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TITLE:

In Vitro Culture of Epithelial Cells from Different Anatomical Regions of the Human Amniotic Membrane

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KEYWORDS:

amniotic epithelium, heterogeneity, reflected amnion, placental amnion, umbilical amnion, pluripotency

SUMMARY:

This protocol describes the isolation of epithelial cells from different anatomical regions of the human amniotic membrane to determine their heterogeneity and functional properties for possible application in clinical and physiopathological models.

ABSTRACT:

Several protocols have been reported in the literature for the isolation and culture of human amniotic epithelial cells (HAEC). However, these assume that the amniotic epithelium is a homogeneous layer. The human amnion can be divided into three anatomical regions: reflected, placental, and umbilical. Each region has different physiological roles, such as in pathological conditions. Here, we describe a protocol to dissect human amnion tissue in three sections and maintain it in vitro. In culture, cells derived from the reflected amnion displayed a cuboidal morphology, while cells from both placental and umbilical regions were squamous. Nonetheless,

all the cells obtained have an epithelial phenotype, demonstrated by the immunodetection of E-cadherin. Thus, because the placental and reflected regions in situ differ in cellular components and molecular functions, it may be necessary for in vitro studies to consider these differences, because they could have physiological implications for the use of HAEC in biomedical research and the promising application of these cells in regenerative medicine.

INTRODUCTION:

Human amniotic epithelium cells (HAEC) originate during the early stages of embryonic development, at around eight days postfertilization. They arise from a population of squamous epithelial cells of the epiblast that derive from the innermost layer of the amniotic membrane[1]. Thus, HAEC are considered remnants of pluripotent cells from the epiblast that have the potential to differentiate into the three germ layers of the embryo[2]. In the last decade, diverse research groups have developed methods to isolate these cells from the amniotic membrane at the term of gestation to characterize their presumptive pluripotency-related properties in a culture model in vitro[3,4].

Accordingly, it has been found that HAEC feature traits characteristic of human pluripotent stem cells (HPSC), such as the surface antigens SSEA-3, SSEA-4, TRA 1-60, TRA 1-81; the core of pluripotency transcription factors OCT4, SOX2, and NANOG; and the proliferation marker KI67, suggesting that they are self-renewing[5-7]. Moreover, these cells have been challenged using differentiation protocols to obtain cells positive for lineage-specific markers of the three germ layers (ectoderm, mesoderm, and endoderm)[4,5,8], as well as in animal models of human diseases. Finally, HAEC express E-cadherin, which demonstrate that they retain an epithelial nature much like the HPSC[5,9].

Apart from their embryonic origin, HAEC have other intrinsic properties that make them suitable for different clinical applications, such as the secretion of anti-inflammatory and antibacterial molecules[10,11], growth factors and cytokine release[12], no formation of teratomas when they are transplanted into immunodeficient mice in contrast with HPSC[2], and immunological tolerance because they express HLA-G, which decreases the risk of rejection after transplantation[13].

However, previous reports have assumed that the human amnion is a homogenous membrane, without considering that it can be anatomically and physiologically divided into three regions: placental (the amnion that covers the *decidua basalis*), umbilical (the part that envelops the umbilical cord), and reflected (the rest of the membrane not attached to the placenta)[14]. It has been shown that the placental and reflected regions of the amnion display differences in morphology, mitochondrial activity, detection of reactive oxygen species[15], miRNA expression[16], and activation of signaling pathways[17]. These results suggest that the human amnion is integrated by a heterogeneous population with different functionality that should be considered for further studies carried out in either in situ or in vitro models. While other laboratories have designed protocols for the isolation of HAEC from the whole membrane, our laboratory has established a protocol to isolate, culture, and characterize cells from different anatomical regions.

PROTOCOL:

This protocol was approved by the ethical committee of Instituto Nacional de Perinatología in Mexico City (Registry number 212250-21041). All procedures performed in these studies were in accordance with the ethical standards of the Instituto Nacional de Perinatología, the Helsinki Declaration, and the guidelines set forth in the Ministry of Health's Official Mexican Standard.

1. Preparation

1.1. Prepare a solution of 1x PBS with EDTA. To do so, add 500 µL of 0.5 M EDTA stock into 500 mL of 1x PBS for a final concentration of 0.5 mM EDTA.

1.2. Prepare the culture medium for HAEC. Take 450 mL of high glucose-DMEM, supplement it with 5 mL of sodium pyruvate (100 mM), 50 mL of heat-inactive fetal bovine serum qualified for stem cells, 5 mL of nonessential amino acids (100x), 5 mL of antibiotic-antimycotic (100x), 5 mL of L-glutamine (200 mM), and 500 µL of mercaptoethanol (1,000x).

1.3. Sterilize the containers for transporting and processing the tissue: a stainless steel container with a lid to transport the whole placenta from the operating theater to the laboratory, a tray (20 cm x 30 cm x 8 cm) to wash and remove blood from the whole placenta before the dissection of the amniotic membrane, and a plastic cutting board to separate the amniotic membrane into the three regions.

