular) and cell pellets (intracellular). For direct detection of NBD-S1P transport (see Sect. 3.4), the supernatant was transferred to a *black* 96-well plate, and the fluorescence of the samples was measured. For NBD-S1P quantification (see Sects. 3.2 and 3.3), the supernatant and cell pellets were transferred to 1.5-mL tubes containing methanol/chloroform ($MeOH/CHCl_3$). NH_4OH solution and $CHCl_3$ were added to the mixture in a stepwise manner. After centrifugation, NBD-S1P and NBD-sphingosine were separated into aqueous (*upper*) and organic (*lower*) phases in the alkaline condition, respectively. The aqueous phases were transferred to a *black* 96-well plate and the fluorescence of the sample was measured after addition of dimethylformamide (DMF)

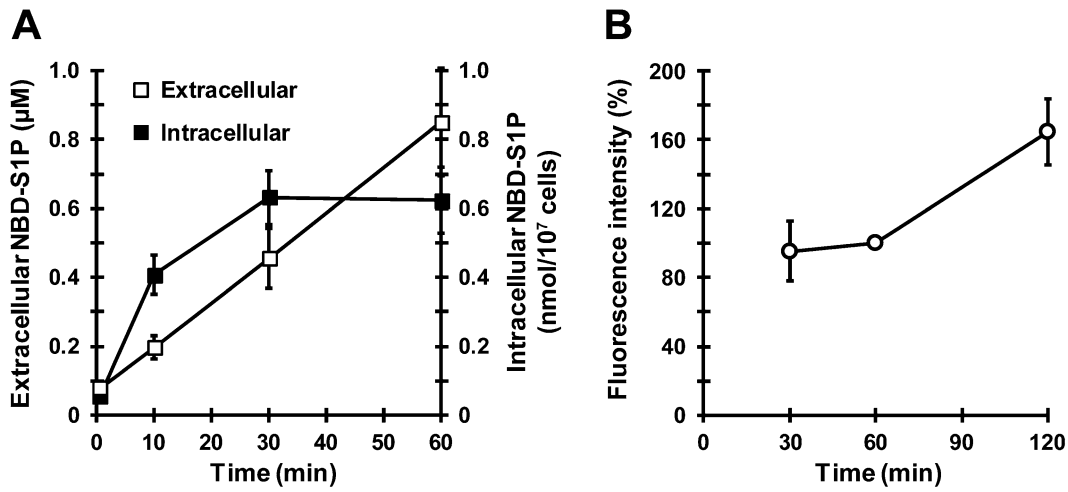

Fig. 3 Measurement of synthesis and release of NBD-S1P in erythrocytes. Erythrocytes were incubated with 5 μM NBD-sphingosine for the indicated times. The reaction mixture was centrifuged and separated to supernatant (extracellular) and cell pellets (intracellular). (**a**) Lipids were extracted from the supernatant and cell pellets. Aqueous phases in lipid extraction were transferred to a *black* 96-well plate and their fluorescence was measured. (**b**) For direct detection of NBD-S1P release, the fluorescence of the supernatants was measured without lipid extraction. The fluorescence intensity of the supernatant at 60 min was set to 100%

To measure NBD-S1P release from erythrocytes, quantification of extracellular NBD-S1P is required. Because the extracellular buffer contains NBD-sphingosine, NBD-S1P must be separated from NBD-sphingosine to quantify the levels of NBD-S1P. Therefore, we adopted an NBD-S1P release assay using lipid extraction in alkaline conditions (Fig. 2). Originally, lipid extraction in alkaline conditions was used as an HPLC method for S1P quantification [3]. In the lipid extraction, S1P and sphingosine are partitioned into aqueous and organic phases, respectively. NBD-S1P and NBD-sphingosine are also separated into aqueous and organic phases, respectively, during lipid extraction (Fig. 2). Thus, the fluorescence derived from the NBD moiety in the aqueous phase corresponds to NBD-S1P. As a result, in the described method, NBD-S1P transport is measured by direct quantification of fluorescence in the aqueous phase of the lipid extraction (Figs. 2 and 3a). Furthermore, we describe a more rapid method to detect S1P transporter activity. When NBD-sphingosine is incubated with erythrocytes for longer than 1 h, most of the NBD-sphingosine in the extracellular buffer is taken up into the cells and phosphorylated to NBD-S1P. Therefore, direct detection of S1P transporter activity is also achieved by quantifying the fluorescence intensity of extracellular buffer (Figs. 2 and 3b).

To date, conventional methods to measure S1P transporter activity in cells require HPLC [3], LC-MS/MS [4], or TLC [1]. In contrast to these chromatography methods, the present method

does not require the time-consuming lipid separation step using chromatography. The present method to measure S1P transporter activity is notable for its low cost, ease of use, and ability to be adapted for multiplexing and high- throughput.

2 Materials

Prepare all buffers and solutions with analytical grade reagents and ultrapure water (resistivity of >18 MΩ cm at 25 °C) at room temperature. Store all buffers and solutions at 4 °C unless otherwise indicated.

2.1 Preparation of Erythrocyte Suspension

1. Acid citrate-dextrose (ACD) solution: dissolve 0.32 g of citric acid, 0.88 g of trisodium citrate dihydrate, and 0.98 g of glucose in 40 mL of water (*see* **Note 1**).
2. Buffer A: 20 mM HEPES, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 138 mM NaCl, and 1 g/L glucose, pH 7.4. Dissolve 4.77 g of HEPES, 0.52 g of NaH_2PO_4, 0.22 g of KCl, 0.2 g of $MgCl_2$ ($6H_2O$), 8.07 g of NaCl, and 1 g of glucose in 1 L of water. Adjust the pH with an NaOH solution.
3. Bovine serum albumin (BSA, fatty acid-free).
4. Buffer A containing 1% BSA. Dissolve 0.1 g of BSA in 10 mL of buffer A (*see* **Note 2**).
5. Buffer A containing 0.1% BSA. Add 1 mL of buffer A containing 1% BSA to 9 mL of buffer A.
6. 1 mL syringe.
7. Needle (26G gauge × 1/2 in.).
8. Rat holder (KN-325-C-4, Natsume Seisakusho, Tokyo, Japan).
9. Female Wistar/ST rats (10–15 weeks old).
10. Anesthetics.
11. Trypan blue.
12. Cell counting chamber.

2.2 NBD-S1P Release Assay

1. Water bath.
2. Vortex mixer.
3. Timer.
4. 1 mM NBD-sphingosine (Avanti Polar Lipids). Dissolve 100 μg of NBD-sphingosine in 209 μL of ethanol.
5. 0.1× Buffer A containing 0.1% BSA. Dilute Buffer A containing 1% BSA with water (1:9, v/v).
6. Refrigerated microcentrifuge.

2.3 Lipid Extraction and Fluorometry

1. 1.5 mL Safe-lock tube (Eppendorf).
2. Vortex mixer.
3. Chloroform.
4. Chloroform/methanol solution (1:1) (*see* **Note 3**).
5. 7 M NH_4OH solution. Dilute 28% NH_4OH with water at 1:2.11 (v/v).
6. Dimethylformamide (DMF).
7. 10 μM NBD-S1P in Buffer A containing 0.1% BSA. Transfer 50 μg of NBD-S1P dissolved in chloroform/methanol (2:1) to a 1.5 mL glass vial and evaporate the solvent. Add 897 μL of Buffer A containing 1% BSA to the vial and dissolve the NBD-S1P by vortexing and sonicating at 37 °C to make a 100 μM NBD-S1P solution. Dilute this solution to 10 μM NBD-S1P by using Buffer A in a 1.5 mL glass vial and store the solution at −20 °C. To quantify NBD-S1P in cells, prepare a 10 μM NBD-S1P solution containing erythrocyte lysate. Centrifuge washed erythrocytes, remove the supernatant, and lyse the cells by pipetting with water at a concentration of 2.78×10^7 cells/mL. Dilute the 100 μM NBD-S1P solution with the cell lysate instead of Buffer A.
8. Black 96-well plate.
9. Fluorescence plate reader.

3 Methods

Perform all the procedures at room temperature unless otherwise indicated.

3.1 Preparation of Erythrocyte Suspension

1. Take up 100 μL of ACD solution into a syringe and attach a needle. Fill the needle with ACD solution. Anesthetize a female Wistar rat. Insert the needle into the rat tail vein and collect approximately 300 μL of blood (*see* **Note 4**). Gently mix the blood and ACD solution by inverting. Remove the needle from the syringe and transfer the blood into a 1.5 mL tube.
2. Centrifuge at 500 × *g* for 15 min at 20 °C (*see* **Note 5**).
3. Remove the upper and middle layer with a micropipette.
4. Transfer 50 μL of the lower layer containing erythrocytes to a new 1.5 mL tube by using a wide-pore tip.
5. Add 1 mL of Buffer A containing 1% BSA.
6. Mix gently by inverting.
7. Centrifuge at 500 × *g* for 10 min at 20 °C. Remove the supernatant.
8. Wash the erythrocytes by repeating steps 5 through 7.

9. Wash the erythrocytes with 1 mL of Buffer A containing 0.1% BSA.
10. Resuspend the erythrocytes in 1 mL of Buffer A containing 0.1% BSA.
11. Mix gently by inverting.
12. Dilute 2 μL of the erythrocyte suspension in 1 mL of Buffer A. After adding the same volume of Trypan blue solution to the diluted cell suspension, perform a cell count.
13. Prepare the erythrocyte suspension (50 μL per sample) at a density of 1×10^8 cells/mL by diluting the samples with Buffer A containing 0.1% BSA.

3.2 NBD-S1P Release Assay in Rat Erythrocytes

1. Prepare 200 μL of chloroform/methanol solution (1:1) in a 1.5 mL Safe-lock tube prior to the assay.
2. Prepare NBD-S1P release buffer (50 μL per sample).

Components	Volume (μL)
1 mM NBD-Sphingosine (*see* **Note 6**)	0.5
Buffer A containing 0.1% BSA	49.5
Total volume	50

3. Preincubate the erythrocyte suspension and NBD-S1P release buffer for 10 min at 37 °C.
4. Add NBD-S1P release buffer to the erythrocyte suspension.
5. Mix gently by pipetting, inverting, or brief vortexing.
6. Incubate the cells for the required time at 37 °C in a water bath (*see* **Note 7**).
7. Centrifuge the cell suspension (100 μL per sample) at 17,000 × *g* for 5 s at 4 °C.
8. Carefully transfer 100 μL of the supernatant to a 1.5 mL Safe-lock tube containing 200 μL of chloroform/methanol solution (1:1).
9. Add 200 μL of ice-cold 0.1× Buffer A containing 0.1% BSA to the erythrocyte pellet and lyse the cells by pipetting.
10. Transfer 100 μL of cell lysate to a 1.5 mL Safe-lock tube containing 200 μL of chloroform/methanol (1:1).
11. Vortex both tubes containing supernatant and cell lysate.

3.3 Lipid Extraction and Fluorometry

1. Prepare the NBD-S1P standards (0, 50, 100, 200, and 400 pmol) by adding 0, 5, 10, 20, and 40 μL, respectively, of 10 μM NBD-S1P solution and Buffer A containing 0.1% BSA (volumes in table below) to 200 μL of chloroform/methanol

solution (1:1) in a 1.5 mL Safe-lock tube (Fig. 4). To quantify the NBD-S1P in cells, prepare the NBD-S1P standards by using the 10 μM NBD-S1P solution containing erythrocyte lysate (Fig. 4) (*see* Sect. 2.3).

NBD-S1P (pmol)	0	50	100	200	400
10 μM NBD-S1P solution (μL)	0	5	10	20	40
Buffer A containing 0.1% BSA (μL)	100	95	90	80	60

2. Add 8 μL of 7 M NH_4OH solution to the mixture containing 200 μL of chloroform/methanol (1:1) and 100 μL of supernatant, cell lysate or NBD-S1P standards in a 1.5 mL Safe-lock tube.
3. Mix well by vortexing.
4. Add 200 μL of chloroform.
5. Mix well by vortexing.
6. Separate the aqueous and organic phases by centrifugation at >14,000 × *g* for 5 min at room temperature.
7. Transfer 80 μL of the upper (aqueous) phase to a black 96-well plate (*see* **Note 8**).

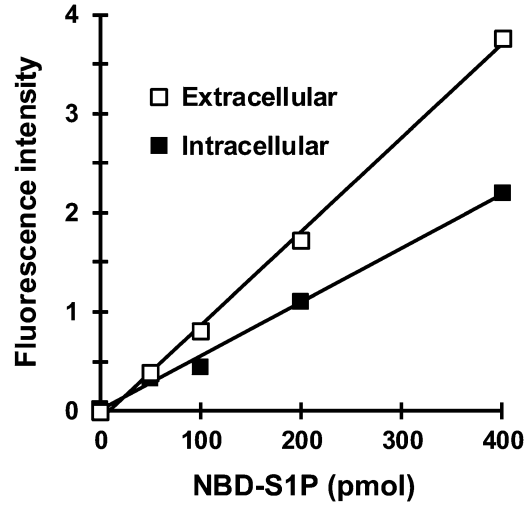

Fig. 4 Typical standard curves for extracellular and intracellular NBD-S1P. Assay buffers containing known amounts of NBD-S1P were applied to lipid extraction, and fluorescence of the resulting aqueous phases was measured (see Sect. 3.3). For the preparation of a standard curve for intracellular NBD-S1P, NBD-S1P was added to the erythrocyte lysates. The erythrocyte lysates attenuated NBD-S1P fluorescence intensity

8. Add 20 μL of dimethylformamide to the upper (aqueous) phase in a black 96-well plate and mix by pipetting.
9. Set the black 96-well plate in a fluorescence plate reader and mix briefly by using the mixer function of the plate reader.
10. Measure the fluorescence of NBD-S1P (excitation: 490/485 nm, emission: 530/538) (*see* **Note 9**).

3.4 Direct Detection of NBD-S1P Transport Without Lipid Extraction

1. Conduct the NBD-S1P release assay as described in Sect. 3.2, steps 1–7 except that the volume of NBD-S1P release buffer and erythrocyte suspension are doubled, and the incubation times are 1 and 2 h.
2. After centrifugation of the cell suspension, transfer 200 μL of supernatant to a black 96-well plate.
3. Measure the fluorescence of the supernatant by using a fluorescence plate reader (excitation: 490/485 nm, emission: 530/538).

4 Notes

1. Prepare the solution in a 50 mL conical tube and dissolve reagents by vortexing.
2. Prepare the buffer in a 15 mL conical tube and completely dissolve the BSA by shaking at room temperature.
3. Prepare 1.5 mL Safe-lock tubes containing 200 μL of chloroform/methanol solution (1:1) prior to the NBD-S1P release assay. The required number of tubes is the sum of the samples and NBD-S1P standards.
4. The tail vein is present on the right side and left side of the rat tail. Wipe the tail vein with a laboratory wipe soaked in 70% ethanol. Compress the base of the tail vein between two fingers. Insert a needle into a location 5 cm away from the base of the tail vein. Slowly pull the plunger of the syringe.
5. Use a swing-rotor and set the brake of the centrifuge to off.
6. The final concentration of NBD-sphingosine is 5 μM after the addition of NBD-S1P release buffer to the erythrocyte suspension.
7. Gently mix the cells by inverting or brief vortexing every 10 min.
8. Quickly add the samples to the black 96-well plate, because the upper phase contains volatile solvents and will drip from the pipette tip.

9. The fluorescence of the samples should be measured at room temperature (>20 °C) because white precipitates form easily in the samples at low temperatures.

Acknowledgement

This work was supported by JSPS KAKENHI (grant number 25870695 to N. K.) and by the Cooperative Research Program of the "Network Joint Research Center for Materials and Devices" and AMED-CREST (to N. K. and T. N.).

References

1. Kobayashi N, Kobayashi N, Yamaguchi A, Nishi T (2009) Characterization of the ATP-dependent sphingosine 1-phosphate transporter in rat erythrocytes. J Biol Chem 284 (32):21192–21200. doi:10.1074/jbc.M109.006163
2. Kobayashi N, Otsuka M, Yamaguchi A, Nishi T (2016) Fluorescence-based rapid measurement of sphingosine-1-phosphate transport activity in erythrocytes. J Lipid Res 57(11):2088–2094. doi:10.1194/jlr.D071068
3. Hisano Y, Kobayashi N, Kawahara A, Yamaguchi A, Nishi T (2011) The sphingosine 1-phosphate transporter, SPNS2, functions as a transporter of the phosphorylated form of the immunomodulating agent FTY720. J Biol Chem 286 (3):1758–1766. doi:10.1074/jbc.M110.171116
4. Hisano Y, Kobayashi N, Yamaguchi A, Nishi T (2012) Mouse SPNS2 functions as a Sphingosine-1-phosphate transporter in vascular endothelial cells. PLoS One 7(6):e38941. doi:10.1371/journal.pone.0038941

Methods in Molecular Biology (2017) 1697: 83–97
DOI 10.1007/7651_2017_24

Published online: 23 April 2017

Analysis of S1P Receptor Expression by Uterine Immune Cells Using Standardized Multi-parametric Flow Cytometry

Jianhong Zhang, Annie Bang, and Stephen J. Lye

Abstract

Flow cytometry is a powerful tool for phenotypic and functional analyses of single immune cells. The increasing capability of flow cytometry technology has driven a more detailed understanding of immune cell subsets and functions in complex cellular systems such as the developing human decidua/placenta. We propose a standardized procedure for the isolation and analysis of human decidual natural killer (dNK) cells and this method can be extended to investigation of other uterine lymphocytes. Here this platform is used to examine the expression of sphingosine-1-phosphate (S1P) receptor and functional growth factors by dNK cells.

Keywords: CD45+ cells, Decidua, Decidual natural killer (dNK) cells, Human pregnancy, Sphingosine-1-phosphate

1 Introduction

Sphingosine-1-phosphate (S1P) is a lipid mediator presented in a variety of species and cell types. The regulation and function of S1P are tightly linked to many physiological and pathophysiological processes, including cancer, atherosclerosis, diabetes, and osteoporosis. Microenvironmental S1P distribution has critical effects on the expression of five S1P receptors (S1PR1-5) [1], which can facilitate the egress of lymphocytes from lymphoid organs into blood and lymphatic fluid. The responsiveness of immune cells to S1P signalling is dependent on their subtypes and activation status [2–4]. Here we demonstrate that the S1PR5-dependent response of decidual natural killer (dNK) cells in human pregnancy can be quantified using a standardized flow cytometry assay.

Flow cytometry is capable of characterizing many features of individual cells in a complex mixture [5, 6]. The accurate measurement of variations in the interaction between human immune system and S1P signalling requires precise and optimized methods to distinguish true biological changes from technical artifacts. Furthermore, biomedical studies using human pregnant specimens require longitudinal assays over a considerable time period needed to recruit suitable subjects. Thus, the standardization of reagents and

protocols becomes crucial particularly when dealing with samples from the uterine lining (endometrium or decidua) that develops heterogeneous texture and cellular components. We proposed a standard procedure which is applicable to multi-parameter flow cytometry technique to study the molecular signature of S1P receptors. The investigation is established on the basis of appropriate isolation, staining, and analysis of innate immune cells in human uterine samples [7, 8]. Meanwhile, this protocol provides a general framework for additional applications. It can be revised and adapted for other biomedical experiments targeting the expression and function of different markers in either lymphoid or non-lymphoid tissue.

2 Materials

This protocol requires handling of freshly isolated human tissue. All components including sample collection, transportation, operation, and disposal must be conducted in compliance with biosafety control policy. Always use freshly prepared working buffers. Store all reagents at room temperature unless otherwise indicated. Sterile instruments and supplies are required.

2.1 Samples Collection and Dissociation

1. A biosafety cabinet.
2. Human decidual samples from early term pregnancy are requested and collected according to the institutional protocol.
3. Hanks' Balanced Salt Solution without calcium and magnesium (HBSS−/−) is used to wash samples and cells before or during digestion steps. Store at 4 °C.
4. EDTA solution (1 M; Invitrogen). Store at 4 °C.
5. HEPES solution (1 M; Invitrogen). Store at 4 °C.
6. Prepare 1 M stock DTT: Weigh 1.54 g of Dithiothreitol (DTT). Add 10 mL of sterile dH_2O. Dissolve completely. Sterilize DTT stock through a 0.22 μm syringe filter. Aliquot into 1.5 mL Eppendorf tubes and store at −20 °C.
7. Predigestion DTT buffer: Add 0.5 mL DTT stock (1 M) into 500 mL RPMI 1640 medium. Store at 4 °C.
8. Digestion solution: For 500 mL HBSS−/− buffer, add 0.5 mL DTT stock (1 M), 1.0 mL EDTA (1 M, pH = 7.6), and 12.5 mL HEPES (1 M). Store at 4 °C (*see* **Note 1**).
9. Ficoll-Paque Plus (GE Healthcare). Store at room temperature.
10. A pair of curved operating scissors. The curved blades enable precise trimming and cutting tissue against the surface petri dishes.
11. Corning® cell strainers (40 and 70 μm).

2.2 Functional Assay

1. Penicillin-Streptomycin stock (Lonza) which contains 10,000 units potassium penicillin and 10,000 μg streptomycin sulfate in per mL solution. Store at −20 °C.
2. Complete RPMI 1640 medium (Invitrogen): Add 50 mL heat inactivated FBS and 5 mL Pen/Strep stock into 500 mL RPMI 1640 medium. Store at 4 °C.
3. HBSS+/+ buffer with calcium and magnesium is used to wash samples and cells after digestion. Store at 4 °C.
4. Fingolimod (FTY720 or hydrochloride; Cayman Chemical): a potent agonist at four of the S1P receptor (S1PR1/2/3/5). Stock solution (5 mM) was prepared by diluting 1 mg FTY720 in 0.5816 mL DMSO. Aliquot and store at −20 °C.
5. Cell Stimulation Cocktail (plus protein transport inhibitors) of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin (eBioscience). Store at −20 °C.

2.3 Immune Staining

1. Variety of antibodies (see Table 1 for the list). For optimal performance of fluorochrome-conjugated antibodies, store vials at 4 °C and do not freeze. Protect from light. In order to recover the maximum volume, quickly centrifuge the antibody vials prior to use.
2. Blocking solution (Dako). Store at 4 °C.
3. BD™ 3× Concentrate stabilizing fixative. Prepare the fixative working buffer by mixing 1 part of BD™ 3× Concentrate with 3 parts room temperature (20 °C) deionized water.
4. LIVE/DEAD fixable dead cell stain kit (Invitrogen). Add 50 μL of DMSO to the vial of reactive dye. Mixed well and make sure all of the dye has dissolved. Aliquot 1 μL of dye using 1.5 mL tubes. Store stock at −20 °C.
5. BD Cytofix/Cytoperm™ solution (BD Biosciences). Store at 4 °C.
6. BD Perm/Wash™ Buffer (10×) (BD Biosciences). Store at 4 °C. Prior to use, dilute it to 1× solution by adding 9 parts of distilled water.
7. BD Stabilizing Fixative solution (BD Biosciences). Store at 4 °C. Prior to use, dilute it to 1× solution by adding 3 parts of distilled water.
8. 5 mL BD Falcon tubes (BD Biosciences) with cell trainer cap.

2.4 Flow Cytometry Analysis

1. 10-Color Gallios flow cytometer (Beckman Coulter) with blue/yellow (488/561 nm collinear), red (638 nm), and violet (405 nm) lasers.
2. Flow-Set™ Fluorospheres (Beckman Coulter). Store at 4 °C.
3. BD™Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (as CompBeads) (BD Biosciences). Store at 4 °C.

Table 1
Staining panel of multicolor flow cytometry (see Note 12)

Fluorochrome parameter	Antigen type	Antibody/ fluorochrome	Manufacturer	Vol. (μL)	Control tubes[a]									Test tubes[b]	
					0	1	2	3	4	5	6	7	8	a	b
FL1	Intracellular	IFNG-FITC	BD Biosciences	8	−	−	+	−	+	+	+	+	+	+	+
FL2	Surface	S1PR5-PE	R&D System	8	−	+	−	+	+	+	+	+	+	+	+
FL5	Surface	CD56-PE/ Cy7	BD Biosciences	1	−	+	+	−	+	+	+	+	+	+	+
FL6	Intracellular	VEGF-APC	R&D System	8	−	+	+	+	−	+	+	+	+	+	+
FL7	Surface	CD3-Alexa Fluor 700	BD Biosciences	4	−	+	+	+	+	−	+	+	+	+	+
FL8	Surface	CD45-APC/ Cy7	BD Biosciences	4	−	+	+	+	+	+	−	+	+	+	+
FL9	Both	Live/dead-violet	Invitrogen	2	−	+	+	+	+	+	+	−	+	+	+
FL10	Surface	CD16-Krome Orange	BD Biosciences	5	−	+	+	+	+	+	+	+	−	+	+

[a]Tube #0 is an unstained control; tubes #1–8 are FMO controls for individual fluorochrome parameter
[b]When examining multiple tubes, we suggest preparing an antibody cocktail (a tube containing a mixture of all testing antibodies) and dispensing equal volume of antibody mixture to individual testing tubes

3 Methods

All work that is subject to the biosafety guidelines must be conducted under the appropriate standard operating procedure. The general experimental procedure is summarized in a flowchart with time frame (Fig. 1). Freshly collected human decidual samples from early or later gestation stage can be used.

3.1 Prepare Single Cell Suspension

1. Upon arrival, visually examine the decidual sample in a 100 mm × 15 mm petri dish. Remove attached fetal membranes and associated placental tissue. Wash thoroughly with ample cold HBSS−/− until liquid is blood free (*see* **Note 2**).
2. Remove remaining HBSS−/− by vacuum and add 10 mL RPMI/DTT buffer.
3. Manually cut tissue into smaller pieces using curved operating scissors. Ideal size of fragments is approximately 1 mm^3.
4. Transfer sample into a 50 mL falcon tube and add RPMI/DTT buffer to 25 mL. Incubate 15 min at room temperature to remove mucus.

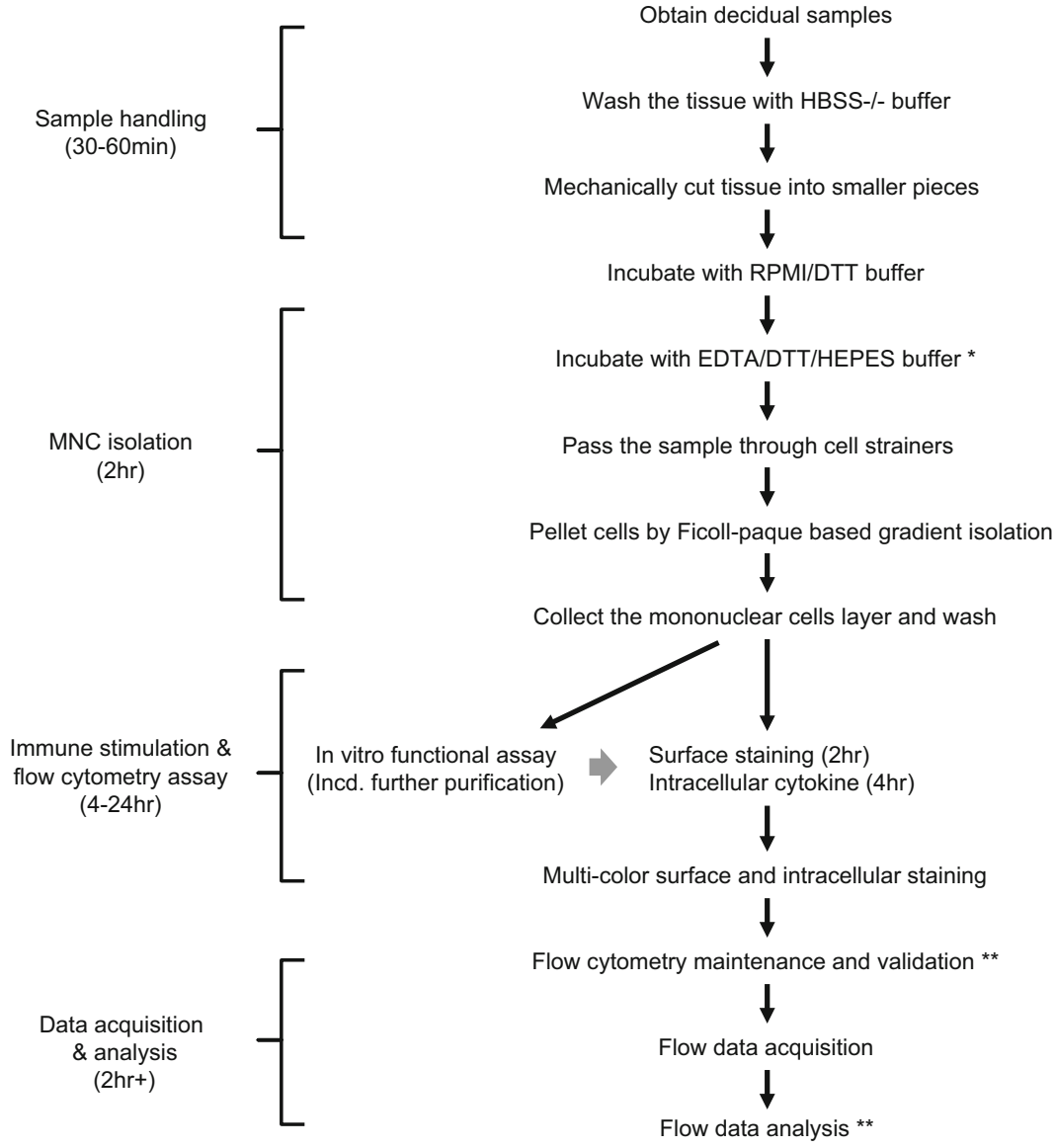

Fig. 1 A workflow for the analysis of dNK response to S1P signalling and timing scale to carry out the experiment. *, A critical step to obtain uterine immune cell suspension. *MNC* mononuclear cells. **, Key steps to standardize the protocol

5. Centrifuge at 500 × *g* for 5 min at room temperature to pellet tissue and cells. Discard the supernatant.
6. Resuspend the cell pellet with 25 mL pre-warmed digestion solution. Shake (140/min) at 37 °C (water bath) for 30 min to dissociate the primary cells (*see* **Note 3**).
7. Vortex the tube at maximum speed for 30 s. Separate isolated cells from remaining tissue using a 70 μm cell strainer to obtain uniform single-cell suspension (*see* **Note 4**).

8. Pipette cell suspension through a 40 μm cell strainer.
9. Centrifuge at 500 × *g* for 5 min 4 °C to pellet cells (*see* **Note 5**).
10. Resuspend cells with 4 mL HBSS+/+ and carefully layer on the top of 4 mL Ficoll solution in 15 mL falcon tubes (*see* **Note 6**).
11. Centrifuge at 800 × *g* for 30 min at 20 °C to pellet cells (brake should be off).
12. Collect the buffy coat with a pipette and transfer it to a new set of falcon tubes. Wash cells with 10 mL HBSS+/+ and centrifuge the tubes at 800 × *g* for 10 min, 4 °C (*see* **Note 7**).
13. Resuspend cell pellet in 15 mL RPMI1640 and centrifuge at 800 × *g* for 5 min, 4 °C.
14. Wash cells again with 15 mL HBSS+/+ buffer and pelleted cells are ready for further study (*see* **Note 8**).

3.2 Cell Stimulation

1. Isolated decidual leukocytes/dNK cells are suspended with 1 mL RPMI1640 with 10% FBS. Incubate in a 24-well plate.
2. For experimental wells, add 1 μL stock solution of FTY720 (5 mM). Incubate at 37 °C for 1.5 h.
3. Add 2 μL cell stimulation cocktail in each wells and incubate another 4 h.
4. Cells are harvested and washed with HBSS+/+ buffer at 800 × *g* for 5 min at 4 °C.

3.3 Surface Marker Staining

1. Preparing compensation tubes for flow cytometer optimization (*see* **Note 9**).
   (a) Vortex BD™ CompBeads thoroughly before use.
   (b) Mix 3 full drops (approximately 360 μL) of each CompBeads in a 1.5 mL tube. Add 600 μL HBSS+/+ and mix it well.
   (c) Pipette 2 μL of antibody to be tested in the experiment into 1.5 mL tubes, separately. Add 100 μL above CompBeads solution into each tube.
   (d) Incubate 30 min at 4 °C. Protect from light.
   (e) Wash with HBSS+/+ buffer and centrifuge at 500 × *g*, 5 min, 4 °C.
   (f) Resuspend pellets with 200 μL fixative staining buffer.
2. Preparing a viability test tube.
   (a) Remove the LIVE/DEAD® reagent stock solutions from the freezer and warm to room temperature.
   (b) Dilute stock solution of LIVE/DEAD® reactive dye by adding 99 μL HBSS+/+ into a stock tube. Mix it well (*see* **Note 10**).

