Editorial Comments:

**Editorial Comment:** Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Author’s Response:** We have now proofread the manuscript for spelling and grammar.

**Editorial Comment:** JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: IncuCyte, Matrigel, Eppendorf, etc.

**Author’s Response:** Commercial language has been removed from the manuscript and instead references to the Table of Materials and Reagents have now been included.

**Editorial Comment:** Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**Author’s Response:** Numbers have been adjusted throughout the manuscript.

**Editorial Comment:** Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

Lines 99, 148, 163, 185: Please specify the temperature of the incubator. The temperature of the incubator is notated throughout.

Line 112: Please specify the treatment applied in this step. Give an example. The treatment and concentration is now notated, including vehicle used in experiment.

Lines 122-123: It is unclear how to replace treatment. Please specify. Terminology instructing “replace treatment” has been removed. The manuscript now instructs to add media after final wash and treatment as described in earlier step.

Line 129: How to measure and plot the wound density? Is it done by a software? Measurements are made with the software associated with the time-lapse imager. This is now indicated in the manuscript and the software version is listed in the Table of Materials.

Line 133: Please specify where (i.e., from which step) the cells are obtained. What is the culturing temperature? The step where cells are obtained and the culturing conditions are now specified

**Editorial Comment:** Figure 1: Please change “hr”, the time unit, to “h”. Please label the x-axis in panel B.

**Author’s Response:** The time unit “hr” has been changed to “h” in Figure 1 and in the manuscript.

**Editorial Comment:** Figure 3: Please change “hr”, the time unit, to “h”. Please include a space between numbers and their time units (0 h, 24 h, etc.). Please change y-axis label “# of Cells (x10^5)” to “Number of Cells (× 105)”.

**Author’s Response:** The time unit “hr” has been changed to “h” in Figure 3, spaces have been added, and the y-axis has been changed accordingly.

**Editorial Comment:** Discussion: Please discuss any limitations of the technique.

**Author’s Response:** We have now included potential limitations of these techniques in the Discussion, which are found in the second and third paragraph.

Reviewer: 1

We thank Reviewer 1 for their careful and constructive evaluation of our manuscript and suggestions for improving our study.

**Reviewer’s comment:** Previous reports indicate that Swan.71 cells are not bona fide trophoblasts due to a report that these cells are HLA-G negative, HLA-A positive, and do not demonstrate hypomethylation of ELF5. If these cells are indeed HLA-G-negative, the authors should recognize the serious limitations of their study.

**Author’s response:** There are published reports stating the presence, absence, and ability to induce the expression of HLA-G in Swan.71 cells (Straszewski-Chavez S.L., et al. 2009; Apps R., et al. 2009; Barrientos G., et al. 2015). The discrepancies in these reports may reflect the propagation of these cells outside of the laboratory that initially isolated and characterized the Swan.71 cells or cell culture methods. The cells used in these experiments were obtained directly from Dr. Gil Mor, who established and characterized the Swan.71 cells as being HLA-G positive. To further support the general application of the invasion, migration, and proliferation assays for first-trimester trophoblast cells, the authors have now repeated the experiments with HTR-8/SVneo cells, which are immortalized human first-trimester trophoblast cells. We have changed the title to reflect the use of immortalized trophoblast cell lines. We have also removed text defining the Swan.71 cells as extravillous trophoblasts and instead refer to the Swan.71 and HTR-8/SVneo cells more generally as immortalized human first-trimester trophoblast cells.

**Reviewer’s comment:** Why is dexamethasone used to test migration of Swan.71 cells and what is the Vehicle used as a control? An explanation is needed for the biological rationale for this experiment. Do Swan71 cells have steroid receptors?

**Author’s response:** Both the Swan.71 and HTR-8/SVneo cells express the glucocorticoid receptor and are glucocorticoid responsive, as has been previously determined (Straszewski-Chaves, S.L., et al., 2009; Kisanga, E.P., et al., 2018; Ma Y., et al., 2002; Wang B., et al., 2013). The synthetic glucocorticoid dexamethasone was used in these experiments based on our recently published study demonstrating that dexamethasone exposure can alter the function of Swan.71 cells. This is now clarified in the text of the manuscript. We have also indicated that 1X PBS was used as the vehicle control in all experiments.

