Video Article

In Vitro Assays to Evaluate the Migration, Invasion, and Proliferation of Immortalized Human First-Trimester Trophoblast Cell Lines

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Abstract

Cell movement is a critical property of trophoblasts during placental development and early pregnancy. The significance of proper trophoblast migration and invasion is demonstrated by pregnancy disorders such as pre-eclampsia and intrauterine growth restriction, which are associated with inadequate trophoblast invasion of the maternal vasculature. Unfortunately, our understanding of the mechanisms by which the placenta develops from migrating trophoblasts is limited. In vitro analysis of cell migration via the scratch assay is a useful tool in identifying factors that regulate trophoblast migratory capacity. However, this assay alone does not define the cellular changes that can result in altered cell migration. This protocol describes three different in vitro assays that are used collectively to evaluate trophoblast cell movement: the scratch assay, the invasion assay, and the proliferation assay. The protocols described here may also be modified for use in other cell lines to quantify cell movement in response to stimuli. These methods allow investigators to identify individual factors that contribute to the cell movement and provide a thorough examination of potential mechanisms underlying apparent changes in cell migration.

Introduction

Placental development is a crucial step in the establishment of pregnancy, impacting both maternal and fetal health. However, the mechanistic basis by which this process occurs is not fully understood. Cell migration is an important biological process that contributes to the establishment and functions of the placenta during pregnancy. Following blastocyst implantation, the trophectoderm differentiates into villous trophoblasts, which cover the surface of the chorionic villi and are involved in gas and nutrient exchange, and extravillous trophoblasts (EVTs), which migrate out from the villi and invade the maternal decidua and vasculature[1]. Migration of the EVTs is essential for remodeling maternal spiral arteries and establishing uteroplacental circulation to support fetal growth[2]. Inadequate trophoblast invasion during pregnancy results in abnormal placental development and may contribute to pregnancy complications such as preeclampsia, gestational hypertension, intra-uterine growth restriction, and preterm birth[3,4]. Therefore, understanding the factors that affect trophoblast motility is essential to determining the pathways required for normal placentation.

Trophoblast migration is controlled by a complex network of signaling molecules, including growth factors, cytokines, hormones, and angiogenic factors[5]. Due to the limitations of studying the placenta in vivo, in vitro assays utilizing immortalized human trophoblast cell lines have been crucial to identifying factors that contribute to trophoblast motility. Scratch and invasion assays have been used extensively to quantitatively assess the role of individual molecules on trophoblast cell migration[6,7,8]. However, while useful in tandem to investigate how changes in migration may be caused by changes in cell invasiveness, these two assays do not account for additional mechanisms that may contribute to altered rates of cell migration. For example, reductions to the rate of cell proliferation may result in fewer cells available to migrate.

Here, we describe quantitative in vitro methods for assessing trophoblast migration using scratch, cell proliferation, and cell invasion assays. In the scratch assay, a uniform wound is created in a cell monolayer, and the migration of cells to fill the gap is measured by automated, time-lapse imaging of the wound size and density of cells within the wound. The cell proliferation assay is based on calculating the ratio of cells at each time point compared to a known starting quantity of cells. In the cell invasion assay, cells are seeded atop an extracellular matrix-coated cell culture insert chamber (e.g. Transwell), and the number of cells that invade through the extracellular matrix in response to chemo-attractant are counted.

The scratch assay is a simple and effective tool that may be used to determine how different environmental conditions affect cell migration. The proliferation and invasion assays may subsequently be used to determine the contribution of cell proliferation and invasiveness to overall changes in cell migration. Collectively, these assays provide robust measurements of biological processes that may contribute to cell motility. Two immortalized first-trimester trophoblast cell lines were used in the described assays, Swan.71 (Sw.71) and HTR-8/SVneo[9,10]. However, these assays may also be optimized for use in other cell types to identify key modulators of cell migration and invasion.