(c) Prepare a 1.5 mL tube with combination of live cells and heat-killed dead cells (approximately 1:1). Dead cells are obtained by keeping a small aliquot of cells in a 65–70 °C water bath for 10 min.

(d) Add 2 μL diluted LIVE/DEAD work solutions in the tube and mix well.

(e) Incubate the tubes for 30 min at 4 °C.

(f) Wash with 1 mL HBSS+/+. Centrifuge at 500 × *g* for 5 min, 4 °C. Resuspend pellets with 200 μL fixative staining buffer.

3. Aliquot 100 μL of cell suspension (1×10^5 or more) in multiple 1.5 mL Eppendorf tubes according to the experimental design (Table 1) (*see* **Note 11**).
4. Add 2 μL of diluted LIVE/DEAD dye into each test tube. Incubate 30 min at 4 °C. Protect from light (*see* **Note 13**).
5. Wash cells with 1 mL HBSS+/+ buffer and centrifuge at 800 × *g* for 5 min, 4 °C.
6. Resuspend cells in 100 μL Dako Blocking Solution. Incubate 30 min at 4 °C.
7. Add primary antibodies in an appropriate volume to all control and test tubes (Table 1). Incubate 30 min at 4 °C. Protect from light (*see* **Note 14**).
8. Wash cells with 1 mL HBSS+/+ buffer and centrifuge at 800 × *g* for 5 min, 4 °C (*see* **Note 15**).

3.4 Intracellular Cytokine Staining

1. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet (*see* **Note 16**).
2. Fix the cells by adding 200 μL of BD Cytofix/Cytoperm™ buffer per tube and vortex to mix. Incubate 30 min at 4 °C. Protect from light.
3. Wash cells with 1 mL 1× BD Perm/Wash™ buffer and discard the supernatants (*see* **Note 17**).
4. Thoroughly resuspend fixed/permeabilized cells in 50 μL 1× Perm/Wash™ Buffer.
5. Add intracellular staining antibodies of mouse anti-human IFNG and VEGF. Incubate at 4 °C for 30 min.
6. Wash cells twice with 1 mL 1× BD Perm/Wash™ buffer.
7. Resuspend cells with 200 μL 1× BD Stabilizing Fixative and transfer to 5 mL BD round-bottom tubes with cell-strainer cap.
8. Store in dark at 4 °C until flow cytometric analysis (analysis should be conducted within 1 day).

3.5 Flow Cytometric Data Acquisition

1. Perform instrument start-up and verification check following the manufacturer's recommendations (*see* **Note 18**).
2. Dispense 5 drops (about 0.2 mL) of Flow-Set fluorospheres into a test tube.
3. Vortex and aspirate the fluorospheres sample with a Flow-Set acquisition protocol created previously. Adjust cytometer's settings to place each fluorospheres peak within the previously determined reference range (Fig. 2). When necessary, adjust the PMT voltages if the positive signals are off scale or if the negative population is too high.
4. Load compensation tubes in a carousel and initiate the proper acquisition protocol. Run each compensation tube separately.
5. Create a working protocol for sample acquisition by adjusting the compensation values of both positive and negative populations (*see* **Note 19**).
6. Proceed to acquiring the actual experimental tubes using the working protocol created in **step 5**.
7. Save data for offline analysis.

3.6 Offline Data Analysis

1. Depending on the complexity of each experiment, it can take as much time to analyze it as took to acquire the data. Flow data are saved as standard fcs files and multiple software are available to conduct data analysis. We use Kaluza Software (Beckman Coulter)—which is custom designed for Gallios flow cytometer, to analyze data by a manual gating strategy (*see* **Note 20**).
2. Visually inspect the time plots and exclude unstable flow of cells by time gating.
3. Singlet cells are gated based on FSC/SSC vs. TOF plots (Fig. 3a, b). Then lymphocytes are gated for further analysis (Fig. 3c).
4. Dead cells may compromise flow cytometric data analysis by non-specific antibody binding; therefore it is important to exclude dead cells from target population. CD56+CD3-CD16 + NK cells are gated from viable CD45+ population (Fig. 3).
5. Fluorescence-minus-one (FMO) controls can be used to identify the spillover to the background in a given channel [9]. The positively vs. negatively stained NK cells were classified by FMO control and distributional similarity (Fig. 4) (*see* **Note 21**).
6. The standardized gating, composition, and layout of a single analysis can be copied and pasted to all samples, with minor adjustment between groups or batch studies.
7. A higher resolution and more advanced flow data analysis can be achieved by referencing other publications [10, 11].

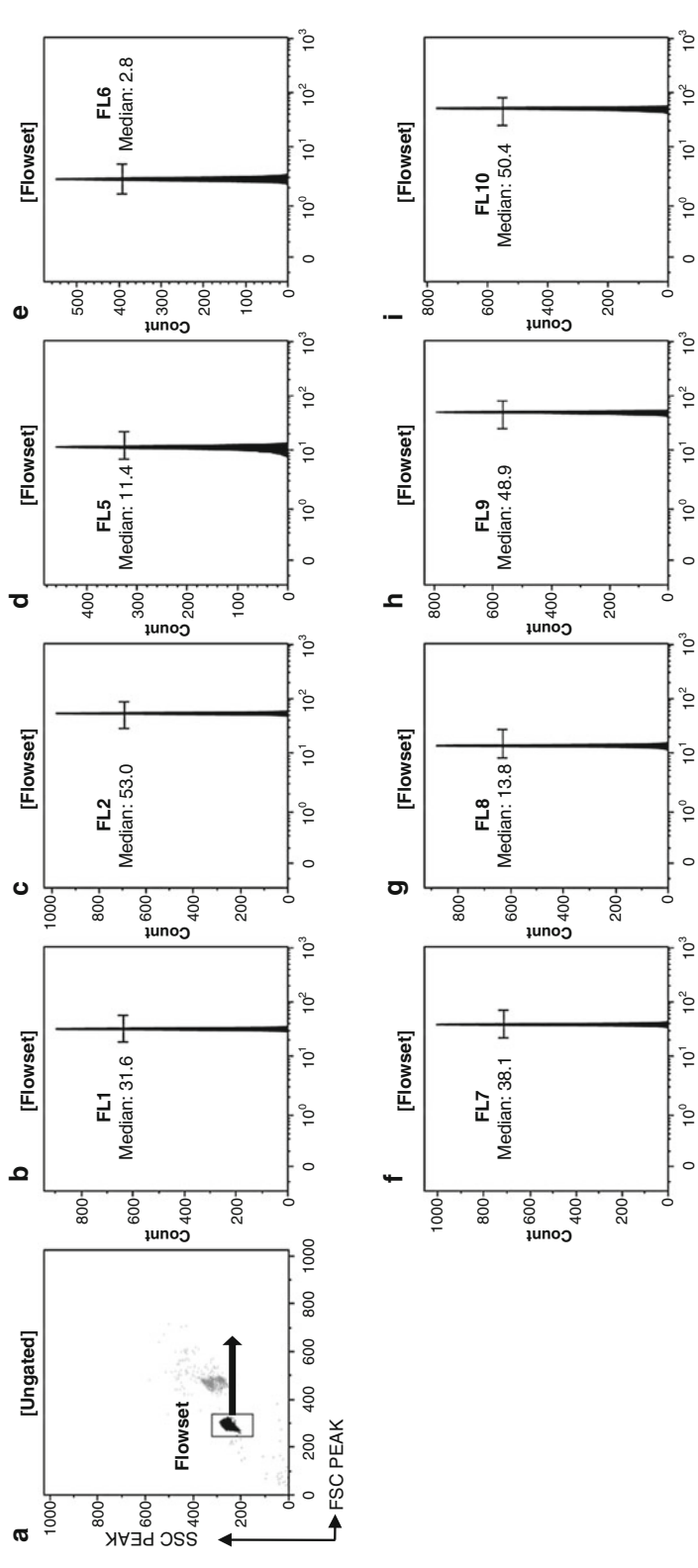

Fig. 2 The standardized flow cytometer setup using Flow-Set™ Pro fluorospheres. To ensure consistency and reliability of flow results over time, fluorescent microbeads (Beckman Coulter) were used to optimize the Gallios flow cytometer for quantitative analysis of human uterine leukocytes. These beads (**a**) have a uniform size and fluorescence intensity allowing quality control of optical alignment, light scatters, fluorescence intensity, and optimal hydrodynamic focusing in different channels (**b–i**). Minimum 5,000 bead events are acquired at low rate. Median fluorescence intensity of each detector is calculated and used for instrumental calibration

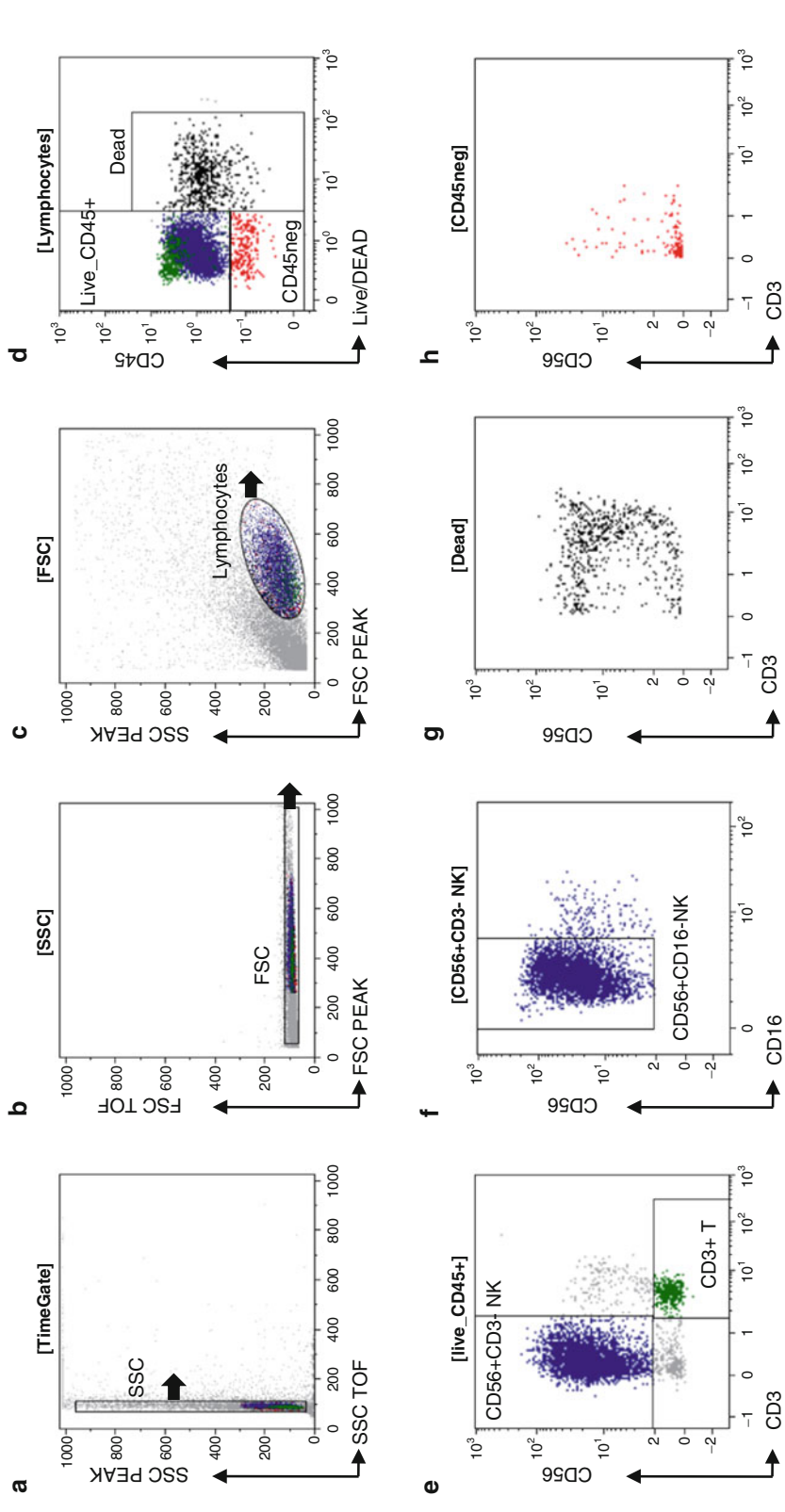

Fig. 3 Prime CD45 and live/dead staining is necessary to accurately characterize decidual immune cells using flow cytometry. Representative results were obtained from an early pregnant decidual sample. Back gating is used to inspect cells of interest in the final population. The stability of the data acquisition was examined by time gating. (**a**, **b**) Singlets were gated out by the combination of SSC and FSC with time-of-flight (TOF). (**c**) Cells of interest—lymphocytes, were gated for further analysis. (**d**–**h**) A combination of CD45 and live/dead staining revealed that a small portion of dead events and/or CD45 negative cells presented in the "lymphocytes" gating. Eliminating these unwanted particles can produce a reliable and accurate result for lymphocyte profiling, particularly for the decidual/endometrial specimens with unique anatomical textures and immunological features

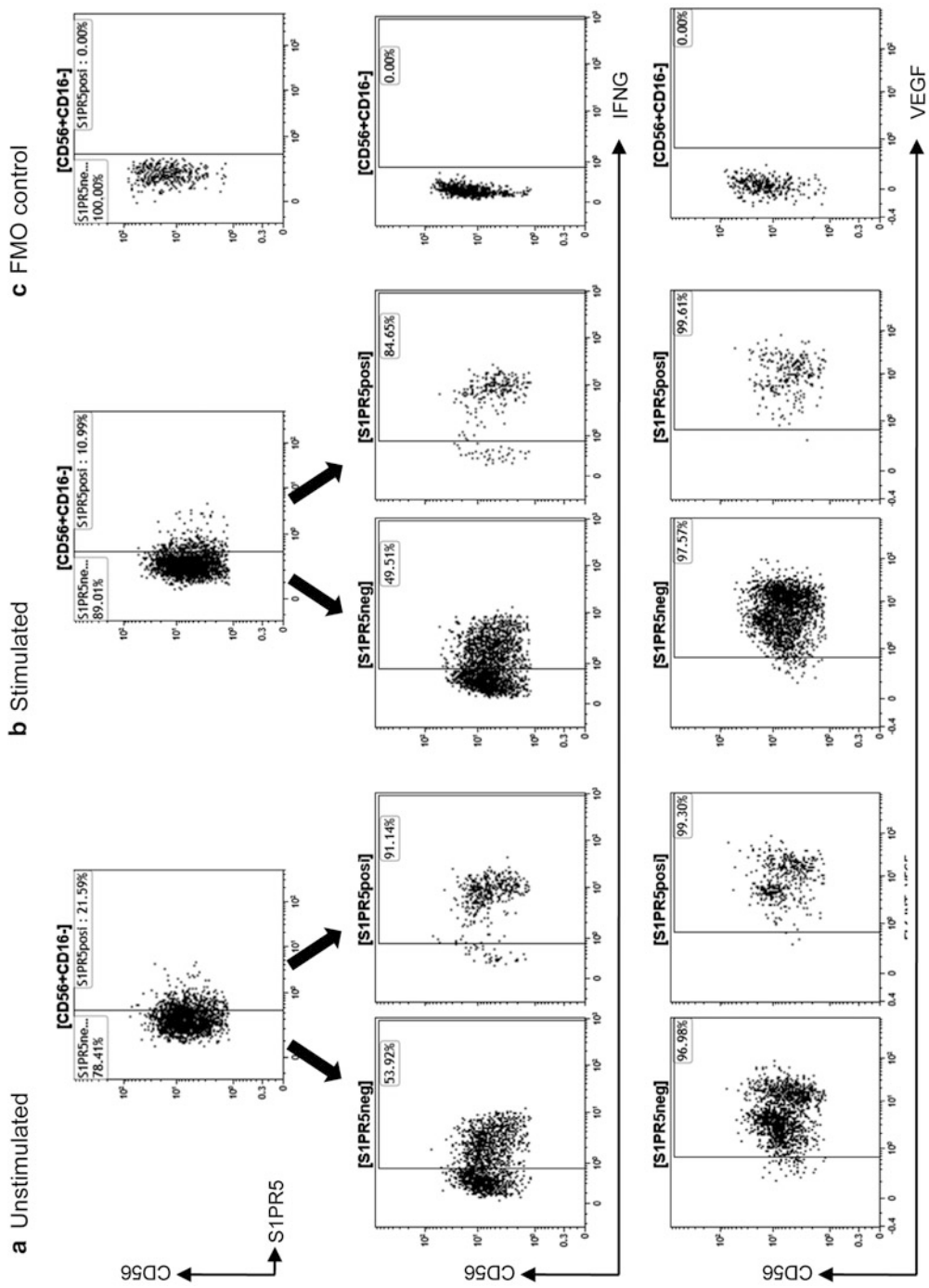

Fig. 4 Decidual NK cell function is associated with S1PR5 expression. Decidual cells from a 14 weeks pregnancy were isolated and stimulated with or without S1PR agonist (Fingolimod) (**a**, **b**). Cells were harvested and stained with surface markers and intracellular cytokines. Viable CD45+CD3-CD56+CD16- NK cells were gated to examine their S1PR5 expression and associated IFNG or VEGF expression. FMO controls for S1PR5-PE, IFNG-FITC, and VEGF-APC were included (**c**)

8. This is an operable protocol for clinical decidual/placental samples collection and data analysis over long periods of biomedical studies. The method has some limitations. For instance, in comparison to the developing CyTOF platform that can readout 20–40 parameters [12, 13], conventional flow cytometry assay has certain limitation on its capacity.

4 Notes

1. Freshly prepared digestion buffer enables optimal cell isolation. Our experience indicates that an ideal cell yield can be reached using buffer stored at 4 °C for 1 week.
2. Human decidual materials from early pregnancy can be obtained following informed consent from healthy women undergoing elective termination of pregnancy. These specimens are able to be visually distinguished from attached placenta or other fetal tissues, which should be removed prior to further processing. For term pregnancy samples, the decidual layer can be scrapped from the chorioamniotic membrane using a disposable sterile cell scraper. A dissecting microscope may help visual inspection.
3. For efficient timing, the water bath should be switched on upon the arrival of human samples as it may take a while to reach stable temperature (37 °C). To prevent cross contamination or infection, appropriate measures such as adding disinfectant to the water bath should be taken. We do not suggest using a dry incubator/chamber because in comparison to water, air has very lower thermal conductivity, which may undermine sample digestion.
4. Cell strainer is a sterile sieving device to obtain real single-cell suspensions or to remove large cell aggregates. To improve working efficiency, in the absence of built-in venting slot, the strainer should be carefully lifted above the tube to let air leave the tube while the filtrated solution flows through the mesh.
5. To maximize the cell yield, decidual clumps can be collected from the top of the strainers and the dissociation procedure (**steps 6–9**) repeated.
6. It is very important when layering the sample not to allow mixing between the Ficoll-Paque media solution and the diluted sample. If done correctly, the Ficoll should remain as a clear layer on the bottom of the tube with the decidual sample as a separate layer on top. An alternative procedure is to load the Ficoll using a long needle to the bottom of the sample solution.

7. When necessary, add 5 mL 1× Erythrocyte Lysis Buffer for 5 min on ice and vortex occasionally. Then wash and centrifuge to remove the excessive red blood cells.
8. The goal of a cell isolation procedure is to maximize the yield of functionally viable and dissociated cells. Human decidual/placental samples have unique textures and cellular compositions. Thus, the final cell yield may vary according to different individuals. Our experience indicates that using this standardized method, 2–10 million mononuclear cells can be isolated from a single sample.
9. This work can be done simultaneously with sample staining. As LIVE/DEAD® viability assay is based on the reaction of a fluorescent reactive dye with cellular amine groups, the compensation tube for this particular channel was prepared using primary cells rather than CompBeads.
10. It is highly recommend to use freshly prepared LIVE/DEAD work solutions for each set of experiments. The signal intensity of reconstituted viability dye is degraded after overnight storage at 4 °C. Do not use previously frozen dye solution.
11. This is a key component of experimental design. The flow cytometry staining panel is constructed on the condition of: (a) A flow cytometer—either Gallios cytometer or other candidate instrument, is selected and standard optical configurations are performed; (b) Appropriate selection of combinations of fluorochromes and antibody conjugates that minimizes compensation and spillover adjustments. Please refer to online resources for the visualization of spectral overlap of fluorophores; (c) Optimized loading volume of each antibody is been titrated according to given experimental conditions; (d) Pilot experiments have been performed to establish a standard acquisition protocol.
12. With increasing reagents and pipetting steps, extra caution is required to avoid human error. Clear labelling, neat layout and undivided attention can greatly minimize or eliminate potential mistakes.
13. The LIVE/DEAD dye we use is amino-reactive, thus the labelling tubes and contents should be free of extraneous proteins that may result in unspecific binding. Washing cells with HBSS or PBS buffer can eliminate these proteins.
14. The fixation and permeabilization of cells can compromise cell surface antigens. It is advised to perform the surface staining first. At this staining step, we add in CD45-APC/CY7, CD56-PE/CY7, CD16-Krome Orange, CD3-Alexa700, and S1PR5-PE.
15. If no intracellular cytokine staining is required, cells can be resuspended in 200 μL BD stabilizing fixative buffer and proceed with flow cytometry analysis.

16. Before adding fixative buffer, cells should be vortexed gently to ensure good penetration of the fixative and reduce cell clumping.
17. After fixative treatment, the cells may turn lighter and become slippery. To improve cell recovery, centrifuge force should be increased to 1,200 × *g* or more. Aspiration of the supernatant with vacuum suction may help to preserve the cells.
18. The hardware and software may vary from cytometer to cytometer. Investigators are free to choose any instrument for their data acquisition under the premise that data accuracy and sensitivity are ensured.
19. Compensation is a critical process for an unbiased analysis of flow cytometry data and becomes increasingly difficult to perform manually as the number of fluorescence parameters increases [14]. We perform computer assisted compensation by using Kaluza software (Beckman Coulter) to build a spillover matrix.
20. Stimulation, fixation, and permeabilization will considerably change the light scatter profiles of immune cells. When necessary, adjust the cell gates with the assistance of control tube and back gating. The implementation of optimized logical transformation is adequate to identify significant features and improve visual effects of the data [15]. Appropriate adjustment of biexponential scaling is important to clearly and completely visualize all populations, particularly for intracellular cytokine staining. For more complex examination of S1P signalling, computational assistance analysis could be an alternative only after critical assessment and verification [16, 17].
21. S1PR expression is sensitive to microenvironmental S1P concentration [18, 19]. Immune cells isolated from different tissues can be used to verify the expression pattern of S1PR signals. In comparison to NK cells, resting T cells have lower expression of S1PR5 [3]. Thus, to identify the S1PR5 positive population, decidual CD3+ T cells can be used as a reference. An alternative method is to include a PBMC sample, T-cell line (Jurkat), NK-cell line (NK92), or monocytic cell line (U937) that have little S1PR5 reactivity [20].

Acknowledgement

This study was supported by Canadian Institutes of Health Research (CIHR) grants (MOP82811, MOP130550, and FDN-143262) to Dr. S. J. Lye. We thank Dr. B. Anne Croy (Queen's University) for her critical review and helpful advice. We thank the donors, the Research Centre for Women's and Infants' Health BioBank Program of Lunenfeld-Tanenbaum Research Institute (LTRI) and the Sinai Health System/University Health Network (Toronto, Canada) for providing human specimens.

References

1. Yanagida K, Hla T (2017) Vascular and immunobiology of the circulatory sphingosine 1-phosphate gradient. Annu Rev Physiol 79. Doi: 10.1146/annurev-physiol-021014-071635
2. Blaho VA, Hla T (2014) An update on the biology of sphingosine 1-phosphate receptors. J Lipid Res 55:1596–1608
3. Walzer T, Chiossone L, Chaix J, Calver A, Carozzo C, Garrigue-Antar L, Jacques Y, Baratin M, Tomasello E, Vivier E (2007) Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. Nat Immunol 8:1337–1344
4. Jenne CN, Enders A, Rivera R, Watson SR, Bankovich AJ, Pereira JP, Xu Y, Roots CM, Beilke JN, Banerjee A, Reiner SL, Miller SA, Weinmann AS, Goodnow CC, Lanier LL, Cyster JG, Chun J (2009) T-bet–dependent s1p5 expression in nk cells promotes egress from lymph nodes and bone marrow. J Exp Med 206:2469–2481
5. Streitz M, Miloud T, Kapinsky M, Reed MR, Magari R, Geissler EK, Hutchinson JA, Vogt K, Schlickeiser S, Kverneland AH (2013) Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the one study. Transplant Res 2:17
6. Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK (2016) A deep profiler's guide to cytometry. Trends Immunol 33:323–332
7. Zhang J, Dunk CE, Lye SJ (2013) Sphingosine signalling regulates decidual NK cell angiogenic phenotype and trophoblast migration. Hum Reprod 28:3026–3037
8. Zhang J, Dunk C, Croy AB, Lye SJ (2016) To serve and to protect: the role of decidual innate immune cells on human pregnancy. Cell Tissue Res 363:249–265
9. Perfetto SP, Chattopadhyay PK, Roederer M (2004) Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol 4:648–655
10. Lugli E, Roederer M, Cossarizza A (2010) Data analysis in flow cytometry: the future just started. Cytometry A 77:705–713
11. Saeys Y, Gassen SV, Lambrecht BN (2016) Computational flow cytometry: helping to make sense of high-dimensional immunology data. Nat Rev Immunol 16:449–462
12. Lai L, Ong R, Li J, Albani S (2015) A cd45-based barcoding approach to multiplex mass-cytometry (cytof). Cytometry A 87:369–374
13. Spitzer Matthew H, Nolan Garry P (2016) Mass cytometry: single cells, many features. Cell 165:780–791
14. Byrd T, Carr KD, Norman JC, Huye L, Hegde M, Ahmed N (2015) Polystyrene microspheres enable 10-color compensation for immunophenotyping of primary human leukocytes. Cytometry 87:1038–1046
15. Parks DR, Roederer M, Moore WA (2006) A new "logicle" display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. Cytometry A 69:541–551
16. Aghaeepour N, Finak G, Hoos H, Mosmann TR, Brinkman R, Gottardo R, Scheuermann RH (2013) Critical assessment of automated flow cytometry data analysis techniques. Nat Meth 10:228–238
17. Verschoor CP, Lelic A, Bramson JL, Bowdish DME (2015) An introduction to automated flow cytometry gating tools and their implementation. Front Immunol 6:380
18. Spiegel S, Milstien S (2011) The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol 11:403–415
19. Fang V, Chaluvadi VS, Ramos-Perez WD, Mendoza A, Baeyens A, Rivera R, Chun J, Cammer M, Schwab SR (2017) Gradients of the signaling lipid s1p in lymph nodes position natural killer cells and regulate their interferon-[gamma] response. Nat Immunol 18:15–25
20. Jin Y, Knudsen E, Wang L, Bryceson Y, Damaj B, Gessani S, Maghazachi AA (2003) Sphingosine 1-phosphate is a novel inhibitor of T-cell proliferation. Blood 101:4909–4915

Methods in Molecular Biology (2017) 1697: 99–115
DOI 10.1007/7651_2017_26

Published online: 30 April 2017

S1P Synergizes with Wall Shear Stress and Other Angiogenic Factors to Induce Endothelial Cell Sprouting Responses

Camille L. Duran, Roland Kaunas, and Kayla J. Bayless

Abstract

Angiogenesis is the process of new blood vessel growth from pre-existing structures. During sprout initiation, endothelial cells (ECs) are activated by pro-angiogenic factors to degrade the basement membrane, migrate into the surrounding matrix, and form structures that anastomose to connect neighboring vessels. Sphingosine 1-phosphate (S1P) is a biologically active lysosphingolipid that is secreted by platelets and promotes angiogenesis under normal and pathological conditions by acting on ECs. In addition to biochemical factors, the endothelium is continuously subjected to mechanical forces in the form of wall shear stress (WSS) from fluid forces. Here, we describe an in vitro, three-dimensional (3D) endothelial sprouting assay that is significantly enhanced by S1P, WSS, angiogenic growth factors (GFs), and fibronectin. This assay is assembled by seeding primary human endothelial cells onto 3D collagen matrices containing S1P and other pro-angiogenic factors. Once attached, physiological levels of WSS are applied to induce robust sprouting responses. This approach promotes the initiation of angiogenic sprouts stimulated by S1P, and allows the study of 3D sprouting of primary human endothelial cells induced in response to these physiological factors.

Keywords: 3D, Collagen, Endothelial cell, Invasion, Sphingosine 1-phosphate, Sprouting, Wall shear stress

1 Introduction

Endothelial cells (ECs) line the entire vascular system and remain dormant for decades. However, in response to the proper signals, new blood vessel growth by activated ECs can be initiated in both physiological and pathological conditions. Although significant progress has been made toward understanding key molecular events that control angiogenesis, additional insights are needed to complete our understanding of the process. As such, information gained from experimental systems is only as reliable as the model systems used. While a number of useful models have been generated to study angiogenesis in vitro and in vivo [1, 2], the system described here establishes a three-dimensional (3D) sprouting system where human ECs oriented on a basal extracellular matrix and

apical fluid compartment respond to sphingosine 1-phosphate (S1P) and other physiologically relevant cues, including flow.

The S1P signaling pathway is highly conserved [3]. S1P is a lysosphingolipid that is continually bioavailable in the circulation [4] but can also be released by activated platelets [5, 6]. As a result, levels of S1P in plasma can range from 100 to 300 nM, while serum levels may reach 1 μM [7–9]. Notably, S1P released by platelets is responsible for the ability of serum to promote EC chemotaxis [10]. Cellular responses to S1P occur through the $S1P_1$–$S1P_5$ G protein-coupled receptors, which were first named endothelial differentiation gene (EDG) receptors [11]. $S1P_1$ was originally discovered in activated ECs [12], which also express $S1P_2$ and $S1P_3$ [13]. S1P binding to $S1P_1$ and $S1P_3$ receptors promotes cell-cell adhesion, adherens junction assembly, and angiogenesis in vitro and in vivo [14–16]. An abundance of data indicate that S1P promotes angiogenic responses. S1P activation of $S1P_1$ and $S1P_3$ receptors promotes various angiogenic activities, including endometrial development, limb development, tumor growth, and placentation [13, 17–23]. In contrast, $S1P_2$ activation can inhibit cell motility [24, 25] and functions as an anti-angiogenic signal [26]. Knockout of *S1p1* in mice results in embryonic lethality due to excessive hemorrhaging [27]. Recent reports found that silencing *S1p1* expression in endothelial cells results in abnormal vascular development consistent with destabilized junctions in various vascular beds [28–30]. Altogether, these data support that S1P promotes junctional stabilization and barrier formation and in doing so contributes to the generation of newly formed sprouting structures.

In addition to S1P, various growth factors have been demonstrated to have pro-angiogenic properties. Two well-recognized angiogenic growth factors used in our S1P + GF model systems are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [31, 32]. VEGF stimulates angiogenesis and promotes EC proliferation, migration, and survival through activation of various downstream kinases [32–36]. Similarly, bFGF enhances EC migration and proliferation and stimulates formation of capillary-like structures with lumens [35, 37–39]. Although a previous study incorporated high levels (40 ng/ml) of VEGF and bFGF into the culture medium in the S1P + WSS model [40], this approach was not sustainable because it was cost-prohibitive. In the current studies, we elected to add tenfold lower amounts of VEGF and bFGF (GFs) selectively into collagen matrices to test if they might synergize with S1P + WSS at low levels. In addition to low levels of GFs, we also tested whether 10 μg/ml fibronectin (FN) would stimulate sprouting as was previously shown by Nicosia and colleagues in aortic ring assays [41]. The results from these studies demonstrate that both GF and FN enhance endothelial sprouting in 3D collagen matrices.

Blood flow is recognized as a physiological stimulator of new blood vessel growth. Although a large portion of the mechanotransduction literature is focused on the effects of flow and WSS on atherosclerosis, fluid forces have historically been recognized to regulate angiogenesis [42]. Notably, angiogenesis occurs in post-capillary venules, where WSS levels have been estimated at 1–8 dyn/cm^2 [43–45]. Independent studies in a variety of model systems have confirmed that modest increases in WSS in the microcirculation enhance angiogenic responses in rat skeletal muscle [46], rabbit ear chambers [43], and the yolk sac of the developing mouse embryo [47]. Thus, given the constant influence of fluid forces and WSS on the EC surface, incorporation of physiological levels of WSS should be considered when designing assays to study angiogenesis.