1.4. Sterilize the surgical instruments (scalpels, scissors, forceps, and clamps), 500 mL beakers, cotton gauzes, and saline solution.

2. **Obtaining placental tissue**

NOTE: The amniotic membranes were obtained from women at full-term gestation (37−40 weeks), under indication of Cesarean delivery, without any evidence of active labor, and no microbiological characteristics of infection. The complete isolation and culture procedures were carried out within a biosecurity cabinet under sterile conditions.

2.1. In the operating room, clamp the umbilical cord to prevent the blood flow to the rest of the tissue. Collect the entire placenta with the umbilical cord clamped in the sterile container.

2.3. Add 500 mL of saline solution to the container to hydrate the placenta.

2.4. Close the container and transport the tissue to the laboratory at room temperature.

2.5. Put the container with the placenta inside the biosecurity cabinet.

NOTE: In case the dissection is not carried out within 15 min of collecting the placenta, store the container with the placenta on ice until processing. Avoid more than 1 h of elapsed time between obtaining the placenta and the start of the dissection.

3. Mechanical separation per region of the amniotic membrane

NOTE: The procedure must be carried out within a biosecurity cabinet under sterile conditions and at room temperature.

3.1. Remove the whole placenta from the container and place it on the tray with the umbilical cord facing upward.

3.2. Using a sterile cotton gauze, clean the blood clots from the surface of the chorion-amnion that covers the placenta.

3.3. Identify the three regions of the membrane: the umbilical amnion enveloping the umbilical cord, the placental amnion covering the decidua basalis, and the reflected region, which is the rest of amnion that is not attached to the placenta (**Figure 1**).

3.4. Dissect the umbilical amnion region (**Figure 2A**).

3.4.1. Using dissecting forceps, hold the portion of amnion membrane that covers the junction of the placenta and umbilical cord.

3.4.2. With a scalpel, dissect the region that surrounds the cord while stretching to separate it from the chorion.

3.4.3. Deposit the separated tissue in a labeled beaker with 100 mL of saline solution.

3.5. Dissect the placental amnion region (**Figure 2B**).

3.5.1. With a sterile cotton gauze, remove the blood clots from the surface of the chorion-amnion that covers the placenta.

3.5.2. Hold the membrane on the border between the placenta and the reflected region with dissecting forceps.

3.5.3. Cut along the circumference of the placenta with the scalpel.

3.5.4. Separate the placental amnion from the chorion, being careful not to cut any vessels from the placenta.

3.5.5. Place the separated tissue in another labeled beaker with 300 mL of saline solution.

3.6. Separate the rest of the amniotic membrane that is not attached to the placenta (i.e., the reflected portion) from the chorion (**Figure 2C**).

3.6.1. Collect the reflected region in another labeled beaker with 300 mL of saline solution.

NOTE: Add saline solution continually during the dissection to prevent tissue from drying out.

4. Washing the membranes

NOTE: The procedure must be carried out within a biosecurity cabinet under sterile conditions at room temperature.

4.1. Discard the saline solution of each membrane region separately.

4.2. Add 100 mL of fresh saline solution to the umbilical region.

4.3. Add 300 mL of fresh saline solution to the placental and reflected regions respectively.

4.4. Shake the membranes with the help of dissecting forceps to remove blood residue.

4.5. Discard the saline solution.

4.6. Repeat the washes and agitation at least 3x until the membranes are translucent.

4.7. Place and extend the membranes on the board to clean with sterile gauze the blood clots that were not removed with the washes.

NOTE: It is very important to remove as many erythrocytes as possible, as their presence affects the trypsin function and the viability of subsequent cell cultures.

5. Enzymatic digestion of the membranes from different regions

NOTE: The procedure must be carried out within a biosecurity cabinet under sterile conditions.

5.1. Cut the reflected and placental regions into two or three fragments.

5.2. Do not cut the umbilical region.

5.3. Place the fragments of each region in centrifuge tubes. Add 20 mL of 0.5% trypsin/EDTA to the reflected and placental regions and 5 mL of 0.5% trypsin/EDTA to the umbilical region, respectively.

NOTE: It is important to cut the reflected and placental regions into smaller pieces because they need to be completely immersed in the trypsin solution.

5.4. Shake the centrifuge tubes lightly for 30 s. Discard the trypsin.

5.6. Add 30 mL of new 0.5% trypsin/EDTA to the reflected and placental regions and 15 mL of new 0.5% trypsin/EDTA to the umbilical region, respectively.

5.7. Place the tubes in a rotator inside the incubator.

5.8. Incubate with rotation (4 rpm) for 40 min at 37 °C.

NOTE: If a tube rotator is not available, shake the tubes lightly manually every 10 min.