Protocol

1. Cell preparation

1. Maintain cells in T-75 flasks in standard growth media at 37 °C, 5% CO_2, and 95% humidity.

NOTE: The cells used in these experiments were the immortalized first-trimester trophoblast cell lines Swan.71 (Sw.71) and HTR-8/SVneo[9,10]. The Sw.71 cells were maintained in DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1.0 mM sodium pyruvate, 10 mM HEPES, 0.10 mM MEM non-essential amino acids, 100 units/mL penicillin, and 100 µg/mL streptomycin. The HTR-8/SVneo cells were maintained in RPMI-1640 supplemented with 5% heat-inactivated FBS.

2. Allow the cells to replicate until they reach 90-100% confluence. Using a sterile tissue-culture flow hood, aspirate the media from the flask and wash cells with 10 mL of sterile 1x phosphate-buffered saline (PBS).
3. Aspirate the PBS and add 5 mL of 1x (0.05%) trypsin-EDTA to the flask, making sure that cells are covered by trypsin. Place flask in the laboratory incubator maintained at 37°C, 5% CO_2, and 95% humidity until all cells have detached from the bottom of the flask (approximately 5-10 min).
4. Add 10 mL of pre-warmed growth media to the flask to deactivate the trypsin-EDTA.

2. Scratch assay

1. Following step 1.4, seed the appropriate number of cells into a 24-well plate to achieve 100% confluence in 48 h.
   NOTE: It is possible to perform the scratch assay with a variety of different plate layouts. The maximum number of wells allowed by the wound maker tool is a 96-well format.
2. The next day (day prior to scratch assay), change media to phenol-red free media supplemented as required, including heat-inactivated, charcoal dextran-stripped FBS.
   NOTE: Other studies have recommended the use of media with low concentration FBS (e.g., 1%) to minimize cell proliferation, which can be used as an alternative in this protocol[11].
3. On the day of the scratch assay, pretreat cells for 30 min by adding desired treatment to the media in each well. The outlined experiments utilized vehicle (1x PBS) and 100 nM dexamethasone diluted in 1x PBS.
4. To create uniform wounds, place sterile 10 µL pipette tips on the pins of the wound maker tool. Lower the tips into the first row of wells and move the plate along the track of the wound maker until the pipette tips reach the other side of the well.
   1. Move the plate back to the starting position, allowing the pipette tips to continuously touch the bottom of the plate to ensure a constant wound across the diameter of the well. Remove and replace the sterile 10 µL pipette tips and lower the tips into the next row of wells.
5. Repeat step 2.4 until all wells are scratched.
   NOTE: If a wound maker tool is not available, wounds can be created by manually scraping the center of the wells with a 10 µL pipette tip.
6. Carefully aspirate media and gently wash cells twice with 1x PBS. Following a final wash, add supplemented phenol-red free, stripped or low-FBS media with desired treatment or vehicle (as directed in step 2.3).
   NOTE: HTR-8/SVneo cells should be washed with supplemented phenol-red media, as washing with 1X PBS will cause cells to detach.
7. Place the 24-well plate containing scratched wells in the automated, time-lapse imager (see **Table of Materials**) housed in a laboratory incubator maintained at 37 °C, 5% CO_2, and 95% humidity. Image wells at regular intervals as needed to allow cells to complete wound healing (e.g., every 2 h for 72 h).
8. Calculate the wound density and width by the software associated with the automated, time-lapse imager (see **Table of Materials**). To calculate the percentage change in wound size relative to the size of the starting wound, set the wound width at time 0 equal to 100%. Divide the wound size in each subsequent timepoint by the wound size at time 0 and multiply by 100 to obtain the percentage (**Figure 1**).