The protocol described here is designed to imitate the sprouting steps of angiogenesis and involves culture and growth of ECs, preparation of 3D collagen matrices, performing 3D cultures, incorporating physiological levels of S1P, GFs, and FN, and applying WSS. Analysis of sprouting responses is discussed, including imaging and quantification of sprouting responses. In these experiments, a monolayer of ECs is attached to a basal 3D collagen matrix containing S1P, GFs, and FN and overlaid with apical medium delivering physiological levels of WSS. S1P combined with GFs to stimulate invasion five- to tenfold over angiogenic growth factors alone [31, 48, 49]. S1P also synergized with WSS to promote sprouting [40, 50, 51]. In the current model system, we have incorporated low amounts of VEGF and bFGF in addition to FN with S1P in the collagen matrix in a defined, serum-free system. These conditions combined with physiological levels of WSS (5.3 dyn/cm^2) stimulate ECs to penetrate and invade into the collagen matrices overnight and form sprouting structures better than S1P alone or S1P + WSS (*see* Figs. 2 and 3). Incorporating various complementary methods, such as biochemical analyses, pharmacological inhibitors, and manipulation of gene expression in conjunction with this 3D culture system as previously described [48, 49, 51–53], is expected to reveal additional molecular signals downstream of S1P, GFs, FN, and WSS that control endothelial sprout initiation.

2 Materials

2.1 Reagents Needed for Propagation of ECs

1. 10× HEPES-buffered saline (HeBS): 200 mM HEPES, 1.5 M NaCl, pH 7.4; autoclave or sterile filter. Solution can be stored at room temperature (room temperature; 20–25 °C) for 1 year.

2. 0.1% Gelatin: Add 1 g gelatin to 900 ml water and 100 ml 10× HeBS; autoclave and sterile filter. Solution can be stored at room temperature for 1 year.
3. 1× HEPES-buffered saline (HeBS): 20 mM HEPES +150 mM NaCl, pH 7.4; autoclave and sterile filter. Solution can be stored at room temperature for 1 year.
4. Trypsin (Gibco, cat. #25200-056): The stock is thawed, divided into 2 ml aliquots, and frozen at −20 °C. Aliquots are stable for 1 year.
5. Fetal bovine serum (FBS, Gibco, cat. #26140-079): The stock is heat inactivated at 56 °C for 30 min, divided into 25 ml aliquots, and frozen at −20 °C. Aliquots are stable for 2 years.
6. Endothelial growth medium: Prepare lyophilized endothelial growth supplement [54] or purchase from Lonza (cat. #CC-4098). Add 0.2 g endothelial growth supplement and 0.05 g heparin (Sigma, cat. #H3393) to 500 ml M199 medium (Gibco, cat. #11150-059) and incubate at 37 °C for 30 min. Sterile-filter mixture (0.2 μm filter unit, Nalgene, VWR cat. # 28199-585) and add 55 ml of sterile heat-inactivated fetal bovine serum, 5.5 ml antibiotic (Gibco, cat. #15240-062), and 0.5 ml gentamicin (Invitrogen, cat. #15710-072). Store the medium at 4 °C for 1 month.
7. Single donor primary umbilical vein endothelial cells (HUVEC) (Lonza, cat. #CC-2517).
8. Tissue culture flasks (75 cm^2, Corning-Costar; VWR cat. #10144-832).

2.2 Reagents Needed for Performing Three-Dimensional Cultures and Applying WSS

1. Collagen solution: Collagen can be purchased commercially or purified from rat tails. Commercially available collagen solution (BD Biosciences) should be adjusted to 7.1 mg/ml with sterile 0.1% (vol/vol) acetic acid in water (*see* **Note 1**). Collagen stocks can be prepared from rat tail tendons as described [55, 56] and resuspended at 7.1 mg/ml with sterile 0.1% acetic acid. Collagen solutions should be stored at 4 °C.
2. S1P: Prepare S1P (Sigma, cat. #73914) according to the manufacturer's instructions as a 125 μM stock in 4 mg/ml FA-free bovine serum albumin (BSA) solution (Sigma, cat. #A8806). The solution is stable for 1 year at 4 °C.
3. 10× M199 (Invitrogen, cat. #11825-015).
4. 5 N sodium hydroxide (NaOH): Add 10 g NaOH to 50 ml sterile distilled water in a sterile 50 ml conical tube. The 5 N NaOH solution can be stored for 1 year at 4 °C (*see* **Note 2**).
5. 2 inch × 3 inch parallel plate flow chambers with 2 inch × 3 inch glass slides.

6. Appropriate tubing and fittings to connect flow chambers in series. Appropriate tubing adaptors to connect tubing to pump head (Cole-Parmer, part # 07003-02) and media reservoir.
7. Motor (Ismatec, Model # BVP-Z).
8. Flow transducer (Transonic, Cat. # T402 A60513) and flow probe (Transonic, Cat. # 2PXL).
9. 2 inch × 3 inch silicone molds with 8 punches (7 mm diameter) to house collagen matrices.
10. Humidified chambers to house collagen matrices during equilibration.
11. VEGF (R&D Systems, cat. #293-VE): Prepare 40 μg/ml solution, aliquot, and store at −80 °C. The stock is stable for 1 year.
12. Basic fibroblast growth factor (bFGF) (R&D Systems, cat. #234-FSE): Prepare 40 μg/ml solution, aliquot, and store at −80 °C. The stock is stable for 1 year.
13. Bovine fibronectin (FN) solution at 1 mg/ml (Sigma, cat. #F1141); aliquot, and store at −80 °C. The stock is stable for 1 year.
14. Ascorbic acid (Sigma, cat. #A4034): Prepare sterile 5 mg/ml solution in PBS. Aliquots can be stored at −20 °C and are stable for 6 months.
15. Reduced serum II (RSII): Prepare a sterile solution of 2 mg/ml BSA, 20 ng/ml human holo-transferrin, 20 ng/ml insulin, 17.1 ng/ml sodium oleate, and 0.02 ng/ml sodium selenite. Aliquots can be stored at −20 °C and are stable for 3 months at 4 °C.
16. Cellometer Auto 1,000 from Nexcelom Bioscience and Cellometer counting chambers (Nexcelom Bioscience cat. # CHT4-SD100).
17. Cold block for collagen gel preparation.
18. Silicone covers (approximately 1 cm^2).
19. 10× PBS: 100 mM Na_2HPO_4, 1.5 M NaCl, pH 7.4; solution can be stored at room temperature (20–25 °C) for 1 year.
20. 1× PBS: 900 ml distilled water +100 ml 10× PBS; autoclave or sterile filter. Solution can be stored at room temperature (20–25 °C) for 1 year.

2.3 Reagents Needed for Analyzing Endothelial Sprout Morphology

1. 4% Paraformaldehyde (25 ml): Combine 10 ml 10% paraformaldehyde (Electron Microscopy Sciences, cat. #15712), 2.5 ml 10× PBS, and 12.5 ml water; store at 4 °C for less than 24 h (*see* **Note 3**).
2. Toluidine blue: Prepare 0.1% toluidine blue (Sigma, cat. #T3260) solution in 30% methanol (*see* **Note 4**).

3. DAPI: Prepare 1 μM DAPI (Molecular Probes, cat. #D1306) solution in water (*see* **Note 5**); store at 4 °C for 1 year.
4. Tris-Glycine buffer: 3.03 g/l Tris base, 14.41 g/l glycine, 2 g/l sodium azide; store at 4 °C for 1 year.
5. 3% Glutaraldehyde in PBS (500 ml): Combine 60 ml 25% glutaraldehyde (Sigma, cat. #G6257), 390 ml distilled water, and 50 ml 10× PBS; store at room temperature for 2 years (*see* **Note 6**).
6. F-actin stain: Phalloidin conjugated to Alexa Fluor 488 (Molecular Probes, cat. # A12379).
7. Stainless steel fine point forceps.
8. Razor blades.
9. Microscope slide.
10. Inverted microscope (Olympus, CKX41) equipped with a set of objectives (4×, 10×, and 20×), eyepiece grid micrometer (10 mm × 10 mm grid, Olympus, code 35047), epi-fluorescence illuminator, Olympus Q Color3 Digital camera (Olympus), and Q Capture Pro 5.1 software.
11. Phalloidin incubation buffer: 10 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 1% BSA and 1% goat serum, pH 7.4.
12. Blocking buffer: 10 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 1% BSA and 1% goat serum, pH 7.4.

3 Methods

3.1 Method for Culturing Endothelial Cells

1. Pre-warm 1× HeBS, M199, FBS, and endothelial growth medium at 37 °C in a water bath.
2. Coat four 75-cm^2 tissue culture flasks with 10 ml of sterile 0.1% gelatin in HeBS for 30 min at room temperature.
3. Thaw 2 ml trypsin and wash ECs with 14 ml warm HeBS. Aspirate HeBS and then add 2 ml trypsin to endothelial monolayer and incubate for 1 min at 37 °C. After removing from incubator, gently tap the flask to loosen cells, and add 2 ml FBS and 6 ml M199 to neutralize the trypsin and suspend the cells (*see* **Note 7**).
4. Collect the cell suspension in a 15-ml centrifuge tube. Spin at 350 × *g* for 3 min.
5. After centrifugation, aspirate supernatant and resuspend in 4 ml endothelial growth medium.
6. Aspirate gelatin and add 14 ml of endothelial growth medium and 1 ml of cell suspension to each flask.
7. HUVEC should be passaged once a week. Three or four days after passaging, change the medium by aspirating conditioned medium and replacing with 15 ml of endothelial growth medium.

3.2 Method for Stimulating EC Invasion with S1P, WSS, GFs, and FN

1. Replace the medium with 15 ml of fresh endothelial growth medium 1 day prior to performing 3D cultures.
2. Calculate the volume of collagen gel needed for the experiment (47 μl of gel per well × number of wells needed), and prepare all components according to guidelines in Table 1.
3. Prepare humidified chambers and silicone molds on 2 × 3 inch glass slides to generate 8 well chambers for collagen matrices per slide.
4. Place silicone molds on cold block to prevent collagen polymerization.
5. Add the needed amount of collagen in a pre-chilled 50 ml conical sterile centrifuge tube and place the tube on ice (*see* **Note 8**).
6. Add 10× M199, mix gently ten times, and place the tube back on ice.
7. Add 5 N NaOH, mix gently ten times, and place the tube back on ice (*see* **Note 9**).
8. Add M199 and mix thoroughly.
9. Add S1P, mix again, and place on ice (*see* **Note 10**).
10. Add VEGF, bFGF, and FN. Mix again and return to ice.

Table 1
Recipe for preparing 3.75 mg/ml collagen I matrices

Reagent	1 ml	2 ml	3 ml	4 ml	5 ml
Collagen I[a]	525 μl	1,050 μl	1,575 μl	2,100 μl	2,625 μl
10× M199	58.5 μl	117 μl	175.5 μl	234 μl	293 μl
5 N NaOH	3.15 μl	6.3 μl	9.45 μl	12.6 μl	15.8 μl
1× M199	413 μl	826 μl	1,239 μl	1,652 μl	2065 μl
S1P[b]	8 μl	16 μl	24 μl	32 μl	40 μl
VEGF[c]	0.1 μl	0.2 μl	0.3 μl	0.4 μl	0.5 μl
bFGF[d]	0.1 μl	0.2 μl	0.3 μl	0.4 μl	0.5 μl
FN[e]	10 μl	20 μl	30 μl	40 μl	50 μl

[a]7.1 mg/ml Collagen I stock
[b]125 μM S1P stock
[c]40 ng/ml VEGF stock
[d]40 ng/ml bFGF stock
[e]1 mg/ml Fibronectin stock

11. Load 47 μl collagen mixture into each open well in the silicone mold (*see* **Note 11**) and cover each well with a single silicone cover prior to polymerization.
12. Place the slides in a humidified chamber and transfer to an incubator (37 °C, 5% CO_2) for 45 min before seeding the cells.
13. Pre-warm HeBS, M199, and FBS for trypsinizing cells.
14. Following equilibration of collagen matrices, trypsinize endothelial cells as described above in Sect. 3.1. Spin at 350 × *g* for 3 min at room temperature.
15. Resuspend the cell pellet with 1 ml M199 and take a 20 μl aliquot for counting using the cell counter. Adjust density to 50,000 cells per 100 μl. If necessary, spin again at 350 × *g* for 3 min at room temperature before resuspending.
16. Allow the cells to attach for 1 h before mixing briefly to allow even population of the surface of the collagen matrices (*see* **Note 12**).
17. Assemble parallel plate flow chambers and pump head and prime the line (*see* **Note 13**) with M199 containing RSII (1:250) and ascorbic acid (50 μg/ml).

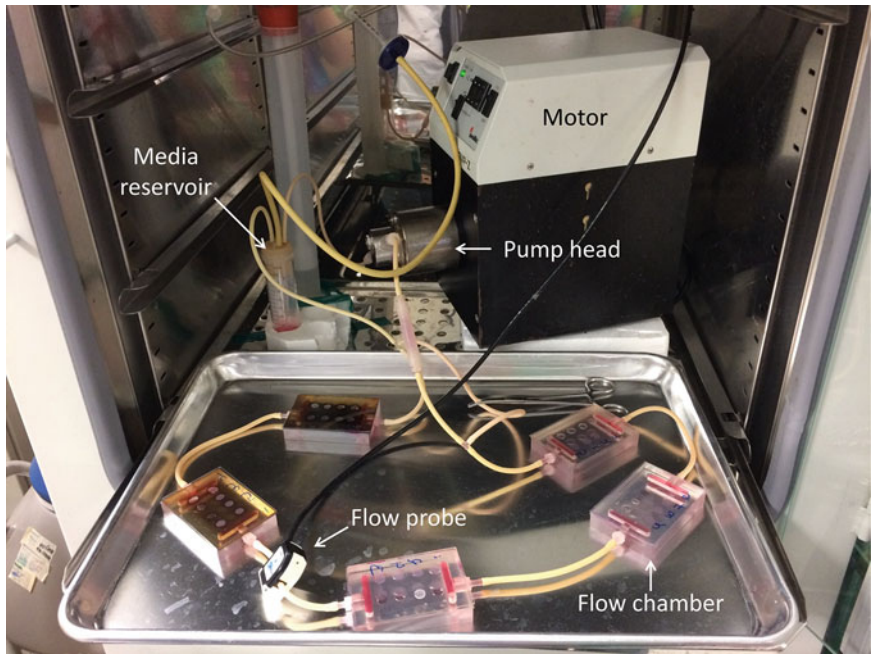

Fig. 1 Photograph illustrating the main components of the system for applying controlled levels of WSS to ECs seeded on the surface of collagen matrices. The motor and pump head are connected to parallel plate flow chambers arranged in series using standard laboratory tubing. A media reservoir is infused with 5% CO_2 to maintain physiological pH. The entire system is housed in an incubator maintaining the temperature at 35.5 °C

18. After 2 h of cell attachment, assemble flow chambers and connect pump head, as shown in Fig. 1.
19. Apply WSS at 5.3 dyn/cm^2 to induce sprouting. The WSS magnitude is calculated as $\tau = \mu Q / wh^2$, where τ is WSS, μ is fluid viscosity (0.7 cP), Q is flow rate, w is the width of the flow channel (29.21 mm), and h is the height of flow channel, dictated by a silicone spacer. The culture medium flow is promoted by a sterile continuous-flow loop, and flow rate is controlled with the pulse-free gear pump (Cole-Parmer) and monitored with the ultrasonic tubing flow sensor (Transonic Systems). The entire system is established in a standard 37 °C tissue culture incubator.
20. Prior to placing in the incubator, infuse the media reservoir with 5% CO_2 to maintain proper pH of culture medium.
21. Fix at various time points to analyze cell morphology and signaling responses.

3.3 Method for Visualizing Endothelial Cell Invasion with Toluidine Blue Staining

1. Take apart flow chamber in 1× PBS solution. Remove excess fluid and fix samples by adding 200 μl of 3% glutaraldehyde to each collagen matrix (*see* **Note 14**). Incubate overnight in a humidified chamber.
2. Remove glutaraldehyde and incubate with toluidine blue solution for 15 min at room temperature. Remove stain and rinse with water for 5 min.
3. Samples are now ready to be photographed and quantify invasion density (*see* Sect. 3.5 below), as shown in Fig. 2a, b, respectively. Note that S1P synergizes with growth factors (GF), fibronectin (FN), and 5.3 dyn/cm^2 WSS to promote the most robust invasion (Fig. 2b).
4. This experimental system allows varying of culture conditions to demonstrate that EC invasion stimulated by S1P and WSS is enhanced by the addition of FN, GF, and GF + FN (Fig. 3).

3.4 Method for Visualizing Endothelial Cell Invasion with Phalloidin Staining

1. Take apart flow chamber in 1× PBS solution. Remove excess fluid and add 2 ml of 4% paraformaldehyde in PBS (*see* **Note 14**) to cover all eight samples in each chamber. Incubate 30 min at room temperature.
2. Collect paraformaldehyde and dispose in the proper container. Quench the fixation reaction with 2 ml Tris-Glycine and incubate for 20 min at room temperature. Perform this step twice.
3. Remove collagen matrices from silicone molds. Slice gels before permeabilizing using a razor blade and glass slide. If necessary, samples can be stored in Tris-Glycine solution long term at 4 °C.

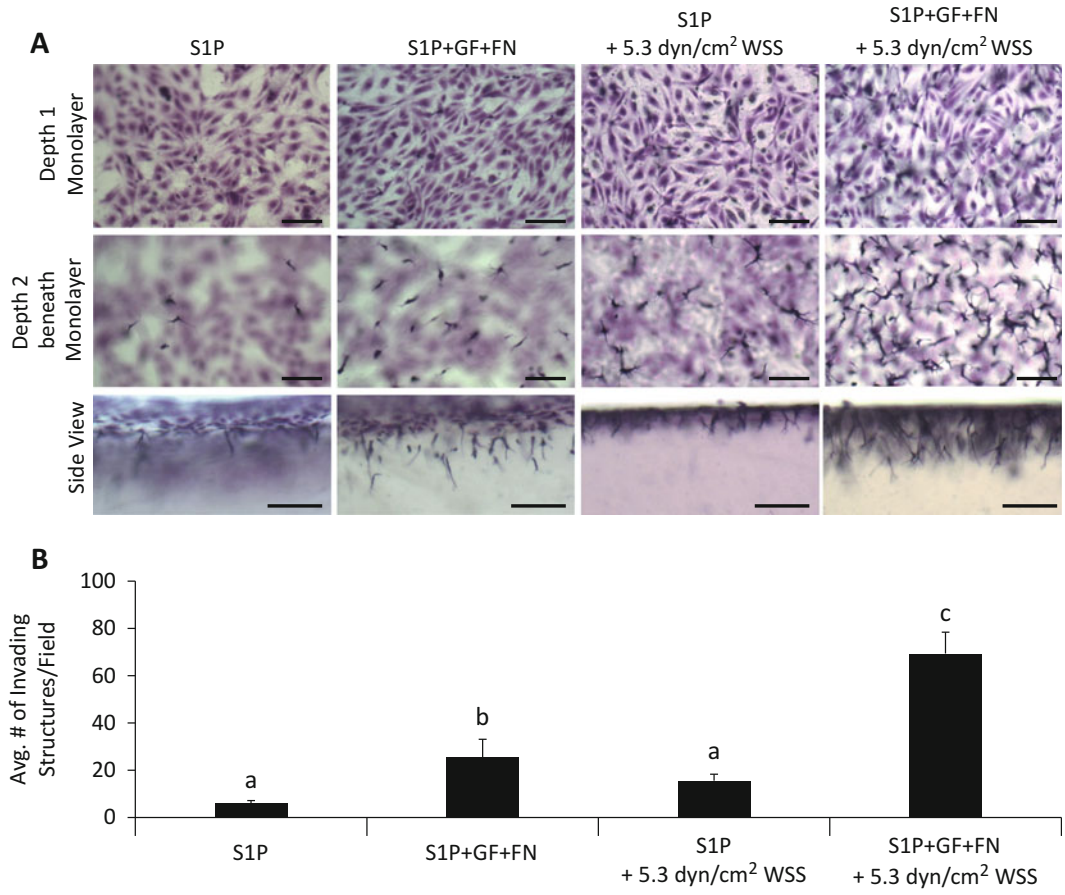

Fig. 2 S1P, GF, FN, and WSS synergize to induce the most robust sprouting responses. A monolayer of endothelial cells (ECs) were seeded on the upper surface of solidified collagen matrices containing 1 μM sphingosine 1-phosphate (S1P) combined with 10 μg/ml fibronectin (FN) and 4 ng/ml VEGF and bFGF (GF). Cells were exposed to no flow (0 dyn/cm^2) or flow (WSS applied at 5.3 dyn/cm^2) for 24 h. Invasion occurs when ECs leave the monolayer and enter the 3D collagen matrix, forming sprouts. (**a**) Representative photographs illustrating invasion responses. Images taken show an *en face* view of the monolayer (Depth 1) and underlying invading structures (Depth 2), as well as a side view of invading ECs after 24 h. Scale bars in *top* and *middle panels* represent 100 μm. Scale bar in *bottom panels* represents 200 μm. (**b**) Quantification of the average number of invading sprouts per field (+/− st.dev.) after 24 h. Distinct lowercase letters indicate statistically significant differences between treatment groups calculated with a one-way ANOVA and Tukey's HSD post-test with $p < 0.05$

4. Transfer gels to a 24 well plate. Permeabilize gels in 500 μl of 0.5% Triton X-100 in PBS with constant rotation for 30 min at room temperature.
5. Block gels overnight in Blocking buffer at 4 °C in 24 well plates, 500 μl/well.
6. Prepare Alexa 488-Phalloidin staining solution (1:200) in blocking buffer containing 10 mM Tris, 150 mM NaCl, 0.1%

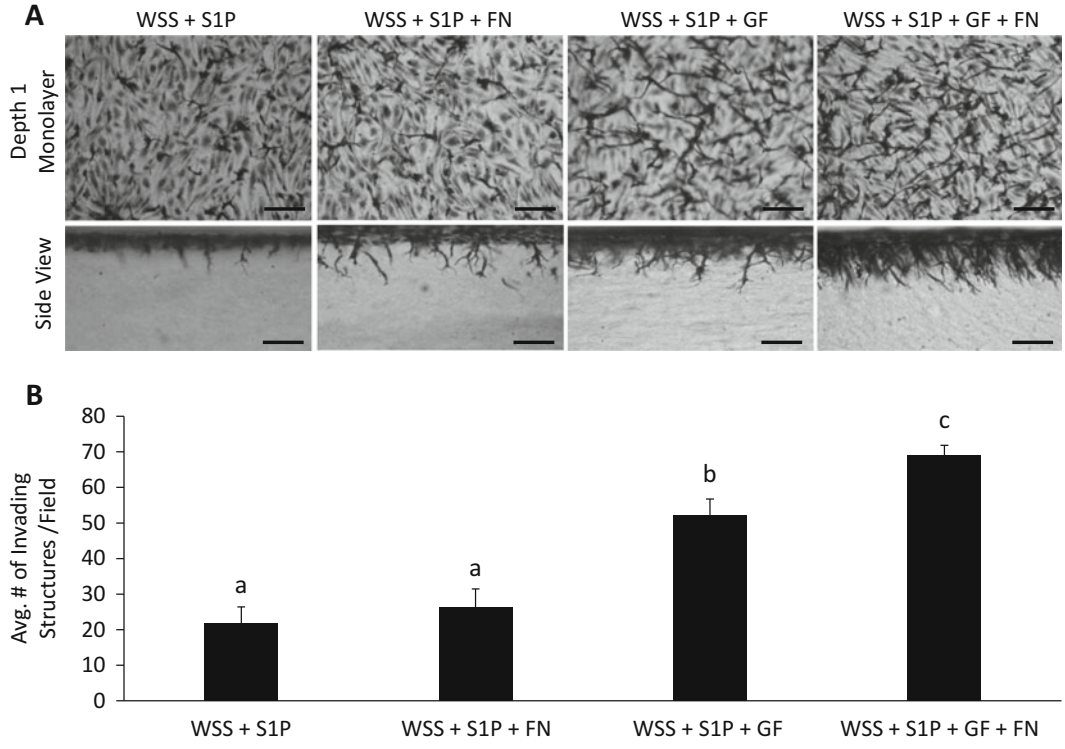

Fig. 3 FN and GF enhance S1P and WSS-induced sprouting responses. Collagen matrices were prepared with S1P, S1P + FN, S1P + GF, or S1P + GF + FN (*see* Table 1). Cells were seeded on surface of polymerized collagen matrices and 5.3 dyn/cm^2 WSS was applied for 22 h. (**a**) Representative photographs illustrating invasion responses. Images taken show an *en face* view of the monolayer, as well as a side view of invading ECs. Scale bars represent 100 μm. (**b**) Quantification of the average number of invading sprouts per field (+/− st.dev.) after 22 h of invasion. Distinct lowercase letters indicate statistically significant differences between treatment groups calculated with a one-way ANOVA and Tukey's HSD post-test with $p < 0.05$

Triton X-100, 1% BSA, and 1% goat serum. Incubate five slices in 100 μl in a 96 well plate. Incubate for 1 h at room temperature with constant mixing and protect from light.

7. Transfer gels to a 24 well plate and wash twice with 500 μl of 0.1% Triton X-100 for 1 h.
8. Remove wash buffer and replace with 500 μl fresh buffer. Add DAPI at 1:10 to each well (*see* **Note 14**). Incubate for 30 min at room temperature with constant rotation and protect from light.
9. Image samples using confocal microscopy or other suitable method.
10. The above-described experiments in Figs. 2 and 3 report invasion responses that were observed after treatment with S1P, WSS, and other pro-angiogenic factors for 24 h. Using

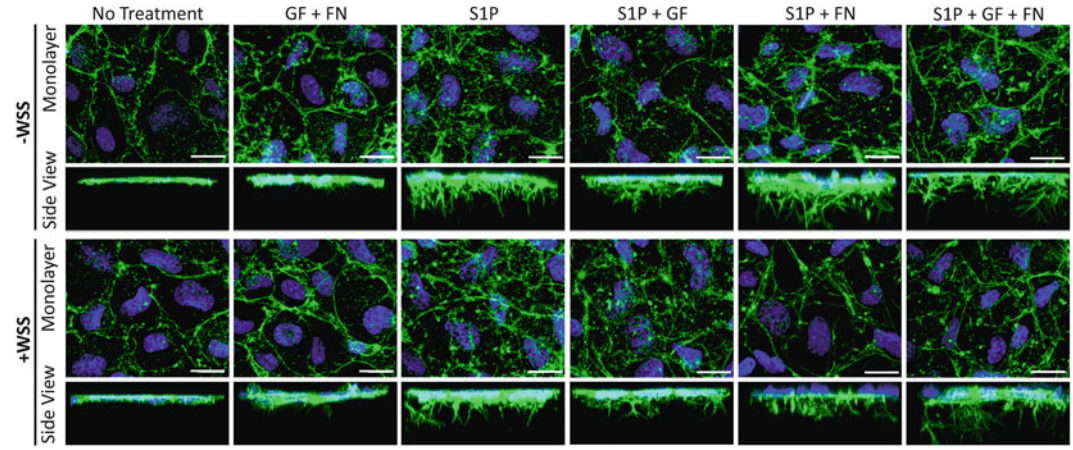

Fig. 4 S1P consistently initiates EC sprouting into collagen matrices. ECs were seeded onto 3D collagen matrices containing nothing (no treatment), or the indicated combinations of GF, FN, and S1P. Cells were treated −/+ 5.3 dyn/cm^2 WSS for 2 h, then fixed and permeablized. Cells were stained with FITC-conjugated phalloidin (*green*) and DAPI (*blue*) and imaged using confocal microscopy. Scale bars represent 10 μm. *Top panels* show *en face* view of EC monolayers and *bottom panels* show a side view of invading sprouts. Images shown are 3D images of the entire field rotated 90 degrees

phalloidin staining as described above, it can be observed that S1P appears to stimulate early invasion responses after only 2 h in culture (Fig. 4), demonstrating S1P provides a necessary stimulus to initiate EC sprout formation.

3.5 Quantifying Invasion Density

1. Remove the destaining water from the wells to be quantified and replace with fresh water.
2. Cover the wells with a clean coverglass to remove meniscus and place the glass on microscope stage.
3. Center the well using low magnification, and then observe area to be quantified using a 10× or 20× objective. Insert eyepiece containing a mounted reticle that displays a 10 × 10 square grid in the field of interest.
4. Record the number of invading cells by visualizing toluidine blue stained cultures. Starting with a focal plane on the monolayer, lower the objective, and count the number of structures underneath the noninvading monolayer in a 2 × 2 grid area.
5. Repeat for the entire grid area. Present data as the average number of invading cells per area analyzed (in mm^2). Using the Olympus microscope and equipment described, a 10× objective with eyepiece reticle quantifies an area of 1 mm^2, while a 20× objective measures 0.25 mm^2.

4 Notes

1. Acetic acid can cause severe skin and eye burns. Vapor is irritating to eyes and respiratory tract. Harmful if swallowed. Wear goggles and gloves.
2. NaOH is highly toxic and caustic, and should be handled with great care. Wear appropriate gloves and a face mask.
3. Paraformaldehyde is highly toxic and may be a carcinogen. It is harmful to the skin, eyes, mucous membranes, and upper respiratory tract if ingested. Avoid breathing the dust or vapor. Wear appropriate gloves and safety glasses and use in a chemical fume hood. Keep away from heat, sparks, and open flame.
4. Toluidine blue solutions will stain skin and clothes. Protective labware is needed.
5. DAPI is a known mutagen and should be handled with care.
6. Glutaraldehyde is highly irritating to eyes and all mucous membranes and should be handled in a fume hood.
7. Do not allow cells to contact trypsin for more than 60 s at 37 °C.
8. Collagen solution is viscous. To add the indicated amount of collagen, first add that volume of M199 in a sterile 50-ml conical centrifuge tube and mark the volume needed with a fine-point marker. Remove M199 and place the tube on ice to chill. The tube should be ice-cold before adding collagen to the line marked on tube.
9. The pH should change completely from acidic (yellow) to basic (magenta color). Make sure that no yellow is visible before continuing.
10. Avoid introducing air bubbles into the collagen mixture. Alternatively, once all components are added, use a hand-held pipette to take up the mixture and mix evenly ten times without introducing air bubbles into the mixture. If there are any bubbles in the tube, make sure that the pipette tip is below the bubbles when taking up the collagen mixture.
11. Avoid introducing bubbles between silicone covers and collagen matrices. If this occurs, remove collagen completely and fill with new collagen solution.
12. Mix cells carefully after seeding by pipetting a 60 μl volume twice to ensure even seeding of the monolayer. Avoid introduction of air bubbles and do not puncture or damage the collagen matrix with the pipette tip.

13. Prime line just before addition of cells. Avoid introduction of air into the tubing and parallel plate flow chambers while assembling the chambers.
14. DAPI, paraformaldehyde, and glutaraldehyde are potentially harmful substances. Handle with care and dispose of waste properly.

Acknowledgements

This work was supported by the Public Health Service and the National Institutes of Health (grant number HL-095786 to K.J.B.) and bridge funding from the Office of the Vice President for Research at Texas A&M University.