**Reviewer’s comment:** Figure 2: Normally when cells invade and move through the Transwell assay their morphology is similar to what is seen on a plastic substrate. The images here show cells that are uniformly circular. Either these cells are dead or is it that the pores of the assay are stained? Can the authors repeat and superimpose an image of the membrane? In addition, is it possible to assess the viability of the cells once they have migrated through?

**Author’s response:** Crystal Violet stains proteins and DNA, highlighting the nucleus of the cells. It is likely that the relatively short Crystal Violet stain utilized in this procedure does not allow for adequate visualization of the cell cytoplasm. Nonetheless, we have now included a negative control for the invasion assay with a Matrigel insert that was not seeded with cells.

**Reviewer’s comment:** It is important that the authors are considering proliferation when studying migration with the scratch assay. However, the method here is too simplified. A proliferation marker is needed (Ki67) to be certain. The lower number of cells counted in Figure 3 with Veh could be due to cell death.

**Author’s response:** The authors have now advised readers in the discussion that cell death may influence the results of these assays. Therefore, trophoblast cells should be evaluated for the induction of cell death by experimental treatment *a priori*.

**Reviewer’s comment:** It is essential to discuss the limitations of these assays. For example, the scratch assay is 2D not 3D and does not measure 'invasion'. Are the wounds created with the scratch assay really uniform? The Transwell assay in particular, is difficult to use with primary trophoblast given the large cell numbers required. The use of microfluidics (Li, Kamm Cancer Res 2017) to study invasion is a major step forward but is not mentioned although has even been used for trophoblast (Abbas J. Roy. Soc. Interface 2017).

**Author’s response:** We have now included a statement addressing assay limitations in the discussion.

**Reviewer’s comment:** Line 112: Unclear if treatment is done on the day prior to the assay. It is assumed this means 30 min before the scratch? Please make it clear.

**Author’s response:** We have now clarified that the pretreatment is performed the day of the scratch assay (30 min prior to scratching plates).

**Reviewer’s comment:** Line 124: Is imaging carried out at 37C and 5% CO2 with this imaging system?

**Author’s response:** We have now clarified that the imaging takes place using an automated, time-lapse imager housed in a standard laboratory incubator maintained at 37oC, 5% CO2, and 95% humidity.

**Reviewer’s comment:** Line 178: The magnification 200x or 400x is specific to the microscope used by the authors. Is this a digital zoom? Can the authors provide information on the objective (5, 10, 20 X et.) or provide image dimensions in microns?

**Author’s response:** We have edited the text to identify the objective used (20X) to obtain images and indicated that scale bars on images are 120 m.

**Reviewer’s comment:** Line 247: The authors discuss the potential for these assays to be used to identify therapeutic targets. This is over-stating the importance of their results for the reasons outlined in point 1 with the use of cell lines. Please remove this.

**Author’s response:** This sentence has been removed from the manuscript.

Reviewer: 3

We thank Reviewer 3 for their careful and constructive evaluation of our manuscript and suggestions for improving our study.

**Reviewer’s comment:** The authors performed Scratch Wound Assay to determine cell migration. However, several studies (e.g. Liang et al. Nature protocol 2007, D'Agostino et al. BMC Cell Biology 2015, Di et al. Sci Rep. 2015) recommended to use low concentration of FBS (e.g. 1% FBS) or mitomycin c to minimize cell proliferation. Normal growth media was used in this study and therefore, this result reflects cell proliferation rather than migration.

**Author’s response:** The reviewer has made a key point that the authors feel that we have addressed by combining the scratch wound, proliferation, and invasion assays. We would anticipate that if treatment resulted in a difference in the scratch wound assay but not the proliferation assay, then one could assume that the treatment alters cell movement but not proliferation. However, if the treatment alters all three assays, then one could assume that differences in the scratch wound assay are a result of both changes to cell movement and proliferation. We have also provided readers a “note” in the scratch assay that an alternative lower concentration of FBS can be used to minimize confounding results with altering rates of cell proliferation.