3. Invasion assay

1. Following step 1.4, seed the appropriate number of cells into 6-well plates to allow cells to reach 90% confluence in 48 h. Maintain cells in a laboratory incubator set to 37 °C, 5% CO_2, and 95% humidity.
2. The next day, change media to phenol-red free media supplemented as required, including heat-inactivated, charcoal dextran-stripped FBS.
   NOTE: Cells can be pretreated at this time depending on experimental design.
3. At this time, place the vial of the 10 mg/mL extracellular matrix (see **Table of Materials**) on ice, and let it thaw in the refrigerator overnight.
4. The next day, pre-cool invasion assay supplies by placing 24-well plates, cell culture inserts, microcentrifuge tubes, and pipette tips in the refrigerator (4 °C) for at least 2 h prior to working with the extracellular matrix.
5. Using chilled supplies in the tissue culture hood, dilute 10 mg/mL extracellular matrix 1:1 with phenol-red-free, serum-free media to a final concentration of 5 mg/mL.
   NOTE: Always keep the stock and diluted extracellular matrix on ice. The ratio of extracellular matrix to media can be adjusted based on the properties of the cell line used in the assay.
6. Using chilled supplies in the tissue culture hood, add 100 µL of diluted extracellular matrix to the inserts (see **Table of Materials**) that have been placed in the 24-well plate. Make sure that the matrix is level without any bubbles. Place the 24-well plate containing matrix-coated inserts in the incubator at 37 °C to allow the matrix to harden.
7. To prep cells for invasion assay, wash cells with 1 mL of 1x PBS, aspirate the PBS, and add 500 µL of 1x trypsin-EDTA to each well. Place the 6-well plate in the laboratory incubator maintained at 37 °C, 5% CO_2, and 95% humidity until all cells have detached from the bottom of the plate.
8. Once cells have detached, add 500 µL of phenol-red-free, serum-free media to each well of the trypsinized cells.
9. Transfer 1,000 µL of detached cells to microcentrifuge tube and spin down for 5 min at 400 x *g* at 4 °C.
10. Aspirate media and resuspend cells in phenol-red-free, serum-free media (volume will depend on concentration of cells). Count cells using an automated cell counter (see **Table of Materials**) and adjust volume of cell suspension for a final concentration of 5 x 10^5 cells per mL.
    NOTE: A hemocytometer and microscope may also be used to count cells.
11. Add 600 µL of complete growth media to the wells of the 24-well plate that will be used for the invasion assay.
    NOTE: Additional chemoattractants or treatments may be added to the complete growth medium in the well (lower chamber). The outlined experiments added vehicle (1x PBS) or 100 nM dexamethasone diluted in 1x PBS to the lower chamber.
12. Place the pre-coated insert into each well containing complete growth medium that will be used for the invasion assay. Then add 200 µL of cells on top of each pre-coated cell insert (upper chamber) for a total of 1 x 10^5 cells.

NOTE: As a negative control for this assay, an equal volume of phenol-red-free, serum-free media (without cells) can be added to the upper chamber.

13. Place 24-well plate in the laboratory incubator maintained at 37 °C, 5% CO_2, and 95% humidity for 24 h.
NOTE: The 24 h incubation period was optimized for the immortalized first-trimester trophoblast cells and further tests may be required to determine the required incubation period in other cell lines.
14. Following overnight incubation, suction the remaining media from the upper and lower chamber without disturbing the extracellular matrix. Then, carefully remove the extracellular matrix and non-invaded cells from the upper portion of the insert with a cotton swab, swabbing as many times as necessary to remove extracellular matrix.
15. Wash each insert 3x with 1x PBS, adding PBS to both the upper and lower chambers of the well. Aspirate PBS after each wash.
16. Fix cells attached to the inserts by placing inserts into 100% ice-cold methanol for 30 min at 4 °C. Wash inserts 3x with 1x PBS and remove PBS after the final wash. Stain the cells attached to the inserts for 10 min with 0.2% crystal violet in 20% ethanol.
17. Rinse 3x with deionized water and aspirate deionized water after the final wash.
18. Air-dry inserts for 1 h at room temperature. Using forceps and a razor blade, carefully cut the bottom membrane of the insert and mount on a glass slide with the bottom side facing up. Allow mounting media to dry at room temperature.
19. Obtain at least 4 unique images of invaded cells per sample using a light microscope with a 20x objective. Count the total number of cells in each image and normalize the number of cells to control samples and plot data (**Figure 2**).