References

1. Chappell JC, Wiley DM, Bautch VL (2012) How blood vessel networks are made and measured. Cells Tissues Organs 195 (1–2):94–107. doi:10.1159/000331398
2. Simons M, Alitalo K, Annex BH, Augustin HG, Beam C, Berk BC, Byzova T, Carmeliet P, Chilian W, Cooke JP, Davis GE, Eichmann A, Iruela-Arispe ML, Keshet E, Sinusas AJ, Ruhrberg C, Woo YJ, Dimmeler S, American Heart Association Council on Basic Cardiovascular Sciences, Council on Cardiovascular Surgery and Anesthesia (2015) State-of-the-art methods for evaluation of angiogenesis and tissue vascularization: a scientific statement from the American Heart Association. Circ Res 116(11):e99–132. doi:10.1161/RES.0000000000000054
3. Spiegel S, Milstien S (2003) Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. Biochem Soc Trans 31(Pt 6):1216–1219
4. Pyne S, Pyne NJ (2000) Sphingosine 1-phosphate signalling in mammalian cells. Biochem J 349(Pt 2):385–402
5. Yatomi Y, Ruan F, Hakomori S, Igarashi Y (1995) Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 86 (1):193–202
6. Yatomi Y, Yamamura S, Ruan F, Igarashi Y (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. J Biol Chem 272 (8):5291–5297
7. Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y, Kume S (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. J Biochem 121(5):969–973
8. Murata N, Sato K, Kon J, Tomura H, Okajima F (2000) Quantitative measurement of sphingosine 1-phosphate by radioreceptor-binding assay. Anal Biochem 282(1):115–120. doi:10.1006/abio.2000.4580
9. Edsall LC, Spiegel S (1999) Enzymatic measurement of sphingosine 1-phosphate. Anal Biochem 272(1):80–86. doi:10.1006/abio.1999.4157
10. English D, Welch Z, Kovala AT, Harvey K, Volpert OV, Brindley DN, Garcia JG (2000) Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J 14 (14):2255–2265. doi:10.1096/fj.00-0134com
11. Kluk MJ, Hla T (2002) Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. Biochim Biophys Acta 1582(1–3):72–80
12. Hla T, Maciag T (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. J Biol Chem 265(16):9308–9313
13. McGiffert C, Contos JJ, Friedman B, Chun J (2002) Embryonic brain expression analysis of lysophospholipid receptor genes suggests roles

for s1p(1) in neurogenesis and s1p(1-3) in angiogenesis. FEBS Lett 531(1):103–108

14. Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, Hla T (1998) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. Science 279(5356):1552–1555
15. Lee M-J, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell 99 (3):301–312. doi:10.1016/s0092-8674(00)81661-x
16. Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D (2001) Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. J Clin Invest 108(5):689–701. doi:10.1172/JCI12450
17. Lee OH, Kim YM, Lee YM, Moon EJ, Lee DJ, Kim JH, Kim KW, Kwon YG (1999) Sphingosine 1-phosphate induces angiogenesis: its angiogenic action and signaling mechanism in human umbilical vein endothelial cells. Biochem Biophys Res Commun 264 (3):743–750. doi:10.1006/bbrc.1999.1586
18. Argraves KM, Wilkerson BA, Argraves WS, Fleming PA, Obeid LM, Drake CJ (2004) Sphingosine-1-phosphate signaling promotes critical migratory events in vasculogenesis. J Biol Chem 279(48):50580–50590. doi:10.1074/jbc.M404432200
19. Kono M, Mi Y, Liu Y, Sasaki T, Allende ML, Wu YP, Yamashita T, Proia RL (2004) The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. J Biol Chem 279 (28):29367–29373. doi:10.1074/jbc.M403937200
20. Kaneko-Tarui T, Zhang L, Austin KJ, Henkes LE, Johnson J, Hansen TR, Pru JK (2007) Maternal and embryonic control of uterine sphingolipid-metabolizing enzymes during murine embryo implantation. Biol Reprod 77 (4):658–665. doi:10.1095/biolreprod.107.061044
21. Mizugishi K, Li C, Olivera A, Bielawski J, Bielawska A, Deng CX, Proia RL (2007) Maternal disturbance in activated sphingolipid metabolism causes pregnancy loss in mice. J Clin Invest 117(10):2993–3006. doi:10.1172/JCI30674
22. Chae SS, Paik JH, Allende ML, Proia RL, Hla T (2004) Regulation of limb development by the sphingosine 1-phosphate receptor S1p1/EDG-1 occurs via the hypoxia/VEGF axis. Dev Biol 268(2):441–447. doi:10.1016/j.ydbio.2004.01.001
23. Chae SS, Paik JH, Furneaux H, Hla T (2004) Requirement for sphingosine 1-phosphate receptor-1 in tumor angiogenesis demonstrated by in vivo RNA interference. J Clin Invest 114(8):1082–1089. doi:10.1172/JCI22716
24. Estrada R, Zeng Q, Lu H, Sarojini H, Lee JF, Mathis SP, Sanchez T, Wang E, Kontos CD, Lin CY, Hla T, Haribabu B, Lee MJ (2008) Up-regulating sphingosine 1-phosphate receptor-2 signaling impairs chemotactic, wound-healing, and morphogenetic responses in senescent endothelial cells. J Biol Chem 283 (44):30363–30375. doi:10.1074/jbc.M804392200
25. Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol Cell Biol 20(24):9247–9261
26. Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Takuwa Y (2002) Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. Circ Res 90 (3):325–332
27. Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL (2000) Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest 106 (8):951–961
28. Gaengel K, Niaudet C, Hagikura K, Lavina B, Muhl L, Hofmann JJ, Ebarasi L, Nystrom S, Rymo S, Chen LL, Pang MF, Jin Y, Raschperger E, Roswall P, Schulte D, Benedito R, Larsson J, Hellstrom M, Fuxe J, Uhlen P, Adams R, Jakobsson L, Majumdar A, Vestweber D, Uv A, Betsholtz C (2012) The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. Dev Cell 23(3):587–599. doi:10.1016/j.devcel.2012.08.005

29. Ben Shoham A, Malkinson G, Krief S, Shwartz Y, Ely Y, Ferrara N, Yaniv K, Zelzer E (2012) S1P1 inhibits sprouting angiogenesis during vascular development. Development 139 (20):3859–3869. doi:10.1242/dev.078550
30. Jung B, Obinata H, Galvani S, Mendelson K, Ding BS, Skoura A, Kinzel B, Brinkmann V, Rafii S, Evans T, Hla T (2012) Flow-regulated endothelial S1P receptor-1 signaling sustains vascular development. Dev Cell 23 (3):600–610. doi:10.1016/j.devcel.2012.07.015
31. Bayless K, Kwak H-I, Su S-C (2009) Investigating endothelial invasion and sprouting behavior in three-dimensional collagen matrices. Nat Protoc 4(12):1888–1898. doi:10.1038/nprot.2009.221
32. Bayless KJ, Davis GE (2003) Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices. Biochem Biophys Res Commun 312 (4):903–913. http://dx.doi.org/10.1016/j.bbrc.2003.11.017
33. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380(6573):435–439
34. Jakeman L, Armanini M, Phillips H, Ferrara N (1993) Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology 133(2):848–859. doi:10.1210/endo.133.2.7688292
35. Pownall ME, Isaacs HV (2010) FGF signalling in vertebrate development. Colloq Ser Dev Biol 1(1):1–75. doi:10.4199/C00011ED1V01Y201004DEB002
36. Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Investig 91(5):2235–2243
37. Beck L Jr, D'Amore PA (1997) Vascular development: cellular and molecular regulation. FASEB J 11(5):365–373
38. Gospodarowicz D, Cheng J, Lui GM, Baird A, Böhlent P (1984) Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. Proc Natl Acad Sci 81 (22):6963–6967
39. Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P (1998) Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol 141(7):1659–1673
40. Kang H, Bayless KJ, Kaunas R (2008) Fluid shear stress modulates endothelial cell invasion into three-dimensional collagen matrices. Am J Phys Heart Circ Phys 295(5):H2087–H2097. doi:10.1152/ajpheart.00281.2008
41. Nicosia RF, Bonanno E, Smith M (1993) Fibronectin promotes the elongation of microvessels during angiogenesis in vitro. J Cell Physiol 154(3):654–661. doi:10.1002/jcp.1041540325
42. Clark ER, Nitschler WJ, Kirby-Smith HT, Rex RO, Smith JH (1931) General observations on the ingrowth of new blood vessels into standardized chambers in the rabbit's ear, and the subsequent changes in the newly grown vessels over a period of months. Anat Rec 50:129–168
43. Ichioka S, Shibata M, Kosaki K, Sato Y, Harii K, Kamiya A (1997) Effects of shear stress on wound-healing angiogenesis in the rabbit ear chamber. J Surg Res 72(1):29–35. doi:10.1006/jsre.1997.5170
44. Kim MB, Sarelius IH (2003) Distributions of wall shear stress in venular convergences of mouse cremaster muscle. Microcirculation 10 (2):167–178. doi:10.1038/sj.mn.7800182
45. Koutsiaris AG, Tachmitzi SV, Batis N, Kotoula MG, Karabatsas CH, Tsironi E, Chatzoulis DZ (2007) Volume flow and wall shear stress quantification in the human conjunctival capillaries and post-capillary venules in vivo. Biorheology 44(5–6):375–386
46. Zhou A, Egginton S, Hudlicka O, Brown MD (1998) Internal division of capillaries in rat skeletal muscle in response to chronic vasodilator treatment with alpha1-antagonist prazosin. Cell Tissue Res 293(2):293–303
47. Lucitti JL, Jones EA, Huang C, Chen J, Fraser SE, Dickinson ME (2007) Vascular remodeling

of the mouse yolk sac requires hemodynamic force. Development 134(18):3317–3326. doi:10.1242/dev.02883

48. Dave JM, Abbey CA, Duran CL, Seo H, Johnson GA, Bayless KJ (2016) Hic-5 mediates the initiation of endothelial sprouting by regulating a key surface metalloproteinase. J Cell Sci 129(4):743–756. doi:10.1242/jcs.170571
49. Su SC, Maxwell SA, Bayless KJ (2010) Annexin 2 regulates endothelial morphogenesis by controlling AKT activation and junctional integrity. J Biol Chem 285 (52):40624–40634. doi:10.1074/jbc.M110.157271
50. Kang H, Duran CL, Abbey CA, Kaunas RR, Bayless KJ (2015) Fluid shear stress promotes proprotein convertase-dependent activation of MT1-MMP. Biochem Biophys Res Commun 460(3):596–602. doi:10.1016/j.bbrc.2015.03.075
51. Kang H, Kwak HI, Kaunas R, Bayless KJ (2011) Fluid shear stress and sphingosine 1-phosphate activate calpain to promote membrane type 1 matrix metalloproteinase (MT1-MMP) membrane translocation and endothelial invasion into three-dimensional collagen matrices. J Biol Chem 286 (49):42017–42026. doi:10.1074/jbc.M111.290841
52. Dave JM, Kang H, Abbey CA, Maxwell SA, Bayless KJ (2013) Proteomic profiling of endothelial invasion revealed receptor for activated C kinase 1 (RACK1) complexed with vimentin to regulate focal adhesion kinase (FAK). J Biol Chem 288(42):30720–30733. doi:10.1074/jbc.M113.512467
53. Kwak HI, Kang H, Dave JM, Mendoza EA, Su SC, Maxwell SA, Bayless KJ (2012) Calpain-mediated vimentin cleavage occurs upstream of MT1-MMP membrane translocation to facilitate endothelial sprout initiation. Angiogenesis 15(2):287–303. doi:10.1007/s10456-012-9262-4
54. Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R (1979) An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc Natl Acad Sci U S A 76(11):5674–5678
55. Rajan N, Habermehl J, Cote MF, Doillon CJ, Mantovani D (2006) Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications. Nat Protoc 1(6):2753–2758. doi:10.1038/nprot.2006.430
56. Bornstein MB (1958) Reconstituted rattail collagen used as substrate for tissue cultures on coverslips in Maximow slides and roller tubes. Lab Invest 7(2):134–137

Methods in Molecular Biology (2017) 1697: 117–131
DOI 10.1007/7651_2017_51

Published online: 29 July 2017

In Vitro Methods to Study the Modulation of Migration and Invasion by Sphingosine-1-Phosphate

Melina G. Castro, Ludmila E. Campos, Yamila I. Rodriguez, and Sergio E. Alvarez

Abstract

Sphingosine-1-phosphate (S1P) is a bioactive lipid that modulates migratory behavior of cells during embryonic development. In addition, S1P might promote tumor progression by enhancing migratory ability and invasiveness of tumor cells. Migration is a complex process that implies cytoskeletal reorganization and formation of structures that enable cell movement. Besides having similar requirements than migration, invasion also involves proteolytic degradation of extracellular matrix (ECM). Matrix metalloproteases (MMPs) have been identified to break down components of the ECM, allowing cancer cells to spread out of the primary tumor. In this chapter, we will describe different techniques to study migration and invasion induced by S1P. To this end, we include detailed protocols of end-point assays to study migration/invasion, and zymography assay to analyze MMP-2 and MMP-9 activity that were standardized in our laboratory in human melanoma cell lines.

Keywords Boyden Chamber, Invasion, Melanoma, Migration, Sphingosine-1-phosphate, Transwell, Wound healing, Zymography

1 Introduction

Over the last years, several studies confirm the participation of sphingosine-1-phosphate (S1P) in enhancing cancer cell proliferation, preventing apoptosis, increasing drug resistance, and stimulating tumor angiogenesis, thus promoting tumor progression [1, 2]. S1P is produced inside the cells by the action of sphingosine kinases (SphK1 and SphK2) and may function as an intracellular messenger or in an autocrine/paracrine fashion to activate five G-protein-coupled S1P receptors called S1PR1-5 [3]. Although many evidences point to a role of S1P in cell motility, its involvement in regulation of migration and invasion is not clear. S1P could positively or negatively regulate cancer cell migration and invasion depending on the S1PRs involved and the intracellular signaling pathways triggered by them [4–6]. For example, activation of

Melina G. Castro and Ludmila E. Campos contributed equally to this work.

S1PR1 and S1PR3 promotes, while engagement of S1PR2 inhibits cell migration [3].

Migration constitutes an essential feature of cells and is required for many biological processes, including embryonic development, immune response, and cancer metastasis. The process of migration in cultured cells involves polarization, change of cell morphology, and the establishment of a front and a rear face. In turn, intense local actin polymerization generates a protrusive structure in the direction of the migration resulting in membrane expansion with the establishment of new contacts to the substratum that trigger the traction to the rest of the cell body [7, 8]. It is important to appreciate that cell migration can implicate augmented random motility due to a chemical stimulus (chemokinesis), or increased migration toward a chemoattractant gradient (chemotaxis).

Once the tumor cells start to move, invasive mechanisms are activated to lead the malignant cells to extravasation and metastasis. Therefore, it is clear that migration is a necessary condition for invasion: a cell cannot invade if it does not move first. Metastasis causes the majority of deaths by cancer and is a complex process that involves many steps, essentially invasion of the extracellular matrix (ECM), migration through blood vessels, and colonization of distant tissue to form a secondary tumor [9, 10]. This pattern requires expression of several proteins like metalloproteases MMP-2 and MMP-9, which are responsible to break down the ECM where the tumor mass is established.

In virtue of the previous evidences, it is apparent that the development of methods to examine cell migration and invasion are critical to achieve a better understanding of cancer progression. Here, we describe different in vitro methods to study S1P-regulated cell migration and cell invasion of melanoma cells available in our laboratory. It is important to have in mind that the assays described here do not completely resemble the processes of migration and invasion, but may offer a significant hint of the role of S1P in both mechanisms.

Wound Healing Assay (WHA): This method is an economical test that resembles cell migration during wound healing in vivo. The assay is reproducible and does not require any special equipment; thus, it may be performed in most laboratories. The method involved the generation of a wound in a monolayer of confluent cells with a pipette tip. The cells on the border of the wound will migrate to cover the wound. This method has two main drawbacks: (1) since there is no need for ECM degradation, it is not suitable to evaluate cell invasion and (2) considering that there is no chemical gradient in the culture plate, migration occurs by chemokinesis and not chemotaxis.

Transwell and Modified Boyden Chamber Assays: These are "single cell" migration and invasion assays; there is no need of cell–cell

contacts and cells migrate independently. Both methods can be used to study chemokinesis or chemotaxis and consist in two chambers separated by a porous membrane constituting a physical barrier that cells has to overcome. For chemotaxis analysis, the attractant should be included only in the lower chamber, while for chemokinesis examination is added at equal concentrations on both chambers. The upper chamber always contains the cell suspension that migrates to the lower compartments following a proper incubation time. Once the cells pass through the membrane, they adhere to it and can be fixed and stained for quantification.

The modified Boyden Chamber consists of two chambers separated with a cell-permeable membrane [11]. In our case in particular, we used a 48-well modified Boyden Chamber. On the other hand, the Transwell assay consists of a permeable cell culture insert nested inside the well of a culture plate. The inserts are separated from the wells by a defined-size porous membrane creating a two chamber system. In both methods the membrane is previously coated with fibronectin to assess cell migration or with Matrigel®, a commercially available mix of extracellular proteins, to study cell invasion.

Gelatin Zymography: This is an economic assay that allows the detection of active MMP-2 and MMP-9 secreted to the culture medium. Because no special equipment or reagents are needed, it might be performed in most laboratories. The technique is an electrophoretic approach based on the ability of MMP-2 and MMP-9 to degrade gelatin present in a matrix of polyacrylamide copolymerized with gelatin. This assay does not strictly allow establishing invasiveness capability, but can provide information about the ability of a S1P to induce the reassemble of ECM. Furthermore, it should be noticed that the method described in this chapter permits to detect only active MMP-2 and MMP-9, but other MMPs and proteases might also be secreted and promote ECM changes. Other MMPs members can also be assayed by similar methods using specific substrates.

In summary, the different tests described here are useful to study the role of S1P in migration and invasion of different cultured cells.

2 Materials

All solutions must be prepared with distilled water and analytical grade reagents. Store the solutions at room temperature (RT) unless otherwise indicated.

1. 100 mm culture petri dishes (Corning Incorporated, Corning, NY, USA; Cat. N° 430293).
2. Phosphate buffered saline (PBS): 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 120 mM NaCl, pH 7.4. Store at 4 °C.
3. Dulbecco's modified Eagle's medium (DMEM, Gibco, USA. Cat N° 12100-046). Store at 4 °C.

4. Minimum essential medium (MEM, Gibco, USA; Cat. N° 61100-061). Store at 4 °C.
5. Fetal bovine serum (FBS, Natocor, Córdoba, Argentina). Store at −20 °C.
6. Trypsin solution (1/250 activity, 0.5 mM EDTA). Store at 4 °C.
7. Neubauer chamber.
8. Sphingosine-1-phosphate, D-erythro (Enzo Life Sciences, Farmingdale, NY, USA. Cat N° BML-SL140). Prepare a *10 μM S1P working solution* according to the manufacturer recommendations. Briefly, dissolve lyophilized S1P in absolute methanol (Merck, Germany) to obtain a *1 mM S1P stock solution*. Sonicate stock solution until S1P is completely suspended (the solution will be cloudy but no obvious suspended particles should be visible). S1P stock solution can be aliquoted and conserved at −20 °C for at least 1 year. Before the assay, transfer the desired amount of S1P stock solution into a siliconized glass tube and evaporate methanol under an atmosphere of dry nitrogen or in the hood. Add DMEM supplemented with bovine serum albumin (BSA) 0.4% (BSA, fatty acid, and γ-globulin free; Sigma, St. Louis, MO, USA) to prepare a *10 μM S1P work solution*. Perform four cycles of vortex and bath sonication, 1 min each. *S1P working solution* can be later properly diluted in serum-free DMEM or MEM to perform each assay. In our experience this solution is stable for 1 week when stored at −20 °C.

2.1 Migration Assays

1. SkMel2 melanoma cell line.

2.1.1 Wound Healing Assay

2. 12-well plates.
3. Yellow pipette tips (20–200 μl).
4. Photographic camera.
5. Image Analysis Software (ImageJ).

2.1.2 Transwell Assay

1. Lu1205 melanoma cell line.
2. Transwell® Permeable Supports, polycarbonate inserts, 6.5 mm membrane diameter, 8 μm membrane pore size (Corning Incorporated, Corning, NY, USA; Cat. N° 3422).
3. 0.1% crystal violet in 20% ethanol.
4. Ice-cold absolute methanol.
5. Distilled water.
6. Cotton swabs.
7. 10% acetic acid/PBS (v/v) solution.
8. Plate spectrophotometer (Epoch, Biotek Instruments, USA).

2.1.3 Modified Boyden Chamber Assay

1. M2 melanoma cell line.
2. 48-well Micro Chemotaxis Chamber (Neuroprobe, Gaithersburg, MD, USA; Cat. N° AP48).
3. 8 μm diameter pore polycarbonate membranes 25 × 80 mm (Neuroprobe, Cat. N° 417-0014). Diameter pore of the membrane should be selected empirically to suit the shape and size of the cells to be used (see Note 1).
4. 10 μg/ml fibronectin (Sigma, St. Louis, MO, USA; Cat. N° F-0895) solution prepared in PBS. To perform the membrane coating, add fibronectin solution in a container and place the shiny side of the membrane in contact with the solution overnight at 4 °C. Next day hang the membrane with a clamp and air-dry at RT.
5. Absolute methanol.
6. Toluidine blue staining solution: 1% toluidine blue, 1% $Na_2B_4O_7 \cdot 10H_2O$.
7. Cotton swabs.
8. Forceps.
9. Containers to perform the membrane staining.

2.2 Invasion Assays

2.2.1 Modified Boyden Chamber

1. M2 melanoma cell line.
2. 48-well Micro Chemotaxis Chamber (Neuroprobe, Gaithersburg, MD, USA; Cat. N° AP48).
3. Staining solution: 0.5% Coomassie R-250 dissolved in 5% methanol/10% acetic acid solution.
4. Decoloring solution: 10% methanol/5% acetic acid.
5. 8 μm diameter pore polycarbonate membranes 25 × 80 mm (Neuroprobe, Cat. N° 417-0014) (see Note 1). Membrane should be coated with Matrigel® prepared according to manufacturer instructions (Corning, Matrigel® Growth Factor Reduced Cat N° 356231). Matrigel® is a solubilized basement membrane preparation extracted from a mouse sarcoma [12]. It is hazardous and very thermosensitive; thus it should be prepared in sterile conditions and using an ice bath. Appropriate aliquots should be preserved at 20 °C.
   (a) *Membrane coating.*

   Before coating the membrane, be sure to have all the sterile materials at 4 °C since Matrigel® is very sensitive and polymerize at RT. First, thaw a Matrigel® aliquot ON at 4 °C on an ice bath. Then, dilute Matrigel® in serum-free (SF) culture medium (appropriate dilution range is between 1:3 and 1:10 and should be determined empirically according to the cell line employed; see Note 2) and keep the solution on ice. Next, place the Matrigel® solution

using cooled tips on a glass surface since plastic may interfere with Matrigel® polymerization. Quickly set the polycarbonate membrane onto the Matrigel® with the brilliant surface in contact with the solution. Immediately, remove the membrane and place on a glass surface with the brilliant surface up. Allow Matrigel® to polymerizate during 48 h in the hood. Before using, rehydrate the membrane in serum-free culture medium.

(b) *Polymerization control.*
Cut a piece of the Matrigel®-coated membrane to stain with a solution of Coomassie Brilliant Blue R-250 (see Sect. 2.2.1, item 3). After rehydration step, stain the membrane with 0.5% Coomassie Brilliant Blue R250 solution for 2 h and then perform destaining with decoloring solution (see Sect. 2.2.1, item 4). Membrane should be uniformly blue stained indicating that the membrane is properly coated.

6. Absolute methanol.
7. Toluidin blue staining solution: 1% toluidine blue, 1% $Na_2B_4O_7{\cdot}10H_2O$.
8. Cotton swabs.
9. Forceps.

2.2.2 Gelatin Zymography Assay

1. Fresh M2 melanoma cell line soluble supernatant (see Note 3). To obtain the supernatant, culture 1×10^5 M2 melanoma cells in MEM 10% FBS for 48 h (the amount of cells will depend on the cell line employed and should be determined in each case). Serum starve the cells ON and stimulate with 100 nM S1P for 24 and 48 h. Collect supernatant in sterile microtubes and centrifuge at $500 \times g$ 5 min at 4 °C to eliminate cells and debris. Finally, transfer the supernatant to a microtube and preserve the samples at −80 °C (see Note 4). *Do not add protease inhibitor.*
2. Sample buffer 4×: 250 mM Tris–HCl pH 6.8 containing 40% glycerol, 8% SDS, 0.01% bromophenol blue. Store aliquots at −20 °C.
3. 8% Polyacrylamide gels copolymerized with porcine gelatin type A (final concentration 1 mg/ml, Sigma; Cat N°G1890). Prepare a stock solution of 10 mg/ml gelatin in distilled water and incubate at 37 °C for 30 min. Before use, make sure that gelatin is completely dissolved. This solution is stable for 1 week. Polyacrylamide gels should be prepared according to standard protocols [13].
4. Cold running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS.
5. Renaturing buffer: 50 mM Tris–HCl pH 7.6, 2% Triton X100.

6. Developing buffer: 50 mM Tris–HCl pH 7.6, 5 mM $CaCl_2$, 150 mM NaCl, 0.02% NaN_3.
7. Staining solution: 0.5% Coomassie R-250 dissolved in 5% methanol/10% acetic acid solution.
8. Decoloring solution: 10% methanol/5% acetic acid.
9. Plastic material: tips and plastic recipients.

3 Methods

3.1 Migration Assays

3.1.1 Wound Healing Assay

1. In a 12-well culture plate establish the area that will be used to asses migration. To this end, mark the bottom of the plate with two indelible lines for each well (Fig. 1a). Seed a homogeneous suspension of 20×10^4 SkMel2 cells/well in the labeled plate. Cells are cultured in DMEM supplemented with 10% FBS. Be sure that next day the cell culture is 100% confluent (see Notes 5–8).
2. Next day, make a scratch with a yellow pipette tip in one flowing movement on the center of the cell layer positioning the tip at 45° from the plate surface. The scratch should be in perpendicular direction respect to the indelible marks (Fig. 1a and Note 9).
3. Wash twice with PBS to remove the detached cells and the culture medium. This step should be done very quickly and carefully. Attention must be paid to add the PBS by the walls of the well, *never* onto the cell layer.
4. Add the corresponding stimuli to the cells. We used S1P 100 nM, 1 μM, 0.4% BSA and 10% FBS as positive control. Afterward, take pictures above and below the two indelible mark (4 pictures by well) to calculate the size of the beginning wound (0 h) (Fig. 1a). Place the plate in a CO_2 incubator at 37 °C.
5. After 18 h, take pictures to establish the migrated space.
6. Analyze the pictures with imaging software. We used the ImageJ software (https://imagej.nih.gov/ij/index.html). Measure the scratch width (SW) in three different positions and calculate the mean for each picture (Fig. 1b).
7. Subtract the final width over the initial width and analyze the data respect to the control (0.4% BSA) (see Note 10) (Fig. 2a).

3.1.2 Transwell Assay

1. Seed Lu1205 melanoma cells in DMEM supplemented with 10% FBS and allow them to grow until 95% confluence.
2. Starve Lu1205 cells from FBS overnight (ON).
3. Detach cells from the plate by washing with PBS and incubating 2–3 min with trypsin solution. Inactivate the trypsin with

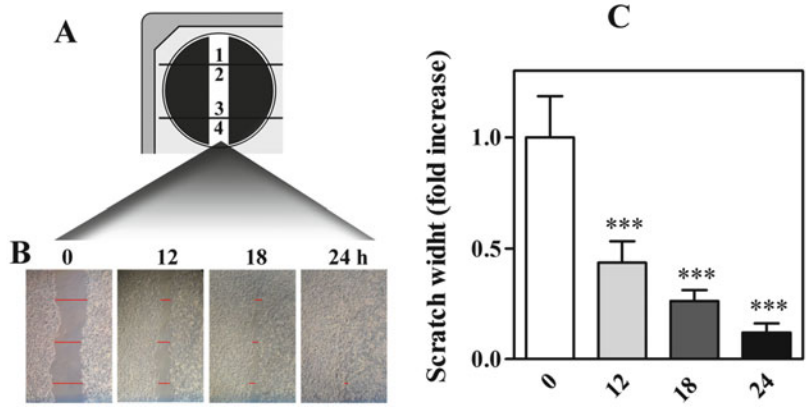

Fig. 1 Establishment of SkMel2 melanoma cell line migration time by wound healing assay. (**a**) Two indelible marks were made in each well on the bottom of the plate. SkMel2 cells were cultured until 100% confluence and scratch performed as described in the text. Four photographs were taken above and below each indelible mark (1–4). (**b**) Representative pictures taken after 0, 12, 18, and 24 h incubation. The scratch width (*red lines*) was measured at three different parts along the scratch. (**c**) Quantification of the data. According to this data we established the closure time at 18 h. The results are shown as the mean ± SD of three independent experiments and were analyzed with one-way ANOVA and Bonferroni's post test (***$p < 0.001$)

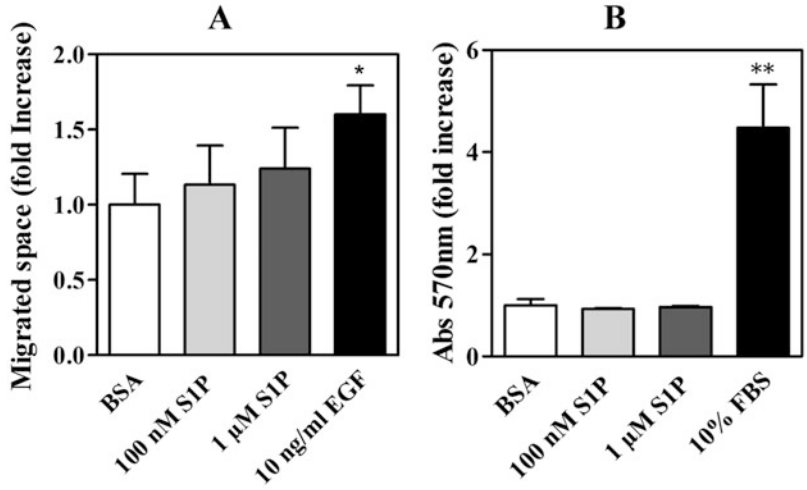

Fig. 2 Effect of S1P on melanoma cell migration. SkMel2 (**a**) and Lu1205 (**b**) melanoma cells were subjected to migration assay by Wound Healing or Transwell assay respectively. Cells were treated with S1P 100 nM and 1 μM during 18 (**a**) or 6 h (**b**). 10 ng/ml EGF was used as positive control. The results are shown as the mean ± SD of three independent experiments and were analyzed with one-way ANOVA and Bonferroni's post test (*$p < 0.1$; **$p < 0.01$)

DMEM 10% FBS and dilute in DMEM SF. Centrifuge 8 min at $700 \times g$ to collect the cells. Count cells in Neubauer Chamber and adjust with DMEM SF to prepare homogenous suspensions of 1.25×10^5 cells/ml.

4. Plate 200 μl of cell suspension (25×10^4 cells) on each insert. Incubate 10 min at 37 °C to allow the cells to settle down.
5. Add 750 μl of S1P at the desired concentrations on the lower compartment; the stimuli should be in contact with the insert. Avoid bubble formation and do not move the insert.
6. Incubate 6 h in a CO_2 incubator at 37 °C. The migrated cells will attach to the bottom of the insert.
7. Remove the insert and perform a crystal violet staining as follows:
   (a) Submerge the insert in PBS solution and clean the upper side containing the cells that did not migrate with a cotton swab. Be careful to not break the membrane. Repeat it twice.
   (b) Place the insert in a clean well filled with 500 μl of cold methanol to fix the cells. Incubate during 15 min at 20 °C.
   (c) Wash the insert as described in step **a**.
   (d) Place the insert in a clean well filled with 500 μl of 0.1% Crystal. Incubate 20 min at RT.
   (e) Repeat step **a** and let the insert dry at RT.
8. Observe the inserts under an inverted microscope at 20× magnification. Count the cells in at least five fields and average the number (see Note 11) (Fig. 2b).

3.1.3 Boyden Chamber

1. Since this method may be used to estimate both migration and invasion, we will describe it only once and remark the differences when proper.
2. Culture M2 melanoma cells in MEM 10% FBS in 100 mm plates during 48 h or until 80–90% confluence. To optimal stimulation of migration, cells should be serum-starved ON. Detach cells from the plate by washing with PBS and incubating 2–3 min with trypsin solution. Inactivate the trypsin with 1 ml of MEM 10% FBS. Quickly dilute the cell suspension in 9 ml of MEM SF since FBS is a source of S1P that may interfere in the experiments. Centrifuge 5 min at $500 \times g$ to collect the cells. Count and dilute the cells in MEM SF to prepare homogenous suspensions of 1×10^6 cells/ml.
3. Add in triplicates 29.5 μl (28–30 μl are optimal) of S1P in the lower wells of the chamber. This step should be performed quickly and carefully to avoid bubbles formation (see Note 12). Care should also be taken to avoid overloading the wells since cross-contamination may occur.
4. Use different concentrations of S1P ranging from 0.1 nM to 1 μM dissolved in MEM SF.

5. Include wells containing MEM SF with the appropriate amount of BSA (according to the S1P concentrations to be tested) to use as negative controls.
6. To establish whether S1P induces chemotaxis, chemokinesis, or both, you can add different gradients or identical concentrations in the upper and lower wells of the Boyden Chamber (see Note 13).
7. Carefully take the membrane, coated with fibronectin to study migration (see Sect. 2.1.3) or with Matrigel® to analyze invasion (see Sect. 2.2.1) with two clean forceps. Place the membrane with the brilliant surface up on the top of the wells already filled with the chemo-attractants. Once the membrane is on place, it should not be moved because it could lead to cross-contamination of the wells.
8. Quickly assemble the upper chamber, applying pressure and adjust the device with the screws provided.
9. Load 50 μl of the cell suspension prepared in step 1 (5×10^4 cells/well) on the upper wells. Care should be taken to avoid bubble formation.
10. To study migration, incubate 6 h in a humidified atmosphere (37 °C and 5% CO_2). To analyze invasion, incubate 24 h in the same conditions. The proper incubation time should always be determined experimentally.
11. Disassemble the device and mark the membrane to remember the adequate position (see Note 14).
12. Place the membrane on a container and fix with methanol during 5 min.
13. Discard the methanol, add the Toluidin staining solution, and incubate 5 min.
14. Wash several times with distilled water and use a cotton swab to remove the cells that did not migrate or invade (brilliant face of the membrane).
15. Hang the membrane with a clamp and air-dry at RT.
16. Scan the membrane and analyze the results using imaging software (Fig. 3). In our laboratory, we use the software Image Studio Lite 5.2 (LI-COR Biosciences, Lincoln, NE, USA), which is available for free download.

