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

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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

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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

2.1.1 Wound Healing Assay

1. SkMel2 melanoma cell line.
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

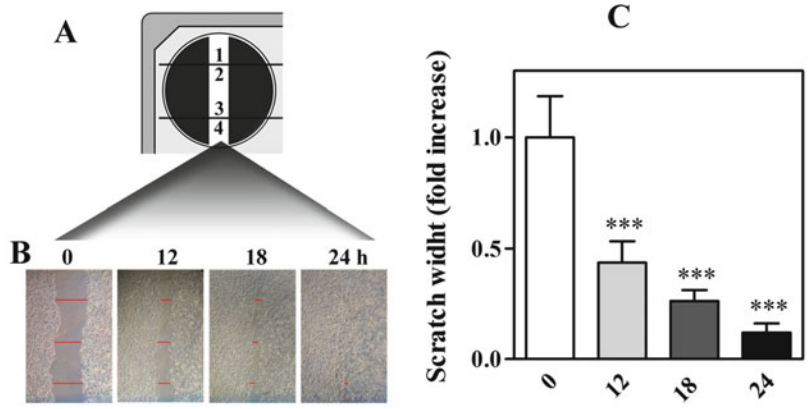

Fig. 1 Establishment of SkMel2 melanoma cell line migration time by wound healing assay. (**a**) Two indelible marks were made in each well on the bottom of the plate. SkMel2 cells were cultured until 100% confluence and scratch performed as described in the text. Four photographs were taken above and 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 $\pm$ SD of three independent experiments and were analyzed with one-way ANOVA and Bonferroni's post test (***$p < 0.001$)

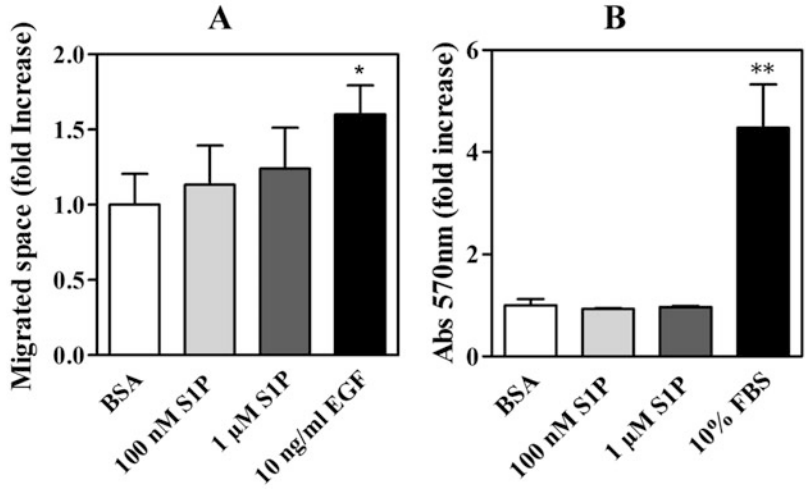

Fig. 2 Effect of S1P on melanoma cell migration. SkMel2 (**a**) and Lu1205 (**b**) melanoma cells were subjected to migration assay by Wound Healing or Transwell assay respectively. Cells were treated with S1P 100 nM and 1 μM during 18 (**a**) or 6 h (**b**). 10 ng/ml EGF was used as positive control. The results are shown as the mean $\pm$ 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).

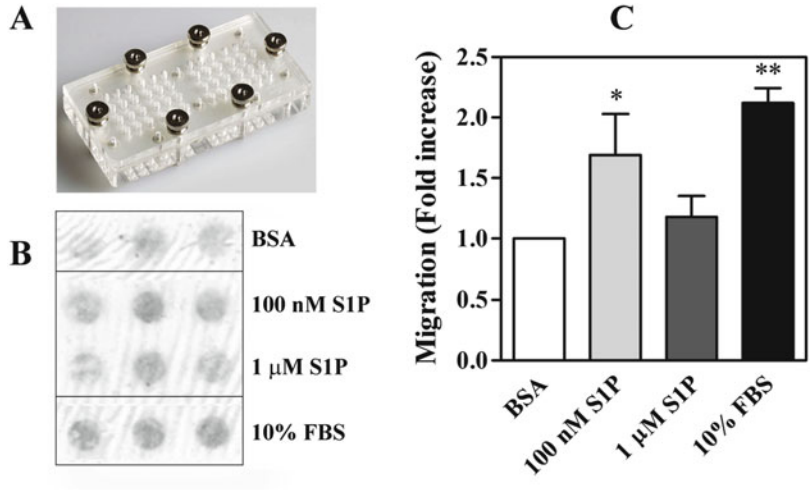

Fig. 3 Analysis of migration in the modified Boyden Chamber. (**a**) Migration assay was performed as described with M2 melanoma cells in a 48-well Boyden Chamber. (**b**) Representative photography of a stained membrane after 6 h incubation. (**c**) Quantification of migration using Image Studio Lite 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).

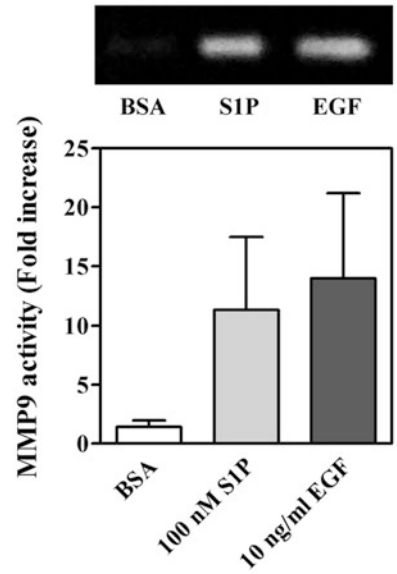

Fig. 4 Zymography assay. M2 melanoma cells were stimulated with 100 nM S1P or 10 ng/ml EGF for 48 h. Fresh supernatant culture medium were obtained and subjected to electrophoresis in polyacrylamide gels copolymerized with gelatin. After MMP-9 activity development, gel was stained and photographed (a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

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