4. Proliferation assay

1. Following step 1.4, count trypsinized cells from the flask using an automated cell counter and seed into 6-well plates at a density of 2 x 10^5 cells per well in standard growth media.
2. Place cells in a laboratory incubator maintained at 37 °C, 5% CO_2, and 95% humidity for 4 h or until cells have adhered.
3. Change media to phenol-red free media supplemented as required, including heat-inactivated, charcoal dextran-stripped FBS and add desired treatment. The outlined experiments added vehicle (1x PBS) or 100 nM dexamethasone diluted in 1x PBS. Place the 6-well plates in a laboratory incubator maintained at 37 °C, 5% CO^2, and 95% humidity.
4. Remove media and replace with new media and treatment every 48 h.
5. After 24, 48, or 72 h treatment, wash cells with 1 mL of 1x PBS and add 500 µL of trypsin-EDTA to each well. Place 6-well plate in incubator until cells have detached from plate.
6. Once cells have detached from the plate, add 500 µL of the supplemented phenol-red free media to deactivate trypsin.
7. Add 5 µL of trypsinized cells to 5 µL of Trypan blue in a separate tube and mix by pipetting.
8. Count cells from each well in duplicate using an automated cell counter.
9. Average the number of cells per well at each time point and plot as a function of time (**Figure 3**).

Representative Results

The immortalized human first-trimester trophoblast cell lines Sw.71 and HTR-8/SVneo were used in these experiments to determine the role of glucocorticoids in trophoblast cell migration[12]. Glucocorticoids were used in these experiments as they have recently been shown to alter trophoblast functions, although alternative treatments could be used[12]. Both cell lines were treated with vehicle (1x PBS) or 100 nM of the synthetic glucocorticoid dexamethasone (Dex) for all experiments. Wound density and size were measured every 2 h for 72 h using an automated, time-lapse imaging system. **Figure 1** shows an example of images and results obtained from the scratch assay at different timepoints. Sample images from 0, 8, and 18 h timepoints for both treatments have been provided (**Figure 1A**). The wound density and wound size are graphed over time for samples treated with vehicle control (Veh) or 100 nM Dex (**Figure 1B**). Dex treatment reduced the rate of wound closure as determined by both wound density and wound size, indicating that glucocorticoids inhibit cell migration in first-trimester trophoblast cells.

Figure 2 shows an example of images taken of the cell culture insert following the invasion assay. Cells were treated for 24 h with Veh or 100 nM Dex. Invaded cells were counted from four independent replicates per treatment using four unique fields of view per replicate. Glucocorticoid treatment reduced cell invasiveness, as shown by a 15% reduction in the number of invaded cells.

Figure 3 shows an example of the cell proliferation assay. Cells were counted at 24, 48, and 72 h following treatment with Veh or 100 nM Dex. Glucocorticoid treatment reduced cell proliferation, as indicated by fewer cells at each time point. Overall, these assays demonstrate that glucocorticoid exposure reduces cell motility by inhibiting both cell proliferation and invasion.