3.2 Invasion Assays

3.2.1 Zymography Assay

1. Mix the M2 supernatant culture medium with sample buffer 4× and incubate 15 min at RT. Do not heat.
2. Load the samples (30 μl/well) in an available protein electrophoresis apparatus. Run the gel at 4 °C (start with 80 V for 15 min and then 100 V for 90 min or until the bromophenol blue dye reaches the bottom of the gel) (see Note 15).

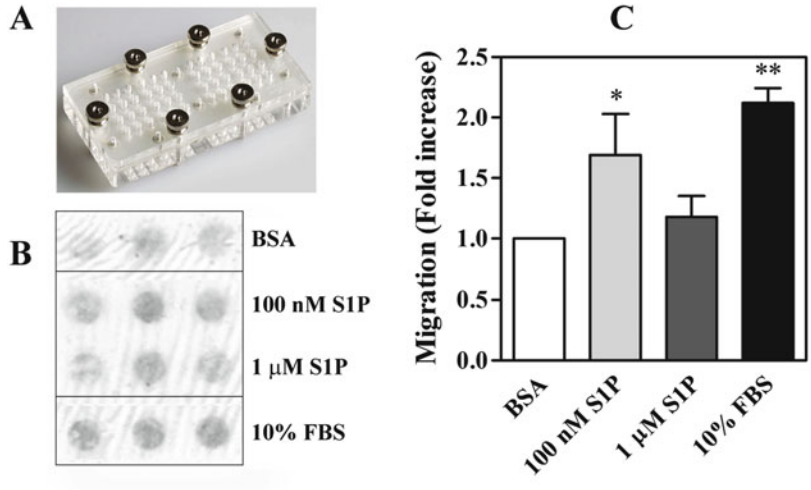

Fig. 3 Analysis of migration in the modified Boyden Chamber. (**a**) Migration assay was performed as described with M2 melanoma cells in a 48-well Boyden Chamber. (**b**) Representative photography of a stained membrane after 6 h incubation. (**c**) Quantification of migration using Image Studio Lite 5.2 software. The results are shown as the mean ± SD of three independent experiments and were analyzed with one-way ANOVA test (*$p < 0.1$; **$p < 0.01$)

3. Carefully remove the gel from the electrophoresis apparatus and wash it gently with distilled water to eliminate SDS residues. Then, incubate the gel with renaturing buffer in a rocking platform for 1 h at RT. Replace the buffer every 15 min.
4. Place the gel on developing buffer and incubate in a rocking platform for 30 min at RT. Replace the buffer and incubate the gel at 37 °C for 18–48 h in a closed tray. In our case, for M2 melanoma cells, we incubated for 48 h, but the right time should be determined empirically.
5. Decant developing solution and wash the gel with distilled water. Add Coomassie R-250 solution and perform the staining for 2 h at RT with mild agitation until the gel gets uniformly blue dark. Save the staining solution because it might be reused several times.
6. Destain the gel with decoloring buffer until gelatinolytic activity is evidenced as clear bands in the blue background of the gel (Fig. 4a) (see Note 16).
7. Scan the gel (see Note 17) and analyze the results with imaging software (Fig. 4b). We employed Image Studio Lite 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

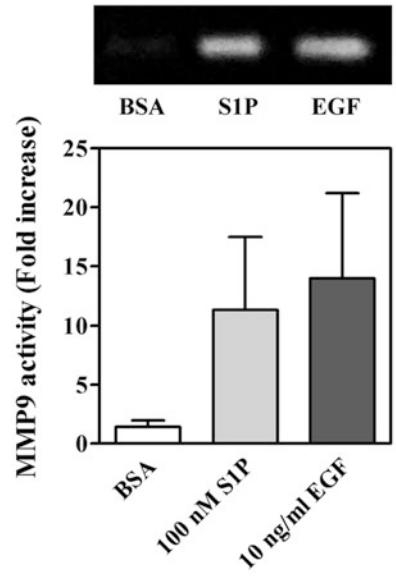

Fig. 4 Zymography assay. M2 melanoma cells were stimulated with 100 nM S1P or 10 ng/ml EGF for 48 h. Fresh supernatant culture medium were obtained and subjected to electrophoresis in polyacrylamide gels copolymerized with gelatin. After MMP-9 activity development, gel was stained and photographed (a representative gel is shown)

4 Notes

1. Choosing the right pore diameter is critical for the analysis. If the pores are too big, nonspecific dropping of the cells will occur; conversely, pores too small will avoid any migration since cells will not be able to squeeze through them.
2. We suggest trying several dilutions of the Matrigel® solution to determine the best concentration for any particular cell. High concentrations of Matrigel® solution will make difficult for invasive cells to go through. On the contrary, too low concentrations will not offer a logical barrier to differentiate between migration and invasion.
3. Although we employed supernatant of culture medium to perform the assay, it is also possible to use total cell lysates or tissue homogenates. In these cases, it is unlikely to distinguish between active MMP-2/9 and zymogene forms.
4. To perform zymography, fresh supernatant medium should be used without addition of protease inhibitor or serum since they may inhibit MMPs activity. Furthermore, it is recommendable to keep the samples at −80 °C no more than 2 weeks to avoid a significant decrease in the activity of MMPs.

5. Wound healing assay could be performed with different cell lines. Tests have to be done to determine the time and number of cells to achieve 100% confluence at the time of the assay.
6. Before performing the wound healing assay, it is important to establish the scratch closure time of the cells that will be used. To this purpose, we used normal culture conditions (DMEM 10% FBS) and we took pictures at different hours (0, 12, 18, and 24 h). We established the closure time at 18 h (Fig. 1b and c). In parallel, we carried cell viability assay (MTT assay), to check whether or not proliferation could mask the effect observed in the closure scratch. In our case, proliferation of SkMel2 cells after 18 h is nonsignificant. However, for cell lines that require over 24 h incubation time to detect significant migration, it is convenient to use a proliferation inhibitor (such as mitomycin C) to avoid changes in cell number that could affect the conclusion of the assay.
7. To establish the closure time and determine the filling of the wound, it is advisable to graph scratch width respect to time zero (0 h) (Fig. 1c).
8. Cells that are not properly attached move to the middle of the well and precipitate on the bottom, filling the scratch. This could lead to a false positive migration analysis. Since this effect is more pronounced in small wells, we strongly recommend using plates with no more than 12 wells to perform the assay.
9. Although the method is reproducible, practice in making the scratch is absolutely necessary. Thus, we strongly advice to take at least four photos along the scratch and perform at least triplicate measurements in each photo (Fig. 1a) Alternatively you can perform the scratch with specialized culture inserts from Ibidi (Fitchburg, WI, USA).
10. The size, width, and shape of the scratch will vary. Therefore, after establishing the closure time (see Note 7) we recommend making the calculations using the variation in scratch width ($SW_{initial} - SW_{final}$) and graph as relative migrated space (Fig. 2a). A suitable alternative is to measure the area of the scratch.
11. Alternatively, use 10% acetic acid to elute the dye and measure the absorbance at 570 nm.
12. The volume to load in the lower chamber will be appropriate when a small positive meniscus is formed in the well. To avoid trapping bubbles, the liquid should be ejected with a rapid motion, but not completely from the pipette tip.
13. To study chemokinesis S1P should be loaded at the same concentration in both lower and upper wells. On the other

hand, to examine chemotaxis S1P should be loaded only in the lower well to create a concentration gradient.

14. To keep track of the membrane orientation, you can either cut one of its corners before mounting and orient the membrane according to the Neuro Probe trademark or make a cut after disassembling the Boyden Chamber.
15. It is advisable to carry out the electrophoresis at 4 °C to avoid MMPs degradation. Although it is possible to use a higher voltage, we do not recommend exceed 120 V to prevent overheating the samples.
16. The proper amount of sample will depend on the MMP activity. Since the technique is not very sensitive, we recommend the following:
    (a) *Always use a positive control.* Several growth factors may induce MMP activity. To this end, in our laboratory we employ epidermal growth factor (EGF).
    (b) If no clear bands are visible, load a higher amount of sample. Eventually, concentrate the samples by ultracentrifugation using an appropriate filter.
    (c) To improve the sensitivity of the assay, a fluorescent matrix of FITC or rhodamine-gelatin can be used. The gelatinolytic activity may be evidenced as dark areas in a fluorescent background [14].
17. Alternatively, take a photograph of the destained gel and analyze.

Acknowledgments

We thank Y. Ohta and T. Stossel (Brigham and Women's Hospital) for the M2 cells and Pablo L. Bergami for the Lu1205 and SkMel2 cells. This work was supported by grants from ANPCyT-Argentina PICT 1659-2010 and 2378-2014 (S. Alvarez).

References

1. Maceyka M, Spiegel S (2014) Sphingolipid metabolites in inflammatory disease. Nature 510(7503):58–67. doi:10.1038/nature13475
2. Pyne NJ, Ohotski J, Bittman R, Pyne S (2014) The role of sphingosine 1-phosphate in inflammation and cancer. Adv Biol Regul 54:121–129. doi:10.1016/j.jbior.2013.08.005
3. Rodriguez YI, Campos LE, Castro MG, Aladhami A, Oskeritzian CA, Alvarez SE (2016) Sphingosine-1 phosphate: a new modulator of immune plasticity in the tumor microenvironment. Front Oncol 6(218). doi:10.3389/fonc.2016.00218
4. Campos LS, Rodriguez YI, Leopoldino AM, Hait NC, Lopez Bergami P, Castro MG, Sanchez ES, Maceyka M, Spiegel S, Alvarez SE (2015) Filamin A expression negatively regulates sphingosine-1-phosphate-induced NF-kappaB activation in melanoma cells by inhibition of Akt signaling. Mol Cell Biol 36 (2):320–329. doi:10.1128/MCB.00554-15

5. Maceyka M, Alvarez SE, Milstien S, Spiegel S (2008) Filamin A links sphingosine kinase 1 and sphingosine-1-phosphate receptor 1 at lamellipodia to orchestrate cell migration. Mol Cell Biol 28(18):5687–5697
6. Goparaju SK, Jolly PS, Watterson KR, Bektas M, Alvarez S, Sarkar S, Mel L, Ishii I, Chun J, Milstien S, Spiegel S (2005) The S1P2 receptor negatively regulates platelet-derived growth factor-induced motility and proliferation. Mol Cell Biol 25(10):4237–4249
7. Bretscher MS (2008) On the shape of migrating cells—a "front-to-back" model. J Cell Sci 121(Pt 16):2625–2628. doi:10.1242/jcs.031120
8. Vicente-Manzanares M, Horwitz AR (2011) Cell migration: an overview. Methods Mol Biol 769:1–24. doi:10.1007/978-1-61779-207-6_1
9. Quail DF, Joyce JA (2013) Microenvironmental regulation of tumor progression and metastasis. Nat Med 19(11):1423–1437. doi:10.1038/nm.3394
10. Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9(4):239–252. doi:10.1038/nrc2618
11. Boyden S (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 115:453–466
12. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21(24):6188–6193
13. Toth M, Sohail A, Fridman R (2012) Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods Mol Biol 878:121–135. doi:10.1007/978-1-61779-854-2_8
14. Bowden ET, Coopman PJ, Mueller SC (2001) Invadopodia: unique methods for measurement of extracellular matrix degradation in vitro. Methods Cell Biol 63:613–627

Methods in Molecular Biology (2017) 1697: 133–140
DOI 10.1007/7651_2017_4

Published online: 22 March 2017

Maintenance of Human Embryonic Stem Cells by Sphingosine-1-Phosphate and Platelet-Derived Growth Factor

Raymond C.B. Wong, Martin F. Pera, and Alice Pébay

Abstract

Human embryonic stem cells (hESCs) have historically been cultivated on feeder layers of primary mouse embryonic fibroblasts (MEF) in a medium supplemented with fetal calf serum (FCS). However, serum contains a wide variety of biologically active compounds that might adversely affect hESC growth and differentiation. Thus, cultivation of stem cells in FCS complicates experimental approaches to define the intracellular mechanisms required for hESC maintenance. This chapter describes the serum-free maintenance of hESCs in culture by addition of sphingosine-1-phosphate (S1P) and platelet-derived growth factor (PDGF). This complete protocol provides a simple alternative chemically defined serum-free system that is relatively inexpensive and advantageous for studying signaling pathways involved in hESC pluripotency.

Keywords: Human embryonic stem cells, Platelet-derived growth factor, Serum-free medium, Sphingosine-1-phosphate

1 Introduction

Human embryonic stem cells (hESCs) are pluripotent stem cells derived from the inner cell mass of the blastocyst [1, 2]. They have great potential for developing cell replacement therapy and as research tools for understanding early human development. hESCs have a tendency to spontaneously differentiate and are difficult to maintain in vitro. The first hESC lines were initially derived and maintained on a feeder layer of mouse embryonic fibroblasts (MEF) in a medium supplemented with high doses of FCS [1, 2]. However, different serum batches vary widely in their ability to maintain hESCs, rendering the use of serum undesirable. Secondly, serum is a complex mixture that contains compounds that may be harmful to the growth of hESCs. Moreover, there is a potential biosafety concern of cross species transmission of pathogens such as viruses if cells cultured in animal sera are subsequently used for implantation into humans. Alternative approaches toward a serum-free culture system have been described, such as the use of a complex serum replacement, knockout serum replacement (KSR)

plus basic fibroblast growth factor (bFGF) [3], or mTeSR medium [4]. We previously demonstrated that two serum components, sphingosine-1-phosphate (S1P) and platelet-derived growth factor (PDGF), inhibit hESC spontaneous differentiation, maintain hESC proliferation, and inhibit hESC apoptosis in the absence of serum [5, 6]. Furthermore, we showed that hESC express the receptors for S1P and PDGF, $S1P_{1-3}$, PDGFR-α, and PDGFR-β [5]. We also demonstrated a critical role of the ERK1/2 and PI3K/AKT signaling pathways in mediating the antiapoptotic effect of S1P and PDGF on hESCs. This chapter describes the procedures for long-term maintenance of hESCs in a chemically defined serum-free system using S1P and PDGF. This system can be used either for short-term support in the absence of a feeder cell layer or long-term growth in the presence of feeder cells. For short-term experimentation, the system enables mechanistic studies of signaling pathways in the absence of either FGF2 or activing. For long-term propagation, the system combines the advantages of a defined liquid medium with the genetic stability associated with the use of feeder cells.

2 Materials

2.1 General (See Note 1)

1. Stereo microscope (Leica MZ6, Leica Microsystems) and microscope stage (Leica Microsystems).
2. MEF are expanded in T75 tissue culture flasks (e.g., 75 cm^2 Blue Plug Seal Cap Tissue Culture Treated, BD Falcon #353135).
3. For hESC maintenance, we culture hESCs in organ culture dishes, 60 × 15 mm style (35 mm culture dishes, BD Falcon #353037).
4. Sphingosine-1-phosphate, D-erythro (Biomol, #SL140).
5. Human recombinant Platelet Derived Growth Factor-AB (PDGF-AB; Pepro Tech Inc. #100-00AB).
6. Phosphate-buffered saline solution without calcium and magnesium (PBS^-) 10×: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4 or 1× PBS^- from Invitrogen (Invitrogen #14190).
7. Phosphate-buffered saline with calcium and magnesium (PBS^+, Invitrogen #14040).
8. Dispase (Invitrogen, #17105-041, *see* **Note 2**).
9. Ethanol and methanol (Merck).
10. Distilled water (Invitrogen #15230-204).
11. Dulbecco's Modified Eagle's Medium High Glucose (DMEM, Invitrogen #11960).

12. Fetal calf serum (FCS, Invitrogen #16000).
13. Insulin-transferrin-selenium-A supplement (ITS-A supplement, Invitrogen #41400, *see* **Note 2**).
14. L-glutamine (Invitrogen #25030).
15. Nonessential amino acids solution (NEAA, Invitrogen #11140-050, *see* **Note 3**).
16. Penicillin-streptomycin (Invitrogen #15070, *see* **Note 4**).
17. HEPES buffer (Invitrogen # 15630106).
18. Mitomycin C from *S. Caespitosus* (Sigma-Aldrich #M0503-10x2mg). Mitomycin C is cytotoxic, mutagenic, and carcinogenic; appropriate safety measures must be implemented for use and disposal of this agent.
19. β-Mercaptoethanol (Sigma #M7154).
20. 0.22 μm syringe filters (Sartorius) and 500 mL bottle filters (Millipore).
21. Gelatin (Type A from porcine skin, Sigma #G1890). Stocks of 1 % gelatin can be made in distilled H_2O, autoclaved, and stored at room temperature.
22. 0.25 % trypsin-EDTA (Invitrogen #25200-056).
23. Bovine serum albumin, essentially fatty acid-free (BSA, Sigma #A7030) or human serum albumin (Sigma #A3782).
24. All tissue culture procedures are performed using sterile techniques in class II biological safety cabinets.

3 Methods

3.1 Preparation of Mouse Embryonic Fibroblasts (MEF) Feeder

3.1.1 Preparation of Mouse Embryonic Fibroblasts Culture Medium (F-DMEM)

1. Supplement DMEM with 10 % FCS, 25 U/mL penicillin (0.5 %), 25 μg/mL streptomycin (0.5 %) and 2 mM L-glutamine.
2. Mix well, sterilize using a pre-wet 0.22 μm filter, and store at 4 °C.

3.1.2 Preparation of Mitomycin C Solution

1. Dissolve 2 mg of mitomycin C in 4 mL of distilled H_2O (0.5 mg/mL final).
2. Filter sterilize using 0.22 μm filter.
3. Keep solution protected from light and store at 4 °C.
4. When ready to use, dilute to 10 μg/mL in F-DMEM.

3.1.3 Preparation of Trypsin-EDTA Solution

1. Dilute 0.25 % trypsin-EDTA with PBS^- to obtain 0.05 % working stock.
2. Filter sterilize, aliquot, and store at 20 °C. Once thawed, it is recommended that an aliquot should be used within a week.

3.1.4 Gelatinized Plates for Culturing MEF

1. Stock solutions of gelatin are diluted to 0.1 % using distilled H_2O and filter sterilized.
2. Coat plates with 0.1 % gelatin and keep at room temperature for at least 20 min.
3. Aspirate the gelatin solution. Plates are now ready to use for plating cells.

3.1.5 Derivation of MEF

MEF are isolated from decapitated and eviscerated late midgestation mouse fetuses from E12.5–14.5 embryos. Usually about three embryos are used to obtain a confluent T75 flask of fibroblasts:

1. Extract embryos from placental tissue.
2. Remove head and visceral tissue (including fetal liver).
3. Transfer remaining fetal tissue to a clean dish and wash three times with PBS^- by transferring tissue to a new dish with PBS^-.
4. Mince the tissue using either scissors or scalpel blade.
5. Add 2 mL of 0.5 % trypsin-EDTA and continue mincing.
6. Add an additional 5 mL of trypsin-EDTA and incubate for 20 min at 37 °C.
7. Pipette to break up the embryos in the trypsin-EDTA until only a few chunks remain. Incubate for another 10 min at 37 °C. The aim is to get a single cell suspension.
8. Add 10 mL F-DMEM to neutralize the trypsin and transfer contents to a 50 mL tube.
9. Mix well and transfer all to T75 flask (*see* **Note 5**).
10. The next day, change medium to remove cell debris and dead cells.
11. When the flask reaches 70–80 % confluency, freeze down the cells and labelled as passage 0. At this stage the cells grow very quickly and can be expanded at a passage ratio of 1:5.
12. Culture for 2 days until confluent. Dissociate into single cells by treatment with 0.05 % trypsin-EDTA for 3 min at 37 °C, then inactivate trypsin by fresh F-DMEM and passage on to new gelatinized flasks (becomes passage 1).
13. Expand the MEF stock by passaging with 0.05 % Trypsin-EDTA every 2 days until passage 3 (*see* **Note 6**).
14. We typically use MEF at passage 3 as feeder layer. Prior to this, the MEF need to be mitotically inactivated by mitomycin C treatment as described below.

3.1.6 Mitomycin C Treatment of MEF

After three passages, MEF are treated with mitomycin C in order to arrest cell division:

1. Aspirate existing medium from flask containing MEF cultures.
2. Add mitomycin C (10 μg/mL, Section 3.1.2) made in warm F-DMEM to each flask.
3. Incubate at 37 °C for 2.5 h.
4. Aspirate medium containing mitomycin C.
5. Wash flask once with F-DMEM and twice with PBS^{-}.
6. Add 0.25 % Trypsin-EDTA solution (*see* **Note 7**) and incubate at 37 °C for 3 min.
7. Slightly agitate the flask until cells start to detach.
8. Collect the cells with F-DMEM and transfer into a falcon tube.
9. Spin the cells at 600 × *g* for 2 min.
10. Aspirate the medium and resuspend the cells with 10–15 mL of F-DMEM.
11. Obtain a 10 μL sample and perform a cell count using a hemocytometer to calculate cell number.
12. Plate MEF on gelatinized organ culture dishes at a density of 6×10^4 cells/cm^2, which corresponds to 1.7×10^5 cells/organ culture dish. Allow MEF to attach overnight at 37 °C with 5 % CO_2 in a humidified incubator. The MEF dishes are now ready to be used for passaging of hESCs.

3.2 Maintenance of hESCs in S1P and PDGF Serum-Free Medium

3.2.1 Preparation of S1P and PDGF Serum-Free Medium

1. Dissolve S1P in methanol to obtain a stock concentration of 3.3 mM and store at −20 °C.
2. Extemporaneous dilutions are made in 0.1 % fatty acid-free BSA (final concentration 0.01 % BSA), alternatively human serum albumin can also be used. Mix well and sterilize using a 0.22 μm filter.
3. Supplement DMEM with 0.1 mM β-mercaptoethanol, 1 % NEAA, 2 mM L-glutamine, 25 mM HEPES, 25 U/mL penicillin, 25 μg/mL streptomycin, as well as 10 μM S1P and 20 ng/mL PDGF-AB.

3.2.2 Preparation of Dispase Solution

1. A 10 mg/mL solution of Dispase is made in pre-warmed DMEM supplemented with 20 % FCS, 0.1 mM NEAA, 2 mM L-glutamine, 25 U/mL penicillin, 25 μg/mL streptomycin, 0.1 mM β-mercaptoethanol, and 1 % ITS-A.
2. Incubate at room temperature for 5 min, mix gently, and leave for at least 15 min at room temperature.
3. Sterilize the solution using a 0.22 μm filter.
4. The solution can be used or stored at 4 °C for up to 2 days. Prior to use pre-warm Dispase to 37 °C.

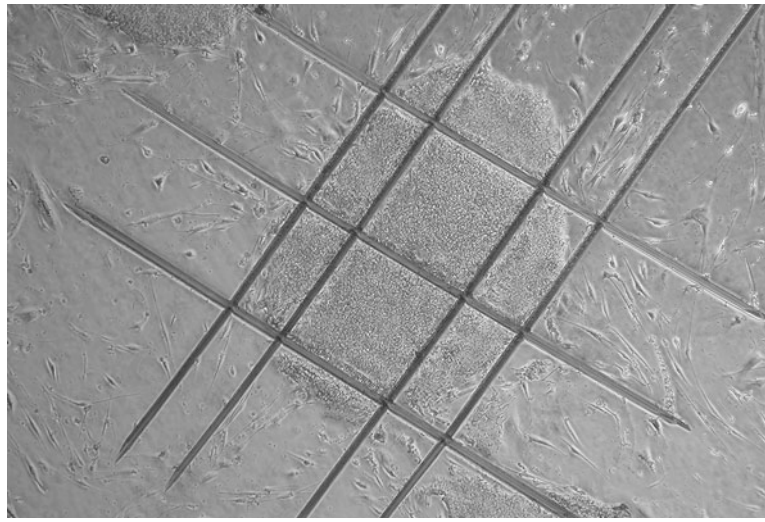

Fig. 1 Microscopic view of a hESC colony being cut into small pieces using a pulled glass pipette during mechanical passaging

3.2.3 Mechanical Passaging of hESC Colonies

hESCs are cultured as colonies on a feeder layer of mitotically inactivated MEF and are routinely passaged once a week. The method used for hESC passage is by mechanical dissociation of colonies, combined with dispase treatment, to dissect the colony into "pieces." Dissection of hESC colonies is performed using a stereo microscope with a 37 °C heated microscope stage. Each colony "piece" is then transferred onto a fresh MEF feeder layer:

1. Prepare 2 × 6 cm Petri dishes with PBS^+ for two washes.
2. Prepare dispase solution and filter sterilize (*see* Section 3.2.2).
3. Change the medium of hESC culture to DMEM.
4. Working with a stereomicroscope, cut hESC colonies into pieces (Fig. 1) using pulled glass pipettes or 27 gauge needles. Avoid cutting differentiated portions of partially differentiated colonies (*see* **Note 8**). To avoid tearing the hESC colony or MEF layer, each stroke should be smooth and only cut in one direction. Aspirate the DMEM.
5. Carefully aspirate DMEM but not hESCs. Add Dispase solution and incubate for 3 min at 37 °C on heated stage or until the edges of the sliced colony fragments start to curl up and detach.
6. Using a 20 μL micro-pipette, nudge the corner of the colony piece until it completely detaches, collect, and transfer to the PBS^+ wash dish.
7. Once all the colony pieces have been collected and transferred to the wash dish, transfer them to the second PBS^+ wash dish.

8. Finally, transfer the pieces to an organ culture dish containing a fresh feeder layer of mitotically inactivated MEF in S1P and PDGF serum-free medium. Usually about eight pieces are placed per organ culture dish, evenly spaced and neatly arranged in a circle.

3.2.4 Long-Term Maintenance of hESC in S1P and PDGF Serum-Free Medium

hESCs can be routinely maintained in the S1P and PDGF serum-free medium on MEF. The dishes of hESCs are kept at 37 °C with 5 % CO_2 in a humidified incubator. Medium is changed every 2 days, and hESCs are passaged weekly by mechanical slicing of the colonies as described in Section 3.2.3. We found that Day 7 hESC colonies are usually big enough and display substantial spontaneous differentiation, rendering them ready to be passage.

Recent evidence raised concerns regarding the genetic stability of hESCs after prolonged maintenance in vitro, including recurrent gain of chromosome 12p and 17q [7–9]. Therefore, all hESC lines should be subjected to regular G-banding karyotype analysis to screen for chromosomal aberrations as quality control. In our experience, we have not observed chromosomal abnormalities in hESCs maintained in the S1P and PDGF serum-free medium as described in this chapter.

4 Notes

1. Formats of culture are reflective of what we found to provide for the greatest ease of handling. These are only indicative and can be modified according to specific needs. In all cases, we culture hESCs with a feeder layer of MEF.
2. Dispase is a protease that cleaves adhesion molecules, thus allowing extraction of hESCs from MEF and the plastic surface of the dish. hESC colonies can be readily aspirated using a pipette, leaving the MEF layer intact on the dish. ITS-A supplement consists of 1 g/L insulin, 0.67 mg/L sodium selenite, 0.55 g/L transferrin, and 11 g/L sodium pyruvate.
3. Nonessential amino acid solution consists of 750 mg/L glycine, 890 mg/L L-alanine, 1320 mg/L L-asparagine, 1330 mg/L L-aspartic acid, 1470 mg/L L-glutamic acid, 1150 mg/L L-proline, and 1050 mg/L L-serine.
4. Pen/Strep solution consists of 5000 U/mL penicillin and 5000 μg/mL streptomycin.
5. In primary cultures of MEF preparations, blood cells, as well as large intact fragments of tissue, may be present along with fibroblasts. Generally these contaminating cell types disappear following subculture as the fibroblasts overgrow them. To use these MEF as feeder layers in hESC culture, it is necessary to

passage them a few times prior to use to minimize contamination of residual non-fibroblast cells.

6. It is not recommended to expand MEF more than passage 4 as their growth rate begins to slow down significantly as the cells senescence. Immortalized fibroblast cell lines may eventually emerge from longer-term cultures of MEF, but these cells do not function well as feeder cells.
7. The volume of trypsin-EDTA solution used for a T75 (75 cm^2)–T175 (175 cm^2) flask is 2–5 mL, respectively.
8. By 7 days after passage, hESC colonies show some regions of cell differentiation, particularly within the center of the colony. These regions are morphologically distinguished by cystic-like structures extending up from the colony.

Acknowledgments

A.P. is supported by an Australian Research Council Future Fellowship (FT140100047). The Centre for Eye Research Australia receives operational infrastructure support from the Victorian Government.

References

1. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399–404
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
3. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227:271–278
4. Ludwig TE, Bergendahl V, Levenstein ME, Yu J, Probasco MD, Thomson JA (2006) Feeder-independent culture of human embryonic stem cells. Nat Methods 3:637–646
5. Pebay A, Wong RC, Pitson SM, Wolvetang EJ, Peh GS, Filipczyk A, Koh KL, Tellis I, Nguyen LT, Pera MF (2005) Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells. Stem Cells 23:1541–1548
6. Wong RC, Tellis I, Jamshidi P, Pera M, Pebay A (2007) Anti-apoptotic effect of sphingosine-1-phosphate and platelet-derived growth factor in human embryonic stem cells. Stem Cells Dev 16:989–1001
7. Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA, Andrews PW (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22:53–54
8. Maitra A, Arking DE, Shivapurkar N, Ikeda M, Stastny V, Kassauei K, Sui G, Cutler DJ, Liu Y, Brimble SN, Noaksson K, Hyllner J, Schulz TC, Zeng X, Freed WJ, Crook J, Abraham S, Colman A, Sartipy P, Matsui SI, Carpenter M, Gazdar AF, Rao M, Chakravarti A (2005) Genomic alterations in cultured human embryonic stem cells. Nat Genet 37:1099–1103
9. Inzunza J, Sahlen S, Holmberg K, Stromberg AM, Teerijoki H, Blennow E, Hovatta O, Malmgren H (2004) Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod 10:461–466

Methods in Molecular Biology (2017) 1697: 141–151
DOI 10.1007/7651_2017_3

Published online: 31 March 2017

Sphingosine-1-Phosphate (S1P) Signaling in Neural Progenitors

Phillip Callihan, Mohammed Alqinyah, and Shelley B. Hooks

Abstract

Sphingosine-1-phosphate (S1P) and its receptors are important in nervous system development. Reliable in vitro human model systems are needed to further define specific roles for S1P signaling in neural development. We have described S1P-regulated signaling, survival, and differentiation in a human embryonic stem cell-derived neuroepithelial progenitor cell line (hNP1) that expresses functional S1P receptors. These cells can be further differentiated to a neuronal cell type and therefore represent a good model system to study the role of S1P signaling in human neural development. The following sections describe in detail the culture and differentiation of hNP1 cells and two assays to measure S1P signaling in these cells.

Keywords: Adenylyl cyclase, cAMP, Differentiation, G-protein-coupled receptor, Inositol phosphates, Neural progenitor cells, Phospholipase C, S1P, S1P receptor, Second messenger, Sphingosine-1-phosphate, Stem cells

1 Introduction

Studies suggest a crucial role for sphingosine-1-phosphate (S1P) and its receptors in the development of the nervous system [1–3] and in neural progenitor cells [4–8]. More detailed understanding of the pharmacology of S1P receptor signaling in human neural precursor cells is necessary to define the role of S1P in neural development. We have reported that S1P regulates the biology of human embryonic stem cell-derived neuroepithelial progenitor cells (previously termed hES-NEP, currently hNP1) through multiple functional S1P receptors. hNP1 cells are derived from WA09 human embryonic stem cells (WiCell Research Institute) [9] and are both morphologically and phenotypically similar to neural progenitor cells in vivo. hNP1 cells are useful for in vitro studies because they are an adherent, self-renewing population of cells that maintain the capacity to differentiate into neurons and glia. These characteristics make hNP1 cells a powerful model system to study S1P signaling in human neural progenitor cells.

Our previous studies have shown that hNP1 endogenous S1P receptors mediate adenylyl cyclase inhibition, phospholipase C activation, Gi-mediated cell proliferation, and Rho-dependent cell

contraction [10]. To study the functionality of S1P receptors in hNP1, we used radiolabeled assays that measure the second messengers cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP_3), generated in cells by adenylyl cyclase and phospholipase C, respectively. Adenylyl cyclase is stimulated by Gαs and inhibited by Gαi G-proteins, and phospholipase C (PLC) is typically activated downstream of activated Gαq but also by Gβγ following activation of Gi-coupled receptors. Given that all S1P receptors couple to either Gi or Gq (or both), the use of these two assays allows complete characterization of endogenous S1P receptor activation. More recently, we have used this approach to compare the metabolism and molecular pharmacology of S1P and its reduced form, dihydrosphingosine-1-phosphate (dhS1P) in human neural progenitors [11]. Finally, hNP1 cells are easily differentiated into neurons in vitro, which expand their role as a model system to include differentiated neurons and the differentiation process. We have used this system to explore the ability of S1P and the related lysophospholipid lysophosphatidic acid (LPA) on neural progenitor survival, neuronal differentiation, and neurite outgrowth [12]. Finally, we have used this cell system to identify regulator of G-protein signaling (RGS) gene expression profiles in neural progenitors and differentiated neurons [13]. Thus, this is a versatile and robust in vitro model for human neural progenitors.