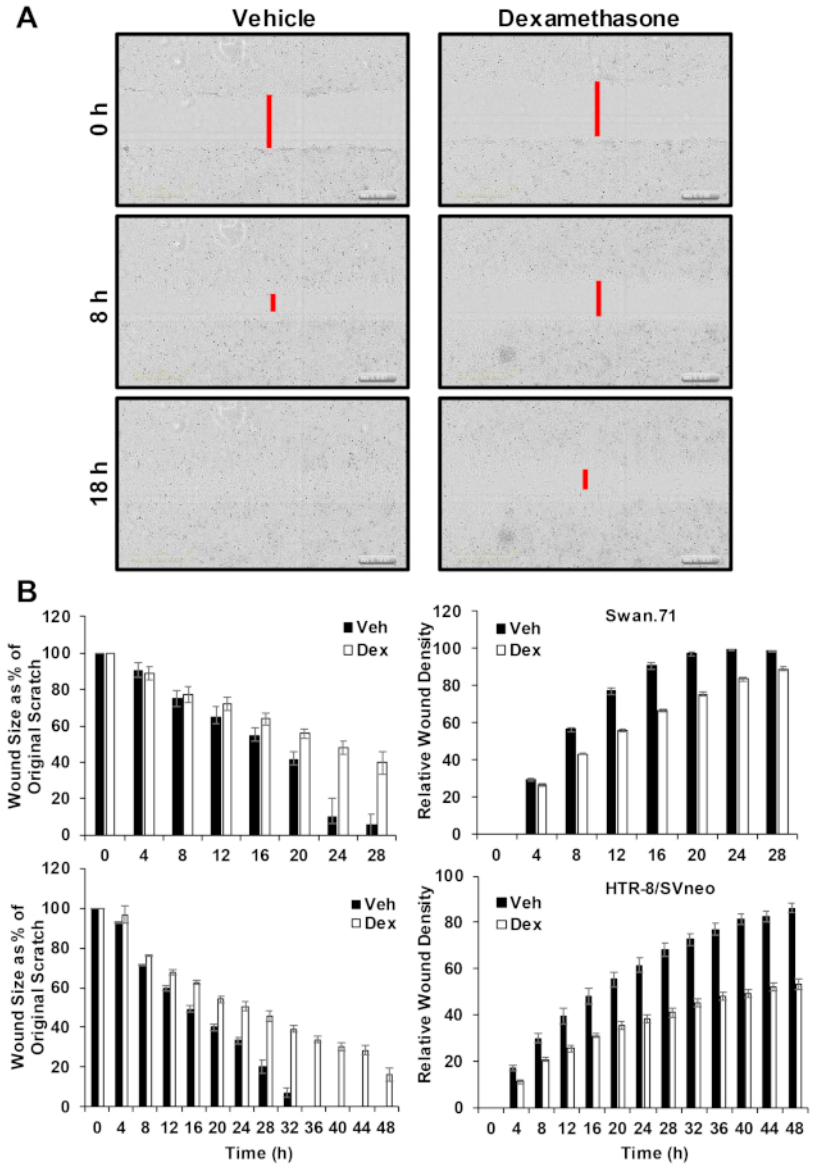

Figure 1: Cell Scratch Assay. (**A**) Representative images of wounds for Veh- and Dex-treated Sw.71 cells at 0, 8, and 18 h are shown. Red lines indicate wound size. (**B**) Wound density and wound size were measured in Sw.71 and HTR-8/SVneo cells treated with vehicle (Veh) or 100 nM Dex. Pictures were taken every 2 h for 72 h using an automated, time-lapse microscope. Data represents the mean of four independent replicates ± SEM. Please click here to view a larger version of this figure.

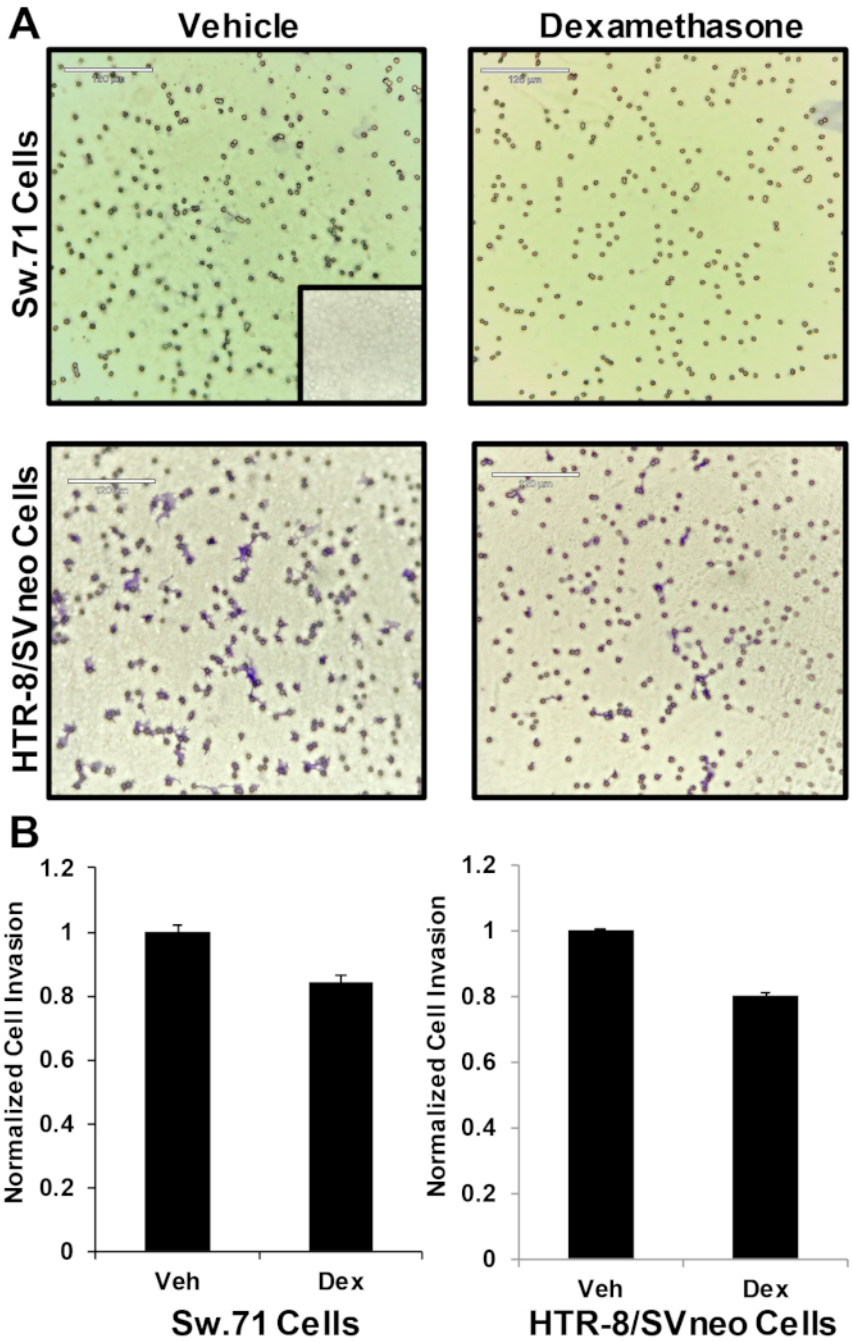

Figure 2: Cell Invasion Assay. Invasion assays were conducted with Sw.71 and HTR-8/SVneo cells treated for 24 h with vehicle (Veh) or 100 nM Dex. (**A**) Representative images from the invasion assay for Veh- and Dex-treated cells after 24 h incubation. Inset image is negative control with no cells added to the upper chamber. Images taken at 200x magnification. Scale bars are 120 µm. (**B**) Invaded cells were counted and the bar graphs represent the normalized mean of four independent replicates ± SEM. Please click here to view a larger version of this figure.