The following sections describe the culture and differentiation of hNP1 cells and the protocols for measuring accumulation of cAMP and IP_3 in hNP1 cell lines.

2 Materials

STEMEZ™ hNP1™ (Aruna Biomedical), poly-ornithine (Sigma-Aldrich), laminin (Sigma-Aldrich), HyClone® DPBS/MODIFIED (1×) containing calcium and magnesium (Thermo Scientific), Matrigel (BD Biosciences), NEUROBASAL™ medium (GIBCO), AB2™ media with ANS™ (Aruna Biomedical), L-glutamine (Sigma), recombinant human b-FGF (R&D Systems), penicillin/streptomycin (Invitrogen), Dowex AG-50W-X4 (200-400mesh) (Bio-Rad, Hercules, CA), [^{3}H]-adenine (PerkinElmer), neutral alumina (Sigma), pertussis toxin (US Biologics), isobutylmethylxanthine (IBMX) (Sigma-Aldrich), adenosine 3′,5′-cyclic monophosphate (cAMP) (Sigma-Aldrich), [^{3}H]-myo-inositol (American Radiolabeled Chemical, St. Louis, MO), [^{14}C]-cAMP (GE Healthcare), 20 mL plastic scintillation vials (Fisher Scientific), concentrated perchloric acid (JT Baker), ScintiSafe 30 % Cocktail (Scintanalyzed) (Fisher Chemical), Dowex® AG 1-X8 (Bio-Rad, Hercules, CA), PBS++ (HyClone). Additional general chemicals (Sigma-Aldrich and/or Fisher Scientific): sodium dodecyl sulfate, imidazole, concentrated hydrochloric acid,

potassium hydroxide, lithium chloride, ammonium formate, formic acid, ammonium hydroxide.

2.1 Solutions

hNP1 Complete Growth Medium: AB2™ media with ANS™, 2 mM L-glutamine, 20 ng/mL b-FGF, 50 U/mL penicillin, 50 g/mL streptomycin. After preparation, media is stored at 4 °C for up to 1 week.

Differentiation Medium: AB2™ media with ANS™, 2 mM L-glutamine, 50 U/mL penicillin, 50 g/mL streptomycin. After preparation, media is stored at 4 °C for up to 1 week.

cAMP Stop Solution: 1.3 mM adenosine 3′,5′-cyclic monophosphate (cAMP) (Sigma-Aldrich), 2 % sodium dodecyl sulfate (Fisher Scientific), and 0.5 μCi/mL of [^{14}C]-cAMP (GE Healthcare).

IP Regenerant: 2 M ammonium formate, 0.1 M formic acid solution.

IP Wash Buffer: 0.5 M ammonium formate.

IP Elution Buffer: 1.2 M ammonium formate, 0.1 M formic acid.

Fixation solution: 8 % paraformaldehyde, 8 % sucrose in PBS++.

Block/permeabilization solution: 1 % BSA/0.1 % saponin in PBS^{++}.

3 Methods

3.1 Human Embryonic Stem Cell-Derived Neuroepithelial Progenitor (hNP1) Cell Culture

Commercially available stocks of hNP1 cells are available as STEMEZ™ hNP1™ from Aruna Biomedical (http://www.arunabiomedical.com/). The cell culture protocol for hNP1 cells is described in detail below.

1. hNP1 cells may be cultured on two different substrates: poly-ornithine/laminin or Matrigel. Tissue culture plates must be coated with these substrates prior to the addition of cells.
   (a) *Poly-ornithine/laminin coating:* Dilute poly-ornithine to 10 μg/mL in distilled, deionized water (ddH_2O). Cover plate with poly-ornithine and incubate at 37 °C for 1 h. Aspirate poly-ornithine and wash plate one time with ddH_2O. Dilute laminin to 5 μg/mL in sterile PBS. Cover plates with laminin and incubate at 37 °C for 1 h. Remove from incubator and store at 2–8 °C for up to 3 weeks. To prepare plates for use, aspirate poly-ornithine/laminin solution and wash one time with HyClone® DPBS/MODIFIED (1×) containing calcium and magnesium. Add media containing cells to flask, taking care not to disturb substrate.
   (b) *Matrigel coating:* Matrigel is diluted 1:2 in NEUROBASAL™ medium, aliquoted and stored at −20 °C. To prepare working solution, the Matrigel aliquot is thawed

on ice and diluted 1:100 in NEUROBASAL™ medium to a final Matrigel dilution of 1:200. Matrigel is then added to the tissue culture plate in a volume sufficient to cover the bottom of the plate. The plates are incubated at room temperature for 1.5 h and can then be used or stored at 4 °C for up to 1 week. Before adding media containing cells to Matrigel-coated plates, aspirate the Matrigel solution, and wash one time with HyClone® DPBS/MODIFIED (1×) containing calcium and magnesium.

2. hNP1 cells are maintained at 37 °C, 5 % CO_2. Cells are grown in hNP1 complete growth medium.
3. Cells are passaged approximately every 48 h and split 1:2 following manual dissociation using a cell scraper.

3.2 Adenylyl Cyclase Activity Assay

This assay measures accumulation of cAMP, which is generated in cells through the action of multiple adenylyl cyclase enzymes that convert ATP to cAMP. Adenylyl cyclase activity is regulated by Gαi and Gαs G-proteins. Gαi subunits inhibit adenylyl cyclase activity resulting in decrease in cAMP production, while Gαs subunits stimulate adenylyl cyclase leading to increased cAMP production.

To measure production of cAMP, cells are first labeled with [^{3}H]-adenine, which is incorporated into the cellular pool of ATP and hence cAMP generated from ATP. To measure accumulation of cAMP, cells are incubated with drugs of interest and a phosphodiesterase inhibitor to block the degradation of generated cAMP. Cells are then lysed, and the [^{3}H]-cAMP is separated from [^{3}H]-ATP precursors using ion exchange chromatography. To measure Gi-coupled inhibitory activity, adenylyl cyclase enzymes are first stimulated by forskolin and then incubated with drug.

The accumulation of cAMP is measured over 30 min in hNP1s. The level of cAMP accumulation is quantified and the recovery of each column is normalized using a [^{14}C]-cAMP label in the stop solution. We have measured cAMP production in hNP1 cells [10] using the following protocol, which is a modified version of previously described protocols [14, 15].

3.3 Dowex AG® 50W-X4 Cationic Exchange Resin Columns

1. Preparation:
   (a) Wash Dowex AG-50W-X4 (200–400 mesh) repeatedly with ddH_2O in a graduate cylinder, allowing the resin to settle and pouring off colored effluent. Repeat until clear.
   (b) Resuspend resin 1:1 with ddH_2O.
   (c) Add 2 mL of slurry to each column and allow water to drain by gravity flow.
   (d) Fill columns with ddH_2O for additional wash.
   (e) Wash columns with 4 mL 1 N HCl and allow to drain by gravity flow.
   (f) Cap and store at room temperature.

2. Equilibration:
   (a) Immediately prior to use, wash each column three times with 10 mL of ddH_2O, allow to drain by gravity flow.
3. Storage:
   (a) Following use of Dowex AG® 50W-X4 cationic exchange resin columns, wash one time with 4 mL of 1 N HCl. Cap and store at room temperature. Columns should be capped during storage to prevent the resin from drying out. Columns can be reused multiple times.

3.4 Neutral Alumina Columns

1. Preparation:
   (a) Add 0.5 g of dry neutral alumina per column.
   (b) Wash each column with 15 mL of 1 M imidazole buffer.
   (c) Wash each column with 15 mL of 0.1 M imidazole buffer.
2. Equilibration:
   (a) Wash each column one time with 8 mL of 0.1 M imidazole buffer.
3. Storage:
   (a) Following use, neutral alumina columns can be stored at room temperature and reused several times.

3.5 Adenylyl Cyclase Assay

1. Plate hNP1 cells in 24-well plates at 80 % confluency and allow to adhere to the plate at 37 °C for 24 h.
2. Aspirate media and add 0.25 mL of hNP1 complete growth media containing 0.6 μCi [^{3}H]-adenine per well. Incubate for 3 h at 37 °C in the presence or absence of 200 ng/mL pertussis toxin. In some experiments, cells are treated with pertussis toxin prior to the assay to define the role of Gi.
3. Aspirate media.
4. Add 0.25 mL of hNP1 complete growth media containing 1 mM isobutylmethylxanthine (IBMX) to each well and incubate for 15 min at 37 °C (*see* **Note 1**).
5. To initiate the assay, add 0.25 mL of hNP1 complete growth media containing 1 mM IBMX plus 2× concentration of drugs (S1P and/or forskolin) and incubate at 37 °C for 30 min (*see* **Note 2**).
6. To terminate the reaction, aspirate media and add 300 μL of Stop Solution to each well.
7. Add 300 μL of Stop Solution to each of three 20 mL plastic scintillation vials (Fisher Scientific) to determine total [^{14}C]-cAMP in Stop Solution (*see* **Note 3**). Add 10 mL of ScintiSafe 30 % Cocktail (Scintanalyzed) (Fisher Chemical) to each vial. Cap and shake vigorously; set aside for scintillation counting at end of experiment.

8. Add 750 μL of distilled, deionized water (ddH_2O) per well.
9. Add 100 μL of 60–62 % concentrated perchloric acid per well.
10. Transfer total contents of well to 1.5 mL microcentrifuge tubes.
11. Add 100 μL of 12 M KOH to each tube (*see* **Note 4**).
12. Cap tubes and vortex each tube for 5 s.
13. Centrifuge tubes at 3000 × *g* for 15 min at 4 °C. Remove tubes from centrifuge and allow samples to equilibrate to room temperature.
14. Load 850 μL of supernatant onto pre-equilibrated Dowex AG® 50W-X4 cationic exchange resin columns, and allow to drain by gravity flow.
15. Wash Dowex AG® 50W-X4 cationic exchange resin column one time with 2 mL of ddH_2O, and allow to drain by gravity flow.
16. Elute the contents of each Dowex AG® 50W-X4 cationic exchange resin column in to a pre-equilibrated neutral alumina column by adding 4 mL ddH_2O.
17. Allow neutral alumina columns to drain by gravity flow.
18. Elute neutral alumina columns into 20 mL scintillation vials by adding 4 mL of 0.1 M imidazole buffer. Allow to drain by gravity flow.
19. Add 10 mL of ScintiSafe 30 % Cocktail (Scintanalyzed) (Fisher Chemical) to each scintillation vial, cap and shake.
20. Load scintillation vials into Beckman-Coulter LS 6500 scintillation counter, and allow samples to dark adapt for 15 min before beginning counting protocol.
21. Count each sample for 5 min using appropriate windows to measure ^{3}H and ^{14}C.

3.6 Calculations

For each column, the percent recovery of cAMP can be calculated by dividing the cpm of [^{14}C]-cAMP eluted from each column by the total cpm of ^{14}C label in 300 μL of the Stop Solution. The total cpm of [^{3}H]-cAMP generated by cells in each well during the 30 min incubation period can then be calculated by dividing the cpm of [^{3}H]-cAMP eluted from each column by the cAMP percent recovery. Thus,

$$\text{cpm } [^{3}H]\text{-cAMP per well} = {}^{3}H_{\text{eluate}} \text{ cpm} / ({}^{14}C_{\text{eluate}}\text{cpm} / {}^{14}C_{\text{stopsoln}}\text{cpm})$$

Finally, cpm of cAMP can be converted to pmol cAMP using the efficiency of scintillation counting (cpm/dpm), the specific activity of the ^{3}H label (Ci/mol), and the conversion factor 2.22×10^{12} dpm/Ci.

3.7 Inositol Phosphate Assay

This assay measures accumulation of [^{3}H]-inositol phosphates (IPs), which are generated by multiple phospholipase C (PLC) enzymes from the substrate phosphatidylinositol bisphosphate. PLC activity is stimulated by Gαq G-proteins and indirectly via the βγ of Gi G-proteins.

To measure production of IPs, cells are labeled with [^{3}H]-inositol, which is incorporated into the cellular pool of phosphatidylinositol. To measure accumulation of IPs, cells are incubated with drug and lithium chloride (LiCl) to prevent the degradation of IPs. Cells are lysed and the [^{3}H]-IPs are separated from precursor ^{3}H using ion exchange chromatography. We have previously reported S1P-mediated production of inositol phosphates in hNP1 cells [10] using the protocol below, which is a modified version of established protocols [16].

3.8 Dowex® AG 1-X8 Anion Exchange Resin Columns

1. Preparation:
   (a) Mix resin and ddH_2O in a 1:1 slurry.
   (b) Pour 1 mL of slurry into each column.
2. Equilibration:
   (a) Wash each column with 5 mL of IP Regenerant.
   (b) Wash each column three times with 10 mL ddH_2O.
3. Storage:
   (a) After use, cap each column.
   (b) Store at 4 °C.

3.9 Inositol Phosphate Assay

1. Plate hNP1 cells in 24 well plates at 80 % confluency and allowed to adhere to the plate at 37 °C for 24 h.
2. Aspirate culture media and add 0.2 mL of hNP1 complete growth media containing 1 μCi/well [^{3}H] myo-inositol per well. Incubate for 18 h at 37 °C. In some experiments, cells are treated with 100 ng/mL pertussis toxin for 18 h prior to assay to define the role of Gi.
3. To initiate the assay, add 50 μL of media containing 50 mM lithium chloride and 5× concentration of S1P or other agonist/antagonist of interest (*see* **Note 5**). Incubate for 30 min at 37 °C.
4. To terminate the assay, aspirate all media.
5. Lyse cells by adding 750 μL of cold, 50 mM formic acid per well, and incubate plates at 4 °C for 30 min.
6. Neutralize by adding 250 μL of 150 mM ammonium hydroxide.
7. Load the contents of each well onto a regenerated Dowex® AG 1-X8 anion exchange resin column.

8. Wash columns with 10 mL of ddH_2O and allow to drain by gravity flow.
9. Wash columns with 10 mL of IP Wash Buffer to remove unhydrolyzed lipids. Allow to drain by gravity flow.
10. Elute [^{3}H]-IPs by adding 2.5 mL of IP Elution Buffer to each column. Allow to drain by gravity flow into 20 mL plastic scintillation vials.
11. Add 10 mL of ScintiSafe 30 % Cocktail (Scintanalyzed) (Fisher Chemical) to each scintillation vial. Cap and shake. Load samples into Beckman-Coulter LS 6500 scintillation counter and allow to dark adapt for 15 min before beginning counting protocol.
12. Count each sample for 1 min measuring [^{3}H].

3.10 hNP Cell Differentiation

Withdrawal of bFGF (basic fibroblast growth factor) inhibits proliferation and induces cell death in the majority of hNP cells; however, a fraction of cells survive, and those that do undergo terminal differentiation toward a predominantly (~95 %) neuronal cell fate [17, 18]. Following 2 weeks of bFGF withdrawal, the majority of hNP cells lose expression of neural progenitor markers Nestin and Sox-2 and induce expression of neuronal markers βIII-tubulin and Map2 [18]. As hNP cells differentiate they also undergo distinct morphological changes that include the formation and elongation of neurites [19]. The protocol below describes neuronal differentiation of hNP1 cells.

1. Plate hNP cells in a T-75 flask (50 % confluent) coated with Matrigel, and incubate for 24 h at 37 °C.
2. Aspirate hNP proliferation media and replace with 10 mL of differentiation media lacking bFGF. Incubate hNP cells for 72 h at 37 °C.
3. Aspirate media from each T-75 flask and replace with fresh differentiation media. Remove cells from plate using a soft edge cell scraper, and plate in 96 well Matrigel-coated plates at 30,000 cells/well or 6 well Matrigel-coated plates at 500,000 cells/well.
4. Incubate cells in hNP differentiation media at 37 °C for a total of 14 days. To assess the effect of pharmacologic agents, apply drugs 24 h after final plating. Aspirate and replace media and drugs every 72 h (*see* **Note 6**).
5. To assess viability of differentiated cells after 14 days, add 7 μL of CellTiter-Blue® reagent (Promega Corporation) to each well-differentiated cells in of 96 well plate, incubate at 37 °C for 4 h, and measure using SpectraMax M2 model microplate reader (Molecular Devices).

6. To assess expression of neuronal markers in differentiated cells:
   (a) Fix cells in 96 well plate with 100 μL warm fixation solution. Incubate plates for 20 min at 37 °C.
   (b) Aspirate media and fixation solution, and wash cells with 200 μL warm PBS^{++}.
   (c) Wash cells three times with 100 μL of block/permeabilization buffer.
   (d) Add 50 μL of primary antibody solution containing primary antibody, 1 % BSA, and 0.02 % Na Azide in PBS^{++} to each well (*see* **Note 7**). Incubate cells in primary antibody at room temperature for 1 h.
   (e) Aspirate primary antibody solution from each well and wash cells twice with 100 μL PBS^{++}.
   (f) Add 50 μL of secondary antibody solution containing secondary antibody, 3 μg/mL DAPI, and 1 % BSA in PBS^{++} to each well (*see* **Note 7**). Incubate cells in secondary antibody solution for 1 h at room temperature.
   (g) Aspirate secondary antibody solution from each well, and wash cells twice with 100 μL PBS^{++}. Finally add 200 μL PBS^{++} to each well, and seal plates with optical plate covers.
   (h) Image cells using high-content imaging system according to the manufacturer's instructions (*see* **Note 8**).

4 Notes

1. IBMX is an inhibitor of phosphodiesterase activity thereby allowing for the accumulation of cAMP in cells.
2. If measuring Gαi activity, add 50 μM forskolin (FSK) to each well. FSK stimulates adenylyl cyclase to produce cAMP. The addition of FSK results in rapid accumulation of cAMP. To measure Gαs G-protein activity, FSK can be excluded from this step.
3. [^{14}C]-cAMP is no longer available from GE Healthcare. As a substitute, use adenosine 3′,5′-[8-^{14}C] cyclic monophosphate ammonium salt (American Radiolabeled Chemicals, Saint Louis, MO). [^{14}C]-cAMP is added to stop buffer to control for recovery of cAMP in each column.
4. After addition of KOH, a white precipitate will form in the tubes.
5. The addition of LiCl inhibits the degradation of inositol phosphates.

6. To assess the role of S1P in differentiation, S1P (0.1 μM) is added every 72 h during the 14 days differentiation.
7. The following primary antibody dilutions may be used: 1:200 Rabbit anti Map2 (Neuromics); 1:200 Mouse anti Tubulin (β-III tubulin, Tuj) (Neuromics). The following secondary antibody dilutions may be used: 1:1000 Donkey anti Rabbit Alexa Fluor® 488 conjugated (Invitrogen); 1:1000 Goat anti Mouse Alexa Fluor® 488 conjugated (Invitrogen).
8. The protocol above describes methods for preparing cells to be imaged using a high-content imaging fluorescent microscope plate reader in order to allow differentiation and imaging on a single surface without replating cells. To perform the analysis on slides using a traditional microscope, coverslips may be added to the bottom of a six-well plate prior to Matrigel coating. Neural progenitors are grown and differentiated on the coverslip and immunostaining performed according to standard protocols.

References

1. Pitson SM, Pebay A (2009) Regulation of stem cell pluripotency and neural differentiation by lysophospholipids. Neurosignals 17:242–254
2. McGiffert C, Contos JJ, Friedman B, Chun J (2002) Embryonic brain expression analysis of lysophospholipid receptor genes suggests roles for s1p(1) in neurogenesis and s1p(1-3) in angiogenesis. FEBS Lett 531:103–108
3. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL (2005) Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol 25:11113–11121
4. Blanc CA, Grist JJ, Rosen H, Sears-Kraxberger I, Steward O, Lane TE (2015) Sphingosine-1-phosphate receptor antagonism enhances proliferation and migration of engrafted neural progenitor cells in a model of viral-induced demyelination. Am J Pathol 185:2819–2832
5. Miron VE, Schubart A, Antel JP (2008) Central nervous system-directed effects of FTY720 (fingolimod). J Neurol Sci 274:13–17
6. Kimura A, Ohmori T, Ohkawa R, Madoiwa S, Mimuro J, Murakami T, Kobayashi E, Hoshino Y, Yatomi Y, Sakata Y (2007) Essential roles of sphingosine 1-phosphate/S1P1 receptor axis in the migration of neural stem cells toward a site of spinal cord injury. Stem Cells 25:115–124
7. Harada J, Foley M, Moskowitz MA, Waeber C (2004) Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells. J Neurochem 88:1026–1039
8. Guo H, Zhao Z, Yang Q, Wang M, Bell RD, Wang S, Chow N, Davis TP, Griffin JH, Goldman SA (2013) An activated protein C analog stimulates neuronal production by human neural progenitor cells via a PAR1-PAR3-S1PR1-Akt pathway. J Neurosci 33:6181–6190
9. Dhara SK, Hasneen K, Machacek DW, Boyd NL, Rao RR, Stice SL (2008) Human neural progenitor cells derived from embryonic stem cells in feeder-free cultures. Differentiation 76:454–464
10. Hurst JH, Mumaw J, Machacek DW, Sturkie C, Callihan P, Stice SL, Hooks SB (2008) Human neural progenitors express functional lysophospholipid receptors that regulate cell growth and morphology. BMC Neurosci 9:118
11. Callihan P, Zitomer NC, Stoeling MV, Kennedy PC, Lynch KR, Riley RT, Hooks SB (2012) Distinct generation, pharmacology, and distribution of sphingosine 1-phosphate and dihydrosphingosine 1-phosphate in human neural progenitor cells. Neuropharmacology 62:988–996
12. Callihan P, Ali MW, Salazar H, Quach N, Wu X, Stice SL, Hooks SB (2014) Convergent regulation of neuronal differentiation and Erk and Akt kinases in human neural progenitor cells by lysophosphatidic acid, sphingosine 1-phosphate, and LIF: specific roles for the LPA1 receptor, ASN Neuro 6(6):1–18
13. Tuggle K, Ali M, Salazar H, Hooks S (2014) Regulator of G protein signaling transcript

expression in human neural progenitor differentiation: R7 subfamily regulation by DNA methylation. Neurosignals 22:43–51

14. Hettinger-Smith BD, Leid M, Murray TF (1996) Chronic exposure to adenosine receptor agonists and antagonists reciprocally regulates the A1 adenosine receptor-adenylyl cyclase system in cerebellar granule cells. J Neurochem 67:1921–1930
15. Salomon Y (1979) Adenylate cyclase assay. Adv Cyclic Nucleotide Res 10:35–55
16. Hepler JR, Nakahata N, Lovenberg TW, DiGuiseppi J, Herman B, Earp HS, Harden TK (1987) Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cytosolic calcium mobilized from intracellular stores in A431 cells. J Biol Chem 262:2951–2956
17. Shin S, Mitalipova M, Noggle S, Tibbitts D, Venable A, Rao R, Stice SL (2006) Long-term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. Stem Cells 24:125–138
18. Dhara SK, Stice SL (2008) Neural differentiation of human embryonic stem cells. J Cell Biochem 105:633–640
19. Dan H, Sanes TAR, Harris WA (2006) Axon growth and guidance. Development of the nervous system, 2nd edn. Elsevier Academic Press, London, UK

Methods in Molecular Biology (2017) 1697: 153–171
DOI 10.1007/7651_2017_43

Published online: 25 May 2017

Ceramide and S1P Signaling in Embryonic Stem Cell Differentiation

Guanghu Wang, Stefka D. Spassieva, and Erhard Bieberich

Abstract

Bioactive sphingolipids are important regulators for stem cell survival and differentiation. Most recently, we have coined the term "morphogenetic lipids" for sphingolipids that regulate stem cells during embryonic and postnatal development. The sphingolipid ceramide and its derivative, sphingosine-1-phosphate (S1P), can act synergistically as well as antagonistically on embryonic stem (ES) cell differentiation. We show here simple as well as state-of-the-art methods to analyze sphingolipids in differentiating ES cells and discuss new protocols to use ceramide and S1P analogs for the guided differentiation of mouse ES cells toward neuronal and glial lineage.

Keywords: Ceramide, Sphingolipid, Sphingosine-1-phosphate, Neuroprogenitor, Oligodendrocyte precursor, Apoptosis, Teratoma

1 Introduction

The use of differentiating stem cells, regardless of being derived from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, is hampered by the persistence of residual pluripotent stem (rPS) cells in the culture of differentiating stem cells. Even though these cells can be present in very low abundance ($<1{:}10^6$), they may impose a significant risk of tumor formation when used for transplantation. From early on, studies showed that stem cell transplantation can lead to the formation of teratomas [1–19]. Teratomas are stem cell-derived tumors that are fatal if they occur in the brain or heart. The potential for tumor formation is thus a major safety concern when using larger numbers of ES or iPS cell-derived cells [16]. We have introduced for the first time a strategy based on small-molecule selective elimination of rPS cells that are at risk to form tumors from stem cells grafts [1, 20, 21]. Our original study used a novel ceramide analog synthesized in our laboratory (N-oleoyl serinol or S18, Fig. 1) to specifically trigger apoptosis in rPS cells [1, 21]. Studies from other laboratories then developed several additional strategies using small molecules to target mitochondrial cell death in rPS cells, either by inhibiting anti-apoptotic proteins or activating inducers of apoptosis [21]. In all of these studies, including our own

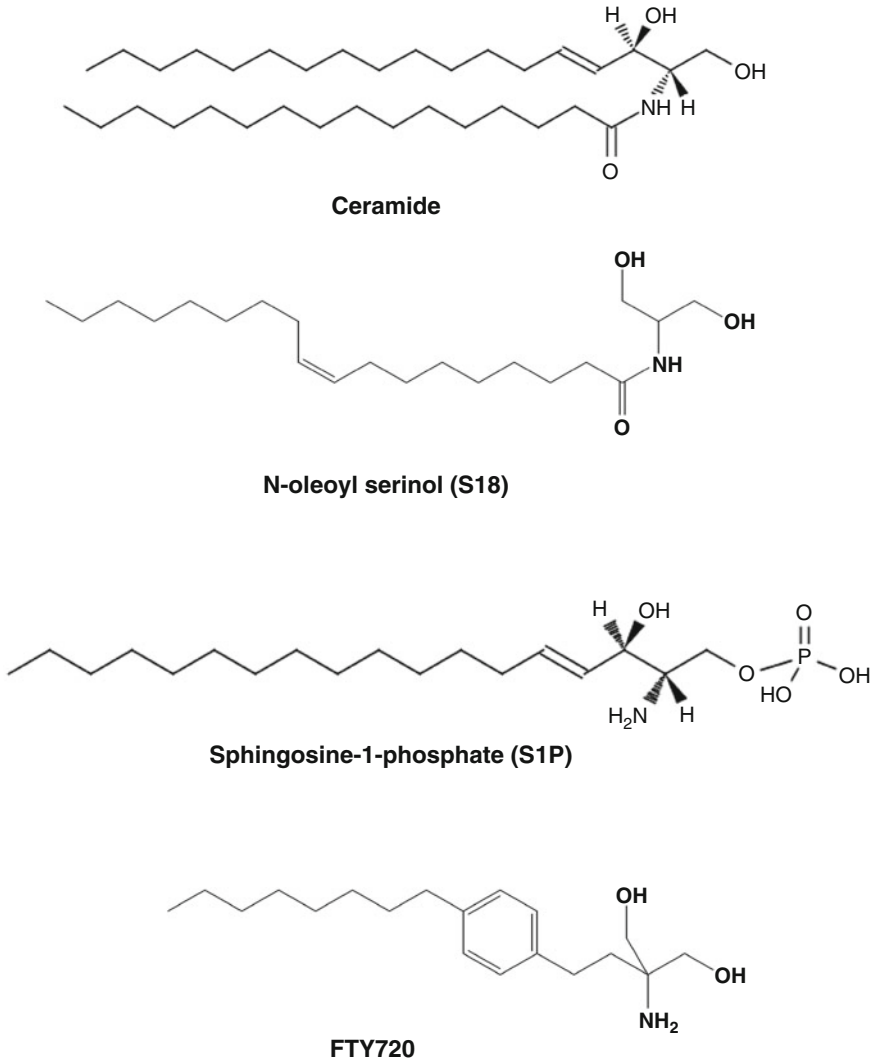

Fig. 1 Structure of the sphingolipids and sphingolipid analogs used for stem cell differentiation

work, it has still to be shown that these strategies are efficient in stem cell therapy applicable to human patients. For the time being, sphingolipids are among the most promising small molecules useful in eliminating rPS cells as well as guiding different lineage specification in surviving progenitors, particularly in neural stem or progenitor cells (NPs). The following section will briefly introduce these "morphogenetic lipids," the molecular mechanisms by which they regulate stem cells, and analytical methods to determine the lipid profile that is associated with stem cell differentiation.

Our studies have shown that rPS cells co-express the pluripotency transcription factor Oct-4 and prostate apoptosis response 4 (PAR-4), a protein that renders rPS cells sensitive toward ceramide and its analogs [1]. PAR-4 is an endogenous inhibitor of atypical

PKC (aPKC) when associated with ceramide or N-oleoyl serinol (S18, Fig. 1). S18 was synthesized and its association with aPKC analyzed in our laboratory [1, 20, 22–26]. If PAR-4 expression is low, S18 or ceramide can activate aPKC. aPKC activation and downstream activation of NF-κB has been found to be critical for neural differentiation of ES cells [27]. However, if PAR-4 expression is high, ceramide or S18 will facilitate PAR-4 inhibition of aPKC and induce apoptosis, a mechanism utilized for small-molecule selective elimination of rPS cells. More recently, studies in our laboratory on the role of ceramide in formation and function of primary and motile cilia identified additional targets of ceramide, particularly in the regulation of the cytoskeleton and cell polarity [28–30]. We have found that long-chain ceramide such as N-nervonoyl sphingosine (C24:1 ceramide) can interact with glycogen synthase kinase 3 (GSK3), a major regulatory protein kinase in the noncanonical Wnt pathway [28, 29]. Incubation with C24:1 ceramide promoted prolonged process formation in hES and iPS cell-derived NPs [28], suggesting that different species of ceramide have distinct functions in ES or iPS cell differentiation, most likely depending on the differentiation stage-specific expression of target or sensitizer proteins interacting with ceramide. These observations suggest that knowledge on the specific sphingolipid profile and using combinatorial approaches adding several sphingolipids to differentiating ES or iPS cells will significantly contribute to safety and efficacy of stem cell therapy.

Sphingosine-1-phosphate (S1P) is a ceramide derivative that has been used to derive or maintain mESCs and hESCs in experimental settings, demonstrating its essential function in stem cell self-renewal and pluripotency [31–36]. In mESCs, the main pathway allowing maintenance of pluripotency appears to be through the activation of the JAK/STAT3 pathway [34, 37, 38]. This notion is supported by studies showing that silencing of the S1P degrading enzyme, S1P lyase (SPL), leads to an increased S1P level concomitant with increased proliferation, and elevated expression of pluripotency markers SSEA1 and Oct-4 in mESCs [39]. The S1P receptor 2/Stat3 signaling has been identified to be the major pathway in SPL knockdown-mediated pluripotency. S1P plays crucial roles in proliferation, migration, and homing of various types of progenitor cells [35, 40–44]. We have shown that ceramide and S1P can act synergistically in ES cell differentiation and have developed relatively simple methods to use ceramide and S1P analogs for the guided differentiation of mouse ES cells toward neuronal and glial lineages [20, 45, 46]. We have also shown that S1P and its prodrug analog FTY720 promote cell survival and differentiation of NPs toward oligodendroglial lineage because these cells express the S1P receptor S1P1 [46]. In particular useful is the combined administration of ceramide/S18 and S1P/FTY720 to in vitro differentiating ES cells (Fig. 1). Since rPS cells express PAR-4 (but not S1P1) and NPs express S1P1 (but not PAR-4), ceramide/S18

eliminates rPS cells and S1P/FTY720 promotes oligodendroglial differentiation of the surviving NPs. NPs can be incubated with C24:1 ceramide, which does not induce apoptosis but promotes neuronal differentiation [28]. In summary, the following protocols supplementing cell culture media with sphingolipids and their analogs can be used to eliminate rPS cells and promote differentiation of ES cells to neuronal or oligodendroglial lineage for in vitro and in vivo studies.