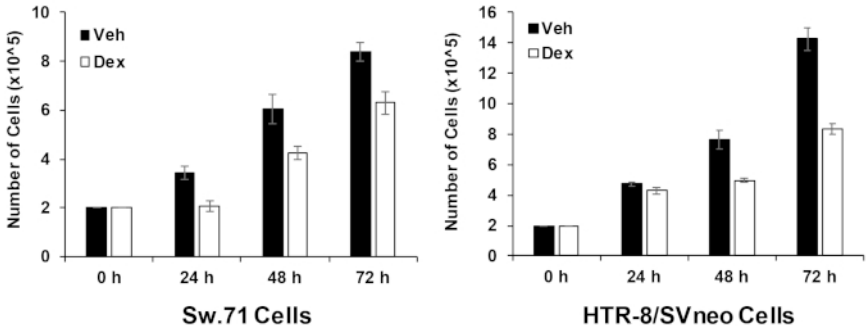

Figure 3: Cell Proliferation Assay. Sw.71 and HTR-8/SVneo cells treated with vehicle (Veh) or 100 nM Dex were counted every 24 h using an automated cell counter. Cell counts are graphed as the mean ± SEM of at least 11 independent replicates. Please click here to view a larger version of this figure.

Discussion

This procedure builds on the use of migration and invasion assays to include the rate of cell proliferation as a potential contributor to measured differences in trophoblast cell movement. Used together, these in vitro assays are simple and effective methods that can be used to identify the cellular pathways that regulate trophoblast migration. As described above, trophoblast cells may be treated with hormones, cytokines, growth factors, or other molecules to determine their impact on trophoblast movement. Alternatively, target proteins may be depleted from cells to elucidate their functional role in trophoblast motility. Identifying key modulators of trophoblast migration may provide a better understanding of the basic mechanisms underlying complications of pregnancy related to placental defects, including preeclampsia, intrauterine growth restriction, and preterm birth.

There are several critical steps in this protocol that are required to obtain accurate results. Because phenol-red has estrogenic activity at concentrations used in cell culture media[13], it is important that phenol-red free media is used for experimental endpoints to prevent undesired activation of the estrogen receptor in cells. During the transwell invasion assay, it is important to ensure that the extracellular matrix is homogenous, level, and that cold supplies are used to prevent premature polymerization of the matrix. When conducting the cell proliferation and invasion assays, it is important to count samples immediately to prevent re-adherence of the cells to tissue culture plates. It should also be taken under consideration that cell death due to experimental treatment may influence the results of all three assays. Therefore, markers of cell death (e.g., lactate dehydrogenase release, phosphatidylserine externalization, or DNA degradation) in response to treatment should be evaluated in the experimental cell line prior to evaluating migration, invasion, and proliferation[14]. Some limitations exist for these assays, for example, the process of creating a wound in a cell monolayer induces a cell injury response than may confound the cell migration process. Furthermore, manual scraping may introduce interexperiment variability, although utilizing a wound-maker tool that creates uniform wound widths can partially mitigate this limitation. The disadvantage of the invasion assay is that it is an endpoint assay, and the kinetics of movement are not easily attained. Despite these limitations, the assays described here provide reproducible, quantitative data at relatively low-cost.

The assays described here can also be modified to measure cell migration in other cell types, although these assays would not be suitable for non-adherent cell types. In the scratch assay, the imaging intervals for time-lapse microscopy may be adjusted to sufficiently capture differences in the rate of cell migration. Automated systems for live cell imaging can capture wound size images as frequently as every 15 minutes. Images taken at least every 30 minutes can be stitched together to create a movie of wound healing. In the invasion assay, the concentration of extracellular matrix, the total number of cells seeded, and the length of incubation prior to fixing and counting cells may be adjusted to account for the specific rate of invasion in different cell types. In the cell proliferation assay, the number of cells seeded onto the plates and the time points for cell counting may be adjusted to account for different rates of cell growth. Overall, these assays are flexible and highly accessible tools that can be used in a wide range of cell types to identify the mechanisms underlying cell migration.

Disclosures

The authors have nothing to disclose.

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