2 Materials

2.1 Media for the Cultivation and Differentiation of Mouse ES Cells (Calculated for 100 ml of Medium)

FM10 (Feeder cell medium)
89 ml of DMEM (with L-glutamine and sodium pyruvate)
10 ml of heat-inactivated FBS
1 ml 100× stock of penicillin/streptomycin/amphotericin B (fungizone)
(*see* **Note 1**)

KSR15 (ES cell medium for cells grown on feeders)
81.72 ml of Knockout-DMEM
15 ml of Knockout Serum Replacement (KSR)
1 ml of 100× L-glutamine (200 mM)
1 ml of 100× nonessential amino acids
1 ml of 100× penicillin/streptomycin/amphotericin B
100 μl of ESGRO (LIF)
180 μl of 2-mercaptoethanol

ES15 (ES cell medium for cells grown feeder-free)
81.72 ml of Knockout-DMEM
15 ml of heat-inactivated ES-qualified FBS
1 ml of 100× L-glutamine (200 mM)
1 ml of 100× penicillin/streptomycin/amphotericin B
1 ml of 100× nonessential amino acids
100 μl of ESGRO (LIF)
180 μl of 2-mercaptoethanol

EB1 (Suspension EB medium)
87 ml of Knockout-DMEM
10 ml of heat-inactivated ES-qualified FBS
1 ml of 100× penicillin/streptomycin/amphotericin B
1 ml of 100× L-glutamine (200 mM)
1 ml of 100× nonessential amino acids

EB2 (Attached EB medium)
96 ml of DMEM/F12 50/50
1 ml of 100× penicillin/streptomycin/amphotericin B
1 ml of 100× L-glutamine (200 mM)
1 ml of 100× nonessential amino acids
1 ml of N-2 supplement (100×)

NP (NP medium)
95.5 ml of DMEM/F12 50/50
1 ml of 100× penicillin/streptomycin/amphotericin B
1 ml of 100× L-glutamine (200 mM)
1 ml of 100× nonessential amino acids
1 ml of N-2 supplement (100×)
500 μl of basic fibroblast growth factor (FGF-2) stock (*see* **Note 2**)

2.1.1 Differentiation Medium

91.75 ml Neurobasal medium
5 ml of heat-inactivated ES-qualified FBS
1 ml of 100× penicillin/streptomycin/amphotericin B
250 μl of L-glutamine (200 mM stock)
2 ml of 50× B27 supplement
(*see* **Note 3**)

2.1.2 Trypsinization

0.25 % trypsin/0.2 % EDTA in PBS (*see* **Note 4**)

2.1.3 Freeze Medium

90 % heat-inactivated ES cell-qualified FBS and 10 % DMSO

2.1.4 Gelatin Coating Solution

Dissolve 2 g of gelatin, 300 Bloom in 100 ml of deionized water and autoclave. Gelatin should be completely dissolved after being autoclaved. The 2 % gelatin stock solution can be kept refrigerated until further use. For gelatin coating, dilute stock solution 1:20 in sterile water and incubate tissue culture dishes for 2 h at room temperature. Then, remove solution and let dishes dry in the hood for 2 h.

2.2 Solutions and Reagents for Lipid Analysis

(Important: *see* Note 5 for precautionary measures to avoid toxic or hazardous conditions)

2.2.1 Reagents for Folch Extraction of Lipids

$CHCl_3/CH_3OH$ (2:1, vol:vol)

2.2.2 Running Solvent for Thin Layer Chromatography

$CHCl_3/CH_3OH$ (95:1, vol:vol)

2.2.3 Staining Solution for Lipid Detection on Thin Layer Chromatography

3 % cupric acetate in 5 % phosphoric acid

2.2.4 Reagents for One Phase Extraction of Lipids for Mass Spectrometry

Ethyl acetate/isopropanol/water (60:30:10 v/v/v)
1 mM ammonium formate in 0.2 % formic acid in methanol
HPLC mobile system: 1 mM methanolic ammonium formate/2 mM aqueous ammonium formate

3 Methods

3.1 Propagation and Differentiation of Mouse Embryonic Stem Cells

Overview: In vitro neuronal differentiation of mouse ES cells (ES-J1, ES-D3) followed a serum deprivation protocol as described previously [47–52].

1. Coat a 100 mm tissue culture dish with 0.1 % sterile gelatin solution (freshly prepared from 2 % stock) by incubation for 2 h at room temperature. Remove the solution and dry for 2 h in hood with lid only partially covering the dish. Rinse once with FM10 medium.
2. Seed the dish with 3×10^6 irradiated mouse embryonic feeder fibroblasts (MEFs). Alternatively, feeder fibroblasts mitotically inactivated with mitomycin c can also be used. Cultivate the fibroblasts for 2 days in 10 ml FM10 medium. Mitotically inactivated MEFs are available from commercial sources.

3.2 Propagation of Undifferentiated ES Cells on Feeder Fibroblasts

1. Thaw frozen ES cells and suspend cells in 10 ml freshly prepared KSR15 medium. Spin cells down at 200 × g for 5 min. Resuspend cells in 20 ml of KSR15 and plate them on top of the feeder fibroblasts. Do not completely remove supernatant after centrifugation to minimize loss of cells.
2. After 1 day of cultivation, replace medium with 20 ml of fresh KSR15. At this stage, the ES cells have attached to the feeder cells, although they may not be clearly visible (small rounded cells on flat fibroblasts).
3. After another day of cultivation (day 3 after seeding of the ES cells), replace medium again with 20 ml of fresh KSR15.
4. On the fourth day after seeding on the fibroblasts, colonies of ES cells should be clearly visible (Fig. 2). If not, change medium again and cultivate for another day. When colonies reach a size as shown in Fig. 2, proceed to feeder-free culture.

3.3 Removal of Feeder Fibroblasts and Propagation of ES Cells in Feeder-Free Culture

1. Prepare four gelatin-coated 100 mm tissue culture dishes without feeder cells.
2. Rinse the ES cells on the feeders with 10 ml sterile PBS.
3. Add 4 ml of 0.25 % trypsin/EDTA and incubate cells for 5 min at 37 °C.
4. Immediately add 10 ml of pre-warmed KSR15 and pipette up and down three times. Try to rid the ES cell suspension of most of feeder cell patches.
5. Pellet the cells by centrifugation at 200 × g for 5 min.

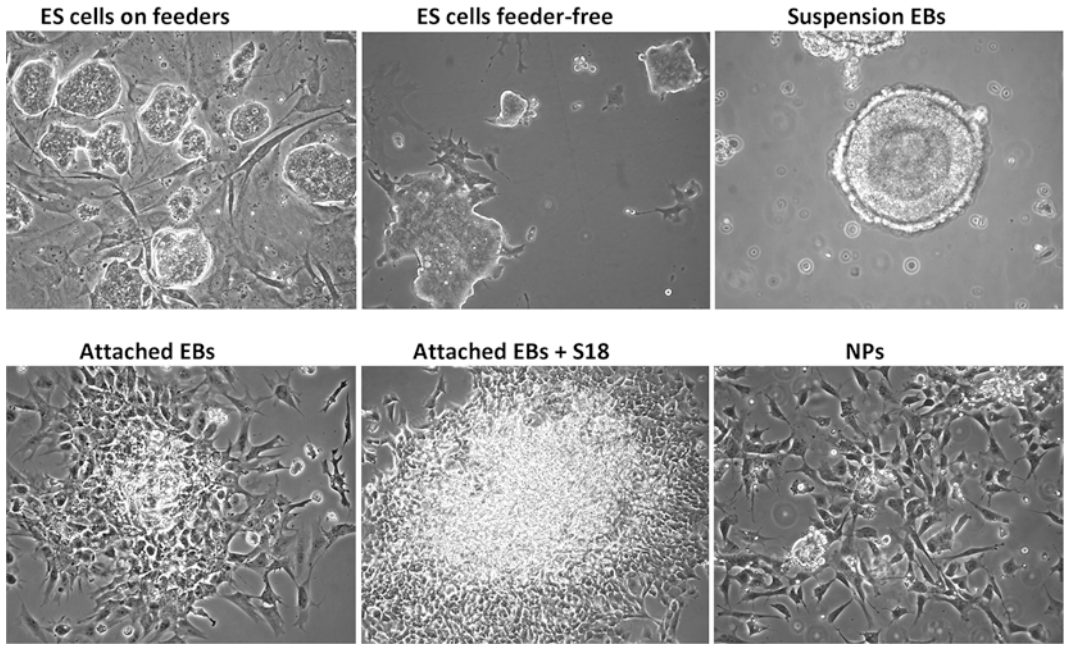

Fig. 2 Different stages of mouse ES cell differentiation

6. Resuspend cells in 20 ml of ES15 and plate them onto a tissue culture dish NOT coated with gelatin. Incubate for 1-2 h at 37 °C. The feeders will attach while most of the ES cells will stay in suspension.
7. Take the medium with the ES cells off the plate and transfer it to a gelatin-coated tissue culture dish.
8. On the next day, change medium to 20 ml of ES15 medium. Change medium every day for another 2 days. DO NOT let the ES cell colonies exceed 50–70 % of the dish surface (Fig. 2). Once cells become too dense, the edges of the colonies start to look ragged. If this happens, cells become prematurely differentiated, which will compromise their pluripotency.

3.4 Preparation of Suspension Embryoid Bodies (EBs)

1. Trypsinize and freeze cells that are not wanted for the preparation of EBs (see freeze medium, Sect. 2.1.3).
2. Trypsinize the residual dishes with 0.25 % trypsin/EDTA for 2 min. Cells should detach, but still form aggregates. Immediately add 10 ml of EB1 medium to neutralize the trypsin. Then transfer cell aggregates to a 50 ml plastic tube using a 25 ml pipette. Rinse dish with another 10 ml of EB1 medium and combine cell suspensions. Centrifuge for 5 min at 50–100 × g or let the cell aggregates settle without centrifugation. Remove medium supernatant and gently wash cell aggregates with 10 ml fresh EB1 medium. Resuspend cell aggregates in 30 ml of EB1 medium and seed them on *bacterial dishes*. It is important not to

use tissue culture dishes to avoid premature attachment of suspension EBs.

3. Change medium and inspect the cell aggregates every day for the next 3–5 days. The cell aggregates should round up and first form a clearly distinguishable epithelial outer cell layer (primitive endoderm), followed by the formation of basal lamina and an inner cell layer (primitive ectoderm) surrounding a cavity (Fig. 2). This cavity is developmentally equivalent to the proamniotic cavity of mammalian embryos. To minimize loss of EBs during medium change, tilt the dish and collect the EBs at one side of the dish. DO NOT attempt to completely remove the old medium, but leave some medium behind to maintain the EB suspension.

3.5 Preparation of Attached EBs and Cultivation of EB-Derived Cells

1. Transfer the suspension EBs to tissue culture dishes. Coating is not necessary. Typically, the suspension EBs from one bacterial dish are sufficient to yield one tissue culture dish with attached EBs. Let the EBs attach overnight (Fig. 2).
2. Change medium to EB2 medium. Keep replacing media daily for 5–7 days. Cells will migrate out to the attached EB. These cells are termed EB-derived cells or EBCs. After sufficient EBCs have been migrated out of the attached EBs, cells can be treated with ceramide analogs or S1P/S1P analogs (*see* Sect. 3.8). Cells can also be used to generate neuroprogenitors (NPs) prior to drug treatment (*see* Sect. 3.8).

3.6 Induction of Neural Differentiation and Cultivation of Neuroprogenitors (NPs)

1. Coat tissue culture dishes with p-ornithin and laminin from commercially available stock solutions (200 µl p-ornithin or 100 µl laminin in 100 ml of sterile water) by first incubating with p-ornithin and then laminin each for 1 h at 37 °C. In between and after the two coating steps, rinse with sterile water, and then store the coated dishes in PBS until use (DO NOT extend storage for more than 1 day).
2. Dissociate EBCs with 2 ml of 0.05–0.25 % trypsin/EDTA for 5 min at 37 °C. Immediately neutralize the trypsin with 10 ml of EB1 medium then harvest cells by centrifugation at 200 × g for 5 min. The dissociation of attached EBs is a critical step that determines the yield of viable NPs (Fig. 2). Harvest cells by centrifugation, resuspend again in NP medium, and seed cells on p-ornithin laminin-coated tissue culture dishes or coated cover slips if immunocytochemistry is to be performed (*see* **Note 6**).
3. Cultivate cells for 3–6 days in NP medium, change medium every day. NPs will form rosettes. After 24–48 h in NP medium, cells can be treated with ceramide or S1P analogs as described in Sect. 3.8 (*see* **Note 7**).
4. Harvest cells by trypsinization with 0.05 % trypsin/EDTA. Neutralize trypsin with soybean trypsin inhibitor. Cells can now be

either replated onto fresh p-ornithin laminin-coated dishes and differentiated or used for transplantation.

3.7 Terminal Differentiation to Neurons and Glia

1. Incubate replated NPs (treated or not treated with ceramide or S1P analogs) with differentiation medium for up to 10 days.
2. Assess differentiation lineage by using the respective markers for neuronal, astrocytic, and oligodendroglial differentiation (*see* Sect. 3.10 for immunocytochemistry protocol).

3.8 Treatment with Ceramide and Sphingosine-1-Phosphate Analogs

3.8.1 Treatment of Attached EBs

1. Two to three days after attachment of suspension EBs to the tissue culture dish, the culture can be incubated with ceramide, S1P, ceramide, or S1P analogs, or a combination of these compounds (Figs. 1 and 2). Typically, the following concentrations are useful to eliminate rPS cells or to direct lineage specification of differentiating cells in the EBs (*see* **Note 8**):

   N-acetyl sphingosine (C2 ceramide): 5–20 μM*

   N-palmitoyl sphingosine (C16 ceramide): 0.5–2.0 μM**

   N-oleoyl serinol (S18): 50–100 μM

   S1P: 0.2–1.0 μM***

   FTY720: 0.1–0.5 μM****

   * C2 ceramide is water-soluble in the given concentration range, however, may have nonspecific and lytic effects. If possible, the use of C16 ceramide or S18 is preferred.

   ** C16 ceramide is not water-soluble in the given concentration range, unless prepared as a 1,000× stock solution in ethanol supplemented with 2 % dodecane. When using a 1:1,000 dilution of this stock, controls with respective final concentration of ethanol and dodecane have to be prepared. If possible, the use of the water-soluble S18 is preferred.

   *** S1P is only partially water-soluble and is to be prepared as 1,000× stock in 1 mg/ml bovine serum albumin/PBS. Alternatively, water-soluble S1P albumin complexes (Huzzah S1P) can be used (Avanti Polar Lipids, Alabaster, AL).

   **** FTY720 is water-soluble in the given concentration range; however, prolonged exposure at higher concentration may downregulate S1P1 receptor expression. At 300 nM for 48 h, S1P1 expression was clearly detectable in OPCs [46].
2. After treatment, EBs and EBCs can be dissociated by trypsinization and replated to generate NPs or used for transplantation.

3.8.2 Treatment of EB-Derived Cells and NPs

1. Dissociate cells from attached EBs by trypsinization and replate as described in Sect. 3.6. These cells may have been treated with the abovementioned compounds or not. However, DO NOT continue or start treatment in the first 24 h after seeding of the cells. Typically, EBCs will first attach and recover before treatment

starts. Treatment is performed with compounds and dosages given in Sect. 3.8.

2. After 2–3 days of drug treatment, NPs can be used for transplantation or further differentiated as described in Sect. 3.7.

3.8.3 Treatment of Other Stages of ES Cell Differentiation

Various stages of ES cell differentiation, including undifferentiated ES cells on feeders, feeder-free ES cells, and suspension EBs have been treated with ceramide or ceramide analogs. The outcomes are not as defined with respect to lineage specification as achievable when later stages of ES cell differentiation are used for treatment. Therefore, treatments of earlier differentiation stages are not included in this protocol. However, a brief summary of treatment outcomes will be discussed to provide the opportunity of using protocols published in other studies. For example, Salli et al. describe the use of nanoliposomal C6 ceramide for the cultivation of undifferentiated ES cells in feeder-free culture [53]. Krishnamurthy et al. describe the use of C16 ceramide or S18 for the restoration of primitive ectoderm in suspension EBs [54].

3.8.4 Treatment of Human ES and Induced Pluripotent Stem Cells

Protocols as detailed in the previous sections have been developed for mES cells. However, our studies have shown that they are, at least in part, applicable to hES and iPS cells. The use of S18 to eliminate rPS cells from a culture of hES cells is described in Ref. [1]. Likewise, hES or iPS cell-derived NPs can be incubated with 2 μM C24:1 ceramide (prepared as 2 mM stock in ethanol/2 % dodecane) to promote neuronal differentiation and process formation in NPs that were cultivated in 5 % KSR as described in Ref. [28]. Detailed methods to maintain and cultivate hES and iPS cells and derive NPs can be found in Refs. [28, 35, 36].

3.9 Determination of Ceramide and Sphingosine-1-Phosphate Using Sphingolipid Analysis

(Important: *see* Note 5 for precautionary measures to avoid toxic or hazardous conditions)

3.9.1 Sphingolipid Extraction

1. Resuspend EBCs or NPs in 500 μl PBS and add 2.5 ml of $CHCl_3$/CH_3OH (2:1, vol/vol) in a Pyrex glass tube capped with Teflon liner. Vortex for 30 s, and then sonicate for 30 min in an ultrasonication bath (e.g., Branson) or shake for 1 h at room temperature (*see* **Notes 5** and **9**).
2. Centrifuge tube at low speed for 20 min. Two phases and the interphase should be clearly visible. The upper phase contains (partially) water-soluble lipids such as gangliosides and a major portion of phospholipids including S1P. If a comprehensive analysis is desired, the upper phase should be removed and kept separately. The lower phase contains ceramide and neutral lipids and should

be carefully recovered without disturbing the interphase. The interphase contains denatured protein which should be kept and air dried. After solubilization in SDS sample buffer, this protein fraction can be used for protein quantification, SDS-PAGE, or other methods of protein analysis.

3. Evaporate the organic solvent from the lower phase with a stream of nitrogen gas. Alternatively, organic solvent can be removed using a rotation evaporator.
4. Dissolve the lipid residue in $CHCl_3/CH_3OH$ (1:1, vol/vol) and store at −20 °C until further analysis. Typically, lipids extracted from 10–50 mg cells are redissolved in 100–500 µl organic solvent to allow for analysis by Thin Layer Chromatography (TLC).

3.9.2 Sphingolipid Analysis by Thin Layer Chromatography

1. Prepare chromatography chamber (alternatively, a glass beaker can be used) and fill it to about 1/2 in. (1 cm) with the running solvent $CHCl_3/CH_3OH$ (95:5, vol/vol). For equilibration of the chamber atmosphere with organic solvent, a filter paper is placed at the wall of the chamber and saturated with organic solvent all the way up to the edge of the chamber (Fig. 3). To avoid loss of organic solvent, the chamber has to be covered with a thick glass plate and a weight placed on top of the plate. Alternatively, aluminum foil can be used to cover the chamber or beaker and secured with a wrapped-around rubber band.
2. Cut a TLC plate using a glass cutter to fit into the chromatography chamber (alternatively, precut plates are commercially available). Draw spotting lines on the silica gel coat of the plate at about 1 in. (2 cm) from the bottom edge of the plate using a soft pencil as shown in Fig. 3. Each line should be about 1/8–1/4 in. (2–6 mm) in width and 1/4 (5–6 mm) apart from each other. Be careful not to hurt the surface of the silica gel.
3. Apply the sample (lipids in organic solvent) and lipid standards with a Hamilton syringe or glass tube. The application should be steady and repeatedly on top of the spotting line. Alternatively, pipette tips can be used, although they tend to form drops that hamper a steady application. Be careful not to directly touch the silica gel surface. The amount of lipid to be applied depends on the normalization method. Typically, a lipid amount equivalent to equal amounts of protein or lipid phosphate is applied to each lane on the plate (*see* **Note 10**).
4. After application of the samples and standards, the plate is placed into the chromatography chamber without letting the running solvent get in immediate contact with the applied lipids (Fig. 3). Also ensure that the application lines are dry before placing the plate into the chamber. The running solvent will be soaked into the silica gel and migrate from the bottom to the top (arrow in Fig. 3). The lipids will dissolve into the migrating solvent and comigrate according to their solubility in organic solvents. More

apolar lipids will migrate faster leading to a separation of the lipid mixture into individual lipid species. After the running solvent has reached the top of the plate, the TLC is complete and the plate will be taken out of the chamber for air drying and subsequent staining of the lipids.

5. Staining of lipid species on TLC plate (important: *see* **Note 5** for precautionary measures).
   Spray TLC staining reagent on the surface of the plate until it is wet. Be careful not to inhale the vapors of the spraying reagent. Alternatively, the plate can be briefly dipped into a bath with staining reagent and the back of the glass plate wiped down with a tissue before heating the plate. The plate will be heated (silica gel surface up!) for 5–15 min at 150–180 °C until the charring reaction leads to the appearance of dark bands. Once standards and sample lipids are visible, the plate is removed from the heater for cooling at room temperature. The plate should be scanned immediately because bands will fade over time. For short-term storage, wrap the plate into aluminum foil and store it at a secure and dark place.
   (*see* **Note 11** for limitations of the staining reaction)

3.9.3 Sphingolipid Analysis by Mass Spectrometry

Overview: Liquid chromatography tandem mass spectrometry analyses allow high quality identification and quantification of S1P and the individual ceramide species [55–57]. Depending on the mass spectrometry instrumentation available, the method might require modifications. The method described below was developed on a Thermo-Fisher TSQ Quantum triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring positive ionization mode and coupled with HP1100 equipped with BDS Hypersil C8 column (150 × 3.2 mm, 3 μm particle size) [55, 56].

1. Lipid extracts from cell pellets corresponding to about 1×10^6 cells are sufficient for reliable measurement of ceramides and S1P. For the lipid extraction, use glass tubes. To control for variation during the extraction, the samples need to be fortified with appropriate internal standards, e.g., C_{17} base D-erythro-sphingosine, C_{17} S1P, N-palmitoyl-D-erythro-C_{13} sphingosine, or N-docosanoyl-D-erythro-C_{13} sphingosine (Avanti Polar Lipids, Alabaster, AL).
2. Lipids are extracted twice with 2 ml ethyl acetate/isopropanol/water (60:30:10 v/v/v) solvent.
3. Combined lipid extracts are dried under a stream of nitrogen and resuspended in 150 μl of 1 mM ammonium formate in 0.2 % formic acid in methanol.
4. Lipid extracts are injected on the HP1100/TSQ Quantum liquid chromatography tandem mass spectrometry system and gradient eluted from the BDS Hypersil C8 column with the mobile phase

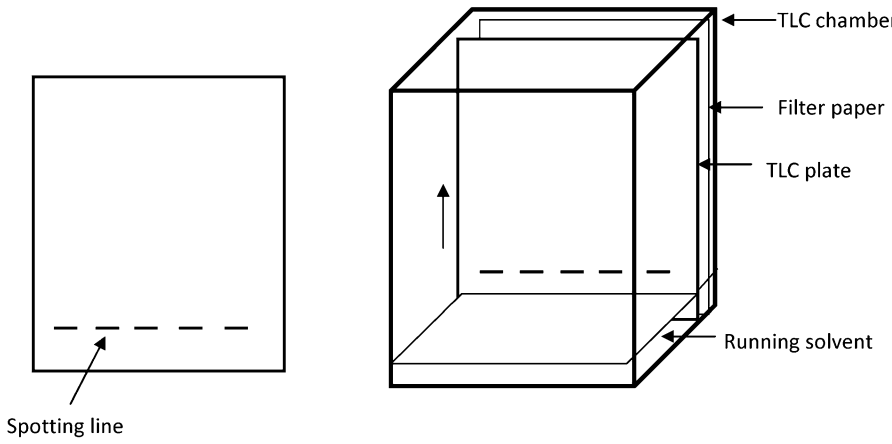

Fig. 3 Assembly for Thin Layer Chromatography (TLC)

system: 1 mM methanolic ammonium formate/2 mM aqueous ammonium formate.

5. Quantitative analysis of S1P and ceramides is based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte, synthetic standards (Avanti Polar Lipids, Alabaster, AL), and an equal amount of the internal standards.
6. The target analyte/internal standards peak area ratios are plotted against analyte concentration.
7. The target analyte/internal standards peak area ratios from the samples are normalized to their respective internal standards and compared to the calibration curves, using a linear regression model.
8. Final results from cells could be normalized to cellular lipid phosphate, which can be measured with a standard curve analysis and a colorimetric assay of ashed phosphate or to protein content [58].

3.10 Immunocytochemistry for Lipids and Lipid Receptors

(*see* cautionary **Note 12** for fixation reagents and **Note 5** for other precautionary measures)

1. Fix cells or sections with freshly prepared 4 % p-formaldehyde in PBS for 20 min at room temperature. Wash twice with PBS.
2. If only surface staining is desired, cells or sections do not need to be permeabilized. However, if immunocytochemistry for lipids or proteins inside of cells is desired, permeabilization can be helpful. For example, a mild permeabilization with 0.2 % Triton X-100 in PBS for 5 min at room temperature has not been shown to significantly remove lipids from the sample, but it may alter the distribution of particular lipids in cellular membranes [59] (*see* **Note 13**).

3. Block binding to nonspecific antigens by incubation of the sample with 3 % ovalbumin/5 % donkey serum (secondary antibodies raised in donkey are used) in PBS for 60 min at room temperature or 37 °C. Wash once with PBS.
4. Incubate with primary antibody diluted in 0.1 % ovalbumin/PBS. The proper dilution will need to be tested. Typically, final antibody concentrations of 5 μg/ml will yield excellent results. The antibody reaction can be performed for 2 h at 37 °C, 4 h at room temperature, or overnight at 4 °C (recommended).
5. Wash sample three times for 10 min with PBS.
6. Incubate with secondary antibody as described for the first antibody. However, shorten incubation time at room temperature to 2 h and avoid overnight incubation with the secondary antibody. Then wash sample three times for 10 min with PBS. Embed in mounting medium. Note that additional staining for nuclei with Hoechst 33258 dye (1 μg/ml) can be included into the first washing step. After drying, the sample is ready for fluorescence microscopy.
7. Additional staining reactions such as TUNEL or BrdU assays can be included prior to antibody staining following protocols provided by the manufacturers.

4 Notes

1. If L-glutamine or sodium pyruvate are not included in the medium, they have to be added separately to a final concentration of 2 or 1 mM, respectively.
2. Dissolve 10 μg FGF-2 in 100 μl sterile PBS, then add 10 μl of this solution to 500 μl of 0.1 % BSA to generate aliquots that can be kept at −20 °C.
3. To eliminate astrocytes, differentiation medium can be supplemented with 20 μM Ara C.
4. It is recommended to be diluted 1:5 when using for plating of EBCs or NPs; alternatively, nonenzymatic dissociation solution can be used instead of trypsin.
5. **CAUTION:** Lipid extraction and analysis requires the use of organic solvents that are toxic for the liver and the nervous system. Therefore, all procedures involving organic solvents ***must*** be carried out in a chemical fume hood. The use of organic solvents produces waste that has to be properly disposed of following institutionally enforced regulations and protocols. When handling organic solvents, glass pipettes and glass containers have to be used. The reagent for staining of lipids on TLC contains caustic

and acidic components. Therefore, extra care and protection is required such as double gloves and face shield. Do not inhale the spraying reagent or organic vapors. The staining reaction uses a heater in the chemical fume hood which requires that organic solvents are placed in safe distance from the heating device to avoid a fire hazard. It is also recommended to handle the hot TLC plate with heat-resistant gloves or tools to avoid burns.

6. Prolonged exposure to trypsin can damage the cells and dramatically reduce survival of EBCs after replating. Therefore, milder methods of dissociation such as nonenzymatic dissociation solutions should be tested. It is also critical to add EB1 as soon as dissociation is visible. However, DO NOT keep cells for extended time in EB1 medium because it will induce premature differentiation otherwise. Remove medium and wash cells with NP medium.
7. If the initial survival of EBCs or NPs is low, double or triple the concentration of FGF-2 in NP medium.
8. Incubation with ceramide or ceramide analogs will eliminate rPS cells and enhance neuronal lineage specification, while the addition of S1P or S1P analogs will direct differentiation toward oligodendroglial lineage.
9. The use of pipette tips should be avoided and glass pipettes used instead. However, if glass pipettes for small volumes are not available, pipette tips (NOT plastic pipettes) can be of short-term use. In this case, it is advisable to equilibrate the atmosphere within the tip and the pipettor by repeatedly pipetting up and down organic solvent before using it with the sample.
10. To detect ceramide, lipid equivalent to 200–300 μg protein (2–3 mg or 3–5 $\times$ 10^6 cells) will yield a detectable band using the staining method described in this protocol. Application of an amount of 0.5–1 μg of lipid standard is recommended.
11. The methods described in this protocol are not suitable for staining of low abundance lipids such as S1P. Further, these methods have to be modified if lipids with a different range of polarity (e.g., phospholipids) are to be analyzed. Many of these modified methods will use radioactive labeling of lipid precursors, which will impose additional hazards. For a more comprehensive lipid analysis, it is recommended to use the commercially available service of sphingolipidomics core facilities. A protocol for mass spectrometric analysis is provided in the section above. However, it is also recommended to contact the core facility prior to sample preparation to receive the proper protocol to be used for the sample analysis in the facility.
12. The following methods are only applicable to cultivated cells or cryofixed tissue sections. Paraffin sections cannot be used for immunocytochemistry because organic solvents will dissolve and

remove the lipids from the sample. Therefore, it is also not possible to use organic solvents such as methanol or acetone for fixation. The recommended fixation reagent is 4 % p-formaldehyde in PBS, which should be prepared fresh. However, frozen stock can also be used for a short period of time. Formaldehyde is a toxic reagent and any steps of the fixation procedure handling open containers should be performed in a chemical fume hood.

13. It is recommended to perform immunocytochemistry with or without permeabilization and compare the results. In the case that permeabilization cannot be avoided, it is advisable to first perform immunocytochemistry for the lipid without permeabilization, then fix the cells again, and perform the permeabilization afterwards. Proteins inside of the cells can then be immunodetected performing a second round of incubation with the respective primary and secondary antibodies.

Acknowledgement

This study was in part supported by the grants R01NS046835, R01AG034389, R01NS095215, and NSF grant 1615874.

References

1. Bieberich E, Silva J, Wang G, Krishnamurthy K, Condie BG (2004) Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. J Cell Biol 167(4):723–734
2. Yanai J, Doetchman T, Laufer N, Maslaton J, Mor-Yosef S, Safran A, Shani M, Sofer D (1995) Embryonic cultures but not embryos transplanted to the mouse's brain grow rapidly without immunosuppression. Int J Neurosci 81(1–2):21–26
3. Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, Watanabe TK, Tanigami A (2003) Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology (Oxford) 42(1):162–165
4. Teramoto K, Hara Y, Kumashiro Y, Chinzei R, Tanaka Y, Shimizu-Saito K, Asahina K, Teraoka H, Arii S (2005) Teratoma formation and hepatocyte differentiation in mouse liver transplanted with mouse embryonic stem cell-derived embryoid bodies. Transplant Proc 37(1):285–286
5. Swijnenburg RJ, Tanaka M, Vogel H, Baker J, Kofidis T, Gunawan F, Lebl DR, Caffarelli AD, de Bruin JL, Fedoseyeva EV, Robbins RC (2005) Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 112(9 Suppl):I166–I172
6. Sanchez-Pernaute R, Studer L, Ferrari D, Perrier A, Lee H, Vinuela A, Isacson O (2005) Long-term survival of dopamine neurons derived from parthenogenetic primate embryonic stem cells (cyno-1) after transplantation. Stem Cells 23(7):914–922
7. Kim D, Gu Y, Ishii M, Fujimiya M, Qi M, Nakamura N, Yoshikawa T, Sumi S, Inoue K (2003) In vivo functioning and transplantable mature pancreatic islet-like cell clusters differentiated from embryonic stem cell. Pancreas 27 (2):e34–e41
8. Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE (2005) Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 166(6):1781–1791
9. Fong SP, Tsang KS, Chan AB, Lu G, Poon WS, Li K, Baum LW, Ng HK (2007) Trophism of neural progenitor cells to embryonic stem cells: neural induction and transplantation in a mouse ischemic stroke model. J Neurosci Res 85(9):1851–1862

10. Choi D, Oh HJ, Chang UJ, Koo SK, Jiang JX, Hwang SY, Lee JD, Yeoh GC, Shin HS, Lee JS, Oh B (2002) In vivo differentiation of mouse embryonic stem cells into hepatocytes. Cell Transplant 11(4):359–368
11. Bielby RC, Boccaccini AR, Polak JM, Buttery LD (2004) In vitro differentiation and in vivo mineralization of osteogenic cells derived from human embryonic stem cells. Tissue Eng 10 (9–10):1518–1525
12. Arnhold S, Klein H, Semkova I, Addicks K, Schraermeyer U (2004) Neurally selected embryonic stem cells induce tumor formation after long-term survival following engraftment into the subretinal space. Invest Ophthalmol Vis Sci 45(12):4251–4255
13. Baker M (2009) Stem cells: fast and furious. Nature 458(7241):962–965. doi:10.1038/458962a. 458962a [pii]
14. Leor J, Gerecht S, Cohen S, Miller L, Holbova R, Ziskind A, Shachar M, Feinberg MS, Guetta E, Itskovitz-Eldor J (2007) Human embryonic stem cell transplantation to repair the infarcted myocardium. Heart 93(10):1278–1284. doi:10.1136/hrt.2006.093161. hrt.2006.093161 [pii]
15. Blum B, Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. Adv Cancer Res 100:133–158. doi:10.1016/S0065-230X(08)00005-5. S0065-230X(08)00005-5 [pii]
16. Lee AS, Tang C, Cao F, Xie X, van der Bogt K, Hwang A, Connolly AJ, Robbins RC, Wu JC (2009) Effects of cell number on teratoma formation by human embryonic stem cells. Cell Cycle 8(16):2608–2612. 9353 [pii]
17. Fong CY, Gauthaman K, Bongso A (2010) Teratomas from pluripotent stem cells: a clinical hurdle. J Cell Biochem. doi:10.1002/jcb.22775
18. Kuznetsov S, Cherman N, Gehron Robey P (2010) In vivo bone formation by progeny of human embryonic stem cells. Stem Cells Dev. doi:10.1089/scd.2009.0501
19. Wang NK, Tosi J, Kasanuki JM, Chou CL, Kong J, Parmalee N, Wert KJ, Allikmets R, Lai CC, Chien CL, Nagasaki T, Lin CS, Tsang SH (2010) Transplantation of reprogrammed embryonic stem cells improves visual function in a mouse model for retinitis pigmentosa. Transplantation 89(8):911–919. doi:10.1097/TP.0b013e3181d45a61
20. Bieberich E (2008) Smart drugs for smarter stem cells: making SENSe (sphingolipid-enhanced neural stem cells) of ceramide. Neurosignals 16(2–3):124–139. doi:10.1159/000111558. 000111558 [pii]
21. Jeong HC, Cho SJ, Lee MO, Cha HJ (2017) Technical approaches to induce selective cell death of pluripotent stem cells. Cell Mol Life Sci. doi:10.1007/s00018-017-2486-0
22. Bieberich E (2008) Ceramide signaling in cancer and stem cells. Future Lipidol 3 (3):273–300. doi:10.2217/17460875.3.3.273
23. Bieberich E, Hu B, Silva J, MacKinnon S, Yu RK, Fillmore H, Broaddus WC, Ottenbrite RM (2002) Synthesis and characterization of novel ceramide analogs for induction of apoptosis in human cancer cells. Cancer Lett 181 (1):55–64
24. Bieberich E, Kawaguchi T, Yu RK (2000) N-acylated serinol is a novel ceramide mimic inducing apoptosis in neuroblastoma cells. J Biol Chem 275(1):177–181
25. Wang G, Krishnamurthy K, Umapathy NS, Verin AD, Bieberich E (2009) The carboxyl-terminal domain of atypical protein kinase Czeta binds to ceramide and regulates junction formation in epithelial cells. J Biol Chem 284 (21):14469–14475. doi:10.1074/jbc.M808909200. M808909200 [pii]
26. Wang G, Silva J, Krishnamurthy K, Tran E, Condie BG, Bieberich E (2005) Direct binding to ceramide activates protein kinase Czeta before the formation of a pro-apoptotic complex with PAR-4 in differentiating stem cells. J Biol Chem 280(28):26415–26424
27. Dutta D, Ray S, Home P, Larson M, Wolfe MW, Paul S (2011) Self renewal vs. lineage commitment of embryonic stem cells: protein kinase C signaling shifts the balance. Stem Cells. doi:10.1002/stem.605
28. He Q, Wang G, Wakade S, Dasgupta S, Dinkins M, Kong JN, Spassieva SD, Bieberich E (2014) Primary cilia in stem cells and neural progenitors are regulated by neutral sphingomyelinase 2 and ceramide. Mol Biol Cell 25 (11):1715–1729. doi:10.1091/mbc.E13-12-0730. mbc.E13-12-0730 [pii]
29. Kong JN, Hardin K, Dinkins M, Wang G, He Q, Mujadzic T, Zhu G, Bielawski J, Spassieva S, Bieberich E (2015) Regulation of Chlamydomonas flagella and ependymal cell motile cilia by ceramide-mediated translocation of GSK3. Mol Biol Cell 26(24):4451–4465. doi:10.1091/mbc.E15-06-0371
30. He Q, Wang G, Dasgupta S, Dinkins M, Zhu G, Bieberich E (2012) Characterization of an apical ceramide-enriched compartment regulating ciliogenesis. Mol Biol Cell 23 (16):3156–3166. doi:10.1091/mbc.E12-02-0079. mbc.E12-02-0079 [pii]
31. Kleger A, Busch T, Liebau S, Prelle K, Paschke S, Beil M, Rolletschek A, Wobus A, Wolf E, Adler G, Seufferlein T (2007) The bioactive

lipid sphingosylphosphorylcholine induces differentiation of mouse embryonic stem cells and human promyelocytic leukaemia cells. Cell Signal 19(2):367–377. doi:10.1016/j.cellsig.2006.07.015

32. Rodgers A, Mormeneo D, Long JS, Delgado A, Pyne NJ, Pyne S (2009) Sphingosine 1-phosphate regulation of extracellular signal-regulated kinase-1/2 in embryonic stem cells. Stem Cells Dev 18(9):1319–1330. doi:10.1089/scd.2009.0023
33. Pebay A, Wong RC, Pitson SM, Wolvetang EJ, Peh GS, Filipczyk A, Koh KL, Tellis I, Nguyen LT, Pera MF (2005) Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells. Stem Cells 23(10):1541–1548. doi:10.1634/stemcells.2004-0338. 2004-0338 [pii]
34. Wong RC, Pera MF, Pebay A (2012) Maintenance of human embryonic stem cells by sphingosine-1-phosphate and platelet-derived growth factor. Methods Mol Biol 874:167–175. doi:10.1007/978-1-61779-800-9_13
35. Callihan P, Alqinyah M, Hooks SB (2017) Sphingosine-1-phosphate (S1P) signaling in neural progenitors. Methods Mol Biol. doi:10.1007/7651_2017_3
36. Wong RC, Pera MF, Pebay A (2017) Maintenance of human embryonic stem cells by sphingosine-1-phosphate and platelet-derived growth factor. Methods Mol Biol. doi:10.1007/7651_2017_4
37. Bradley E, Bieberich E, Mivechi NF, Tangpisuthipongsa D, Wang G (2012) Regulation of embryonic stem cell pluripotency by heat shock protein 90. Stem Cells 30(8):1624–1633. doi:10.1002/stem.1143
38. Burdon T, Smith A, Savatier P (2002) Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 12(9):432–438
39. Smith GS, Kumar A, Saba JD (2013) Sphingosine phosphate lyase regulates murine embryonic stem cell proliferation and pluripotency through an S1P/STAT3 signaling pathway. Biomolecules 3(3):351–368. doi:10.3390/biom3030351
40. Ryu JM, Baek YB, Shin MS, Park JH, Park SH, Lee JH, Han HJ (2014) Sphingosine-1-phosphate-induced Flk-1 transactivation stimulates mouse embryonic stem cell proliferation through S1P1/S1P3-dependent beta-arrestin/c-Src pathways. Stem Cell Res 12(1):69–85. doi:10.1016/j.scr.2013.08.013
41. Arya D, Chang S, DiMuzio P, Carpenter J, Tulenko TN (2014) Sphingosine-1-phosphate promotes the differentiation of adipose-derived stem cells into endothelial nitric oxide synthase (eNOS) expressing endothelial-like cells. J Biomed Sci 21:55. doi:10.1186/1423-0127-21-55
42. Ratajczak MZ, Suszynska M (2016) Emerging strategies to enhance homing and engraftment of hematopoietic stem cells. Stem Cell Rev 12(1):121–128. doi:10.1007/s12015-015-9625-5
43. Adamiak M, Borkowska S, Wysoczynski M, Suszynska M, Kucia M, Rokosh G, Abdel-Latif A, Ratajczak J, Ratajczak MZ (2015) Evidence for the involvement of sphingosine-1-phosphate in the homing and engraftment of hematopoietic stem cells to bone marrow. Oncotarget 6(22):18819–18828. doi:10.18632/oncotarget.4710
44. Kawahara A, Nishi T, Hisano Y, Fukui H, Yamaguchi A, Mochizuki N (2009) The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. Science 323(5913):524–527. doi:10.1126/science.1167449
45. Bieberich E (2012) Ceramide and sphingosine-1-phosphate signaling in embryonic stem cell differentiation. Methods Mol Biol 874:177–192. doi:10.1007/978-1-61779-800-9_14
46. Bieberich E (2010) There is more to a lipid than just being a fat: sphingolipid-guided differentiation of oligodendroglial lineage from embryonic stem cells. Neurochem Res. doi:10.1007/s11064-010-0338-5
47. Hancock CR, Wetherington JP, Lambert NA, Condie BG (2000) Neuronal differentiation of cryopreserved neural progenitor cells derived from mouse embryonic stem cells. Biochem Biophys Res Commun 271(2):418–421
48. Westmoreland JJ, Hancock CR, Condie BG (2001) Neuronal development of embryonic stem cells: a model of GABAergic neuron differentiation. Biochem Biophys Res Commun 284(3):674–680
49. Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD (1996) Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev 59(1):89–102. 0925477396005722 [pii]
50. Bieberich E, MacKinnon S, Silva J, Noggle S, Condie BG (2003) Regulation of cell death in mitotic neural progenitor cells by asymmetric distribution of prostate apoptosis response 4 (PAR-4) and simultaneous elevation of endogenous ceramide. J Cell Biol 162(3):469–479

51. Bieberich E, MacKinnon S, Silva J, Yu RK (2001) Regulation of apoptosis during neuronal differentiation by ceramide and b-series complex gangliosides. J Biol Chem 276 (48):44396–44404
52. Bieberich E (2002) Recurrent fractal neural networks: a strategy for the exchange of local and global information processing in the brain. Biosystems 66(3):145–164
53. Salli U, Fox TE, Carkaci-Salli N, Sharma A, Robertson GP, Kester M, Vrana KE (2009) Propagation of undifferentiated human embryonic stem cells with nano-liposomal ceramide. Stem Cells Dev 18(1):55–65. doi:10.1089/scd.2007.0271
54. Krishnamurthy K, Wang G, Silva J, Condie BG, Bieberich E (2007) Ceramide regulates atypical PKC{zeta}/{lambda}-mediated cell polarity in primitive ectoderm cells: a novel function of sphingolipids in morphogenesis. J Biol Chem 282(5):3379–3390
55. Bielawski J, Pierce JS, Snider J, Rembiesa B, Szulc ZM, Bielawska A (2010) Sphingolipid analysis by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Adv Exp Med Biol 688:46–59
56. Bielawski J, Pierce JS, Snider J, Rembiesa B, Szulc ZM, Bielawska A (2009) Comprehensive quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. Methods Mol Biol 579:443–467. doi:10.1007/978-1-60761-322-0_22
57. Kramer R, Bielawski J, Kistner-Griffin E, Othman A, Alecu I, Ernst D, Kornhauser D, Hornemann T, Spassieva S (2015) Neurotoxic 1-deoxysphingolipids and paclitaxel-induced peripheral neuropathy. FASEB J. doi:10.1096/fj.15-272567. fj.15-272567 [pii]
58. Van Veldhoven PP, Bell RM (1988) Effect of harvesting methods, growth conditions and growth phase on diacylglycerol levels in cultured human adherent cells. Biochim Biophys Acta 959(2):185–196
59. Krishnamurthy K, Dasgupta S, Bieberich E (2007) Development and characterization of a novel anti-ceramide antibody. J Lipid Res 48 (4):968–975

Methods in Molecular Biology (2017) 1697: 173–180
DOI 10.1007/7651_2017_23

Published online: 28 April 2017

3D Stacked Construct: A Novel Substitute for Corneal Tissue Engineering

Shrestha Priyadarsini, Sarah E. Nicholas, and Dimitrios Karamichos

Abstract

Corneal trauma/injury often results in serious complications including permanent vision loss or loss of visual acuity which demands corneal transplantations or treatment with allogenic graft tissues. There is currently a huge shortage of donor tissue worldwide and the need for human corneal equivalents increases annually. In order to meet such demand the current clinical approach of treating corneal injuries is limited and involves synthetic and allogenic materials which have various shortcomings when it comes to actual transplantations. In this study we introduce the newly developed, next generation of our previously established 3D self-assembled constructs, where multiple constructs are grown and stacked on top of each other without any other artificial product. This new technology brings our 3D in vitro model closer to what is seen in vivo and provides a solid foundation for future studies on corneal biology.

Lipids are known for playing a vital role during metabolism and diseased state of various tissues and Sphingolipids are one such class of lipids which are involved in various cellular mechanisms and signaling processes. The impacts of Sphingolipids that have been documented in several human diseases often involve inflammation, neovascularization, tumorigenesis, and diabetes, but these conditions are not yet thoroughly studied. There is very little information about the exact role of Sphingolipids in the human cornea and future studies aiming at dissecting the mechanisms and pathways involved in order to develop novel therapies. We believe that our novel 3D stacked model can be used to delineate the role of Sphingolipids in the human cornea and provide new insights for understanding and treating various human corneal diseases.

Keywords: 3D constructs, Cornea, Extra cellular matrix, Sphingolipids, Stacking

1 Introduction

In recent years tissue engineering applications have garnered great interests across various fields of medical science in order to treat various diseased conditions. The vast implication of tissue engineering using different biomaterials has been a great success, yet there are various limitations when it comes to actual applications due to a number of contributory factors such as immune response to foreign body or material, synthetic materials fail to respond to the changing physiological loads or biochemical stimuli which limit the lifetime of artificial body parts, graft rejections, infection, glaucoma, retinal detachment and extrusion [1, 2]. The application of tissue

engineering in treating ocular dystrophies has also stimulated great interest and has been a great success over the past years [3–7].

Wound healing is one of the major challenges when it comes to treating ocular injuries. It often leads to scarring resulting in either partial vision loss or permanent blindness. The process of corneal wound healing is complex; it involves interactions between the wound-healing epithelium, a temporary "provisional matrix," and cells present in the extracellular matrix (ECM) [8]. During this process the wounds either tend to heal in a regenerative manner, where the tissue returns to its original state, or in a fibrotic manner, where a scar is produced.

Being able to treat corneal injuries without scarring and be able to mimic the actual in vivo process remains elusive. In vitro there have been a number of models investigated and proposed [2, 8–17]. 3D in vitro models are of great interest due to their potential of mirroring cellular and physiological events that are very important during fibrosis and wound healing [8, 9, 18, 19]. In the cornea, the elucidation of using 3D in vitro systems is imperative in order to improve treatments and lead us to the identification of new therapeutic approaches. Our original 3D in vitro model has been well studied and has shown the impeccable ability of recapitulating in vivo events in vitro [18–20] but one of the biggest limitations of our model is that the cells have limited proliferative potential and can only assemble a certain amount of ECM. Such limitations have been partially overcome by stimulating with various growth factors, mainly transforming growth factor-β (TGF-β) isoforms which aid the cells in stimulating, secreting, and assembling double or triple the amount of ECM [18, 21, 22]. Even with TGF-β stimulation, however, the ECM assembled does not exceed 120–150 μm over 4 weeks, when a human corneal stroma is approximately triple in thickness [18, 21, 23]. Thus, a 3D in vitro model that closely mimics the corneal stroma in size would lead to more accurate results and a better understanding of cellular and ECM mechanisms. The herein described 3D self-assembled stacked model represents the latest generation of our promising in vitro model.

Sphingolipids (SPLs) are known to be involved in human diseases associated with inflammation, neovascularization, tumorigenesis, and diabetes; however, their roles associated with these diseases remain understudied and not fully understood [24, 25]. Bioactive SPLs such as Sphingosine-1-phosphate (S1P) and Ceramide (Cer) have been acknowledged as being essential mediators of many basic cellular processes such as cell migration, survival, contraction, proliferation, gene expression, and cell–cell interactions [26]. S1P and Cer actions/levels are regulated by Ceramidase enzymes; their ability to regulate diverse cellular processes has grasped the attention and interest of researchers due to their capabilities of regulating tissue fibrosis in various organ systems by

utilizing S1P and/or Cer [24, 27, 28]. Among the fields of interest pertaining to SPLs, the cornea remains one of the most scarcely studied. There are currently only a few publications that reported the presence of SPLs in the cornea. Swaney et al. [28] reported the presence of Sphingosine kinase-1 (SphK1), Sphingosine kinase-2 (SphK2), and $S1P_{1\text{-}3,5}$ receptor proteins in cultured human primary corneal fibroblasts (HCFs). Watsky et al. [29] observed expression of S1P receptor's mRNA in cultured corneal epithelial cells which mimicked wound healing responses in vivo. In a recent study, our group showed significant differences in total composition and specific SPL subspecies in the healthy cornea compared to the diabetic cornea [30].

The 3D in vitro model described in detail here can be used in order to investigate the role of SPLs in the healthy and the diseased human cornea while providing new insights in treating ocular dystrophies with better clinical results.

2 Materials

Corneal samples obtained should only be used for scientific purposes and ethical permission must be obtained prior conducting any further experiments. The corneal tissue samples should be from donors with no history of ocular trauma or systemic disease. All reagents and media used should be completely sterile and all the protocols must be initiated in a sterile Laminar flow hood. The storage temperature of the media should be at 4 °C. Waste material should be disposed as per the proper disposal regulations.

2.1 Cell Isolation and Culture

1. Healthy corneal tissue samples from donors with no ocular trauma or systemic disease.
2. Dulbecco's Phosphate Buffered Solution (1×).
3. Sterile forceps.
4. Single edge razor blades and sterile surgical scalpel blades No. 10.
5. Eagle's Minimum Essential Medium (American Type Culture Collection, Manassas, VA, USA) containing 10% FBS and 1% antibiotic. 6.0.05% Trypsin-EDTA (1×).

2.2 3D Constructs Assembly

1. Polycarbonate membrane inserts with 0.4-μm pores (Corning Costar; Corning Incorporated, Corning, NY, USA).
2. Eagle's Minimum Essential Medium containing 10% FBS and 1% Antibiotic.
3. 0.5 mM 2-O-α-Dglucopyranosyl-L-ascorbic acid (Vitamin C).

2.3 Stacked Constructs

1. Sterile forceps.
2. Sterile Spatula.

3. Wax block.
4. Dulbecco's Phosphate Buffered Solution (1×).

2.4 S1P Stock Preparation

1. S1P stock solution was prepared at a concentration of 125 μM for all S1P treatments by dissolving S1P powder in 4 mg/ml of BSA in water at 37 °C in a glass vessel.
2. SphKI2 is a selective inhibitor of SphK1 [31] and a stock solution was made at a concentration of 5 mM by dissolving the powder in DMSO.

3 Methods

3.1 Cell Isolation

1. On receipt of the corneal tissue samples, the tissues should be transferred into a petri dish containing DPBS (1×).
2. The corneal epithelium and endothelium should be removed from the stroma by scraping with a razor blade.
3. The corneal stromal tissues are further cut into small pieces of size ~2 × 2 mm and placed into T25 culture flaks.
4. Explants then should be allowed to adhere to the bottom of the flask at 37 °C for about 30–40 min and then EMEM media containing 10% fetal bovine serum and 1% antibiotic needs to be added carefully without disturbing the explants.
5. The explants should be left undisturbed until the cells begin isolating and migrating through the flask and further they require passage into T75 culture flasks upon 100% confluence after 1–2 weeks of cultivation at 37 °C, 5% CO_2.

3.2 Culture of Primary Human Corneal Fibroblast Cells and Assembly of 3D Constructs

1. HCF cells isolated from explants are cultured in Eagle's Minimum Essential Medium containing 10% fetal bovine serum and 1% antibiotic.
2. Fresh media needs to be supplied every other day for the entire duration of culture. The cultures need to be passaged upon 80–100% confluence.
3. For assembly of 3D constructs about 1×10^6 cells/well of HCF cells need to be counted and seeded on polycarbonate membrane inserts with 0.4-μm pores (Fig. 1) (*see* **Note 1**).
4. The constructs need to be grown in Eagle's Minimum Essential Medium containing 10% fetal bovine serum and 1% antibiotic and after 24 h of cell seeding the cultures need to be stimulated with 0.5 mM 2-O-α-Dglucopyranosyl-L-ascorbic acid (Vitamin C) (*see* **Note 2**).
5. The cultures should be maintained for 2 weeks time point and fresh media should be supplied every other day during the entire study period.

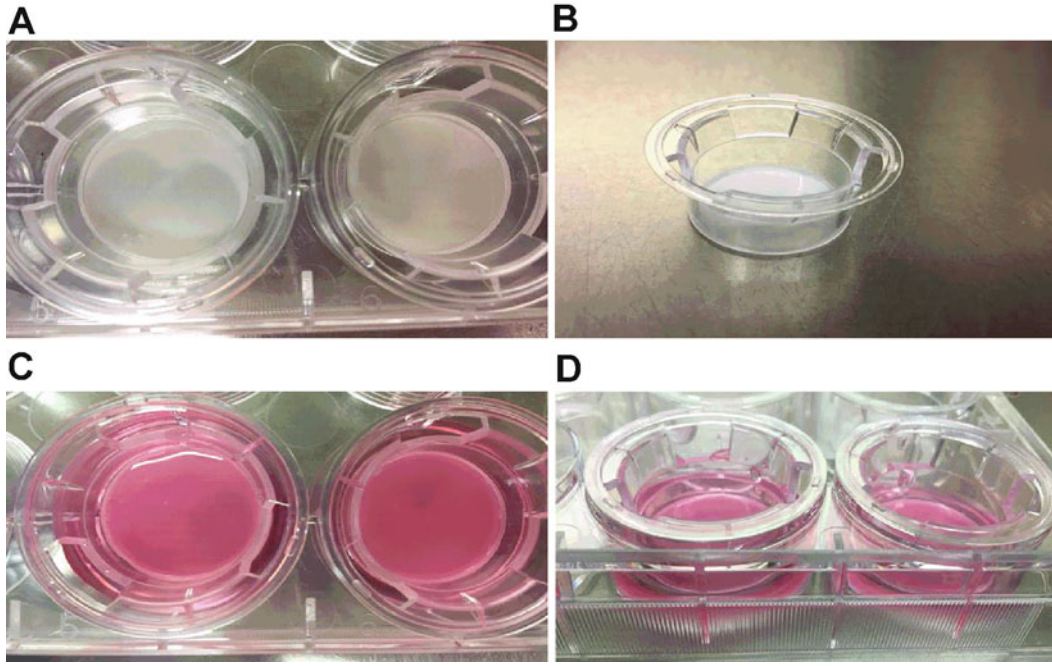

Fig. 1 3D constructs assembly using polycarbonate membrane inserts. (**a**, **b**) Polycarbonate membrane plate and respective inserts with 0.4-μm pores. (**c**) Human corneal fibroblast grown in EMEM media containing 10% fetal bovine serum and 1% antibiotic. (**d**) Cells stimulated with media containing 0.5 mM 2-O-α-Dglucopyranosyl-L-ascorbic acid (Vitamin C)

3.3 Stacking of 3D Constructs

1. 3D constructs maintained for 2 weeks are used for stacking. Firstly, the media needs to be aspirated and the constructs should be washed with sterile DPBS (1×) twice.
2. Further remove the constructs on to a wax block with sterile forceps and gently detach the edges of the membrane from the plastic with the help of a sterile spatula.
3. Now, slowly peel the ECM secreted inwards from one edge of the membrane without any breakage and transfer to a 21.5 cm^2 petri dish containing 2 ml of sterile DPBS (1×) (*see* **Note 3**).
4. Aspirate media from another construct well designated to be stacked upon and wash it with DPBS (1×) twice. Transfer the detached construct on top of the second designated construct well using forceps and ensure the transferred construct is folded inwards (*see* **Note 4**). Spread the construct gently with the help of forceps and spatula ensuring even attachment covering the entire construct well (*see* **Notes 4** and **5**).
5. Incubate the construct for about 15–20 min in about 150–200 μl culture media at 37 °C to allow proper attachment to the base construct and also to prevent floating. Gently add

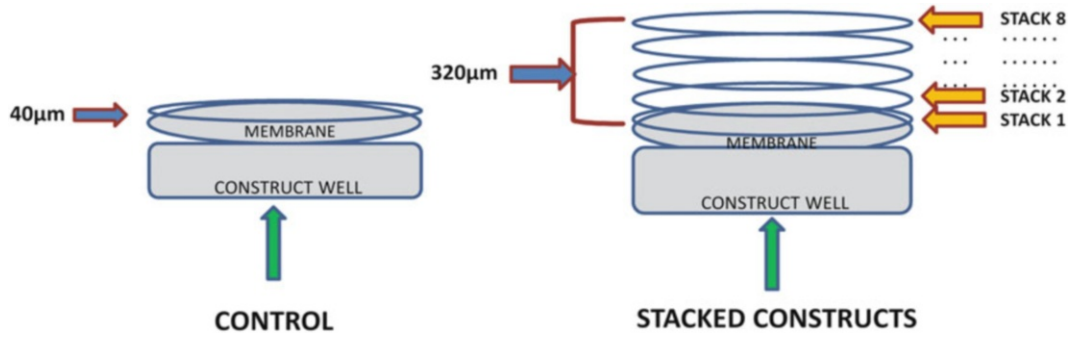

Fig. 2 Stacking of 3D constructs. In controls the cells seeded on the polycarbonate membrane secrete an ECM of about 40 μm thickness whereas in the stacked constructs the total thickness of the ECM makes about 320 μm

media to the stacked constructs without disturbing or detaching the top construct from the bottom layer.

6. Repeat stacking every other day until 8 constructs are stacked all together and change the media 3 times per week (Fig. 2).

3.4 Sphingolipid Analysis

1. Lipid extraction from human corneas needs to follow our previously optimized protocol [30].
2. Samples should be analyzed using targeted LC MS/MS methods. Using targeted lipidomics analysis the changes in SPLs profile in the samples should be identified.

4 Notes

1. During assembly of constructs make sure to have an even cell suspension without any cell lumps and distribute evenly throughout each well by pipetting up and down in order to avoid construct contractions.
2. While preparing the Vitamin C (0.5 mM 2-O-α-Dglucopyranosyl-L-ascorbic acid) media, take about 12 ml of media to which dissolve 0.5 mM 2-O-α-Dglucopyranosyl-L-ascorbic acid and incubate it for about 15 min in order to ensure even dissolving of Vitamin C. Further filter this Vitamin C solution to the entire bottle of culture media.
3. While peeling off the matrix from the membrane try to slowly roll one edge of the matrix initially in order to avoid breakage. When reached half way through the membrane can be slowly peeled off with forceps in one stroke.
4. When the matrix is transferred to the petri dish containing PBS it spreads out flat, exactly the way it's peeled, which clearly gives an

idea about the matrix initial orientation and making it easier to identify the top and bottom of the matrix. Ensure spreading the construct the same manner as that of its initial orientation.

5. Ensure even spreading of the corneal matrix without any creases, this would help in avoiding contraction of the corneal matrix.

Acknowledgement

This work was supported by NIH/NEI EY025256.

References

1. Akpek EK, Alkharashi M, Hwang FS, Ng SM, Lindsley K (2014) Artificial corneas versus donor corneas for repeat corneal transplants. Cochrane Database Syst Rev CD009561
2. Chen FM, Liu X (2016) Advancing biomaterials of human origin for tissue engineering. Prog Polym Sci 53:86–168
3. Karamichos D, Brown RA, Mudera V (2007) Collagen stiffness regulates cellular contraction and matrix remodeling gene expression. J Biomed Mater Res A 83:887–894
4. Griffith LG, Naughton G (2002) Tissue engineering—current challenges and expanding opportunities. Science 295:1009–1014
5. Ruberti JW, Zieske JD (2008) Prelude to corneal tissue engineering—gaining control of collagen organization. Prog Retin Eye Res 27:549–577
6. Guo X, Hutcheon AE, Melotti SA, Zieske JD, Trinkaus-Randall V et al (2007) Morphologic characterization of organized extracellular matrix deposition by ascorbic acid-stimulated human corneal fibroblasts. Invest Ophthalmol Vis Sci 48:4050–4060
7. Karamichos D (2015) Ocular tissue engineering: current and future directions. J Funct Biomater 6:77–80
8. Zieske JD (2001) Extracellular matrix and wound healing. Curr Opin Ophthalmol 12:237–241
9. Karamichos D, Guo XQ, Hutcheon AE, Zieske JD (2010) Human corneal fibrosis: an in vitro model. Invest Ophthalmol Vis Sci 51:1382–1388
10. Priyadarsini S, Sarker-Nag A, Rowsey TG, Ma JX, Karamichos D (2016) Establishment of a 3D in vitro model to accelerate the development of human therapies against corneal diabetes. PLoS One 11:e0168845
11. Karamichos D, Hjortdal J (2014) Keratoconus: tissue engineering and biomaterials. J Funct Biomater 5:111–134
12. Wilson SL, Yang Y, el Haj AJ (2014) Corneal stromal cell plasticity: in vitro regulation of cell phenotype through cell-cell interactions in a three-dimensional model. Tissue Eng A 20:225–238
13. Proulx S, Uwamaliya JD, Carrier P, Deschambeault A, Audet C et al (2010) Reconstruction of a human cornea by the self-assembly approach of tissue engineering using the three native cell types. Mol Vis 16:2192–2201
14. Gonzalez-Andrades M, Alonso-Pastor L, Mauris J, Cruzat A, Dohlman CH et al (2016) Establishment of a novel in vitro model of stratified epithelial wound healing with barrier function. Sci Rep 6:19395
15. Hopkins AM, DeSimone E, Chwalek K, Kaplan DL (2015) 3D in vitro modeling of the central nervous system. Prog Neurobiol 125:1–25
16. Schulz S, Beck D, Laird D, Steinberg T, Tomakidi P et al (2014) Natural corneal cell-based microenvironment as prerequisite for balanced 3D corneal epithelial morphogenesis: a promising animal experiment-abandoning tool in ophthalmology. Tissue Eng Part C Meth 20:297–307
17. Ghezzi CE, Rnjak-Kovacina J, Kaplan DL (2015) Corneal tissue engineering: recent advances and future perspectives. Tissue Eng Part B Rev 21:278–287
18. Karamichos D, Hutcheon AE, Zieske JD (2011) Transforming growth factor-beta3 regulates assembly of a non-fibrotic matrix in a 3D corneal model. J Tissue Eng Regen Med 5: e228–e238
19. Karamichos D, Lakshman N, Petroll WM (2009) An experimental model for assessing fibroblast migration in 3-D collagen matrices. Cell Motil Cytoskeleton 66:1–9

20. Karamichos D, Zareian R, Guo X, Hutcheon AE, Ruberti JW et al (2012) Novel in vitro model for Keratoconus disease. J Funct Biomater 3:760–775
21. Karamichos D, Rich CB, Zareian R, Hutcheon AE, Ruberti JW et al (2013) TGF-β3 stimulates stromal matrix assembly by human corneal keratocyte-like cells. Invest Ophthalmol Vis Sci 54:6612–6619
22. Karamichos D, Hutcheon AE, Zieske JD (2014) Reversal of fibrosis by TGF-beta3 in a 3D in vitro model. Exp Eye Res 124:31–36
23. Saika S (2006) TGFbeta pathobiology in the eye. Lab Invest 86:106–115
24. Coant N, Sakamoto W, Mao C, Hannun YA (2016) Ceramidases, roles in sphingolipid metabolism and in health and disease. Adv Biol Regul 63:122–131
25. Brush RS, Tran JT, Henry KR, McClellan ME, Elliott MH et al (2010) Retinal sphingolipids and their very-long-chain fatty acid-containing species. Invest Ophthalmol Vis Sci 51:4422–4431
26. Shea BS, Brooks SF, Fontaine BA, Chun J, Luster AD et al (2010) Prolonged exposure to sphingosine 1-phosphate receptor-1 agonists exacerbates vascular leak, fibrosis, and mortality after lung injury. Am J Respir Cell Mol Biol 43:662–673
27. Shea BS, Tager AM (2012) Sphingolipid regulation of tissue fibrosis. Open Rheumatol J 6:123–129
28. Swaney JS, Moreno KM, Gentile AM, Sabbadini RA, Stoller GL (2008) Sphingosine-1-phosphate (S1P) is a novel fibrotic mediator in the eye. Exp Eye Res 87:367–375
29. Watsky MA, Weber KT, Sun Y, Postlethwaite A (2010) New insights into the mechanism of fibroblast to myofibroblast transformation and associated pathologies. Int Rev Cell Mol Biol 282:165–192
30. Priyadarsini S, McKay TB, Sarker-Nag A, Allegood J, Chalfant C et al (2016) Complete metabolome and lipidome analysis reveals novel biomarkers in the human diabetic corneal stroma. Exp Eye Res 153:90–100
31. French KJ, Schrecengost RS, Lee BD, Zhuang Y, Smith SN et al (2003) Discovery and evaluation of inhibitors of human sphingosine kinase. Cancer Res 63:5962–5969

Methods in Molecular Biology (2017) 1697: 181–182
https://doi.org/10.1007/978-1-4939-7413-9

INDEX

O

P

Q

S

T

W

Z