5.9. Transfer the trypsin/cell solutions from each region into new centrifuge tubes.

5.10. Add 2x the volume of HAEC media prewarmed at 37 °C per tube to inactivate the enzyme.

5.11. Store the first digestion on ice.

5.12. Repeat steps 5.6–5.8 for a second digestion period.

5.13. For each region, hold one end of the amnion portion using dissecting forceps, and with another pair squeeze along the tissue to remove rows of epithelial cells that did not completely peel off during previous incubation periods.

5.14. Collect the second digestion into another set of centrifuge tubes and inactivate with 2x the volume of HAEC media.

5.15. Discard the digested membranes into a biohazard container.

6. Isolation of the HAEC

NOTE: The procedure must be carried out within a biosecurity cabinet under sterile conditions.

6.1. Centrifuge all tubes at 200 x *g* for 10 min at 4 °C.

6.2. Discard the supernatant and add 10 mL of prewarmed HAEC media (to 37 °C) per tube and pipet gently to disaggregate each pellet.

6.3. Combine the cellular suspensions of the two digestions in an individual tube for each membrane region.

6.4. Filter the cellular suspensions using 100 µm cell strainers to remove the extracellular matrix debris and obtain single cells.

6.5. Prepare three aliquots with 90 µL of trypan blue in a microcentrifuge tube.

6.6. Add 10 µL of each cell suspension per membrane region into the microcentrifuge tubes and

mix.

6.7. Count the cells with a hemocytometer under a light field microscope.

7. Culture of HAEC

7.1. Seed the HAEC from the three regions separately at a density of 3×10^4 cells/cm^2 with prewarmed HAEC media, supplemented with 10 ng/mL of human epidermal growth factor (EGF).

7.1.1. Seed the cells in 100 mm plates to maintain them in vitro or in 24 well plates for immunochemical analysis.

7.2. Incubate the dishes at 37 °C under normoxic conditions (5% CO_2) in a humidified incubator.

7.3. Add EGF daily and change the medium every third day.

NOTE: The cells will become confluent after 4−6 days.

7.4. Use the cells for conventional immunohistochemistry, cell sorting analysis, cryopreserving, RNA and protein extraction, or to continue the passage.

8. Passage of HAEC

8.1. Remove the HAEC medium and wash 2x with PBS/EDTA 0.01M solution.

8.2. Incubate with a PBS/EDTA 0.01M solution for 15 min at 37 °C.

8.3. Remove the PBS/EDTA and add 1.5 mL of 0.5% trypsin/EDTA.

8.4. Incubate for 5−8 min at 37 °C.

8.5. Inactivate the enzyme with two volumes of HAEC media.

8.6. Collect the mixed solution and centrifuge for 5 min at 200 x *g*.

8.7. Count the cells and continue with following passages or use the cells for further analysis.

REPRESENTATIVE RESULTS:

HAEC were isolated from each of the three anatomical regions of the amniotic membrane and individually cultured in vitro. After 48 h of culture, cells with an epithelial phenotype adhered to the surface of the plate, although the media also contained cell debris and floating cells, which were removed once the medium was changed (**Figure 3**).

During the processing of primary culture (passage zero, P0), some complications could arise that

309 can interfere with the experimental data analysis (**Figure 4**): it is advisable to discard the cultures
310 and process another membrane upon identifying the presence of bacteria due to contamination
311 of the reagents or during the isolation process (**Figure 4A**); excessive erythrocytes due to
312 insufficient washing of the membranes (**Figure 4B**); deficient or no adhesion of the cells to the
313 plates (**Figure 4C**); or cells with fibroblast morphology (**Figure 4D**), suggesting that human
314 amniotic mesenchymal cells (HAMC) were isolated instead of HAEC.
315
316 HAEC morphology depends on the origin of these cells: cells from the reflected zone have a
317 cuboidal morphology and grow in a cobbled monolayer, unlike cells from the placental and
318 umbilical regions, which are flatter and squamous (**Figure 5**). These data support that the
319 epithelial layer from the amnion is not uniform throughout the membrane. During the passages,
320 the size of the cells from all regions increases, but they maintain their epithelial nature and do
321 not acquire a fibroblast morphology. Indeed, immunofluorescence against E-cadherin showed
322 that the primary cultures (P0) and subcultures (P1-P2) maintained their epithelial phenotype
323 (**Figure 6**), suggesting that there is no contamination of another cell type, such as mesenchymal
324 stromal cells, or epithelial-mesenchymal transition. In addition, these cells show no evidence of
325 cell death according to the TUNEL assay (**Supplementary Figure 1**) but are positive for the KI-67
326 proliferation marker (**Figure 6**), although our previous results found no significant differences for
327 this marker between each passage[5].
328
329 The number of cells obtained varies according to the region: 61.6 x 10^6 and 71.8 x 10^6 cells from
330 the reflected and placental regions, respectively, and less than 1 x 10^6 per sample from the
331 umbilical region (**Table 1**). It has been reported with this protocol that placental and umbilical
332 region are very similar in their expression profiles, as opposed to the reflected and placental
333 regions, especially in genes that participate in ECM receptor interaction, focal adhesion, and the
334 PI3K-Akt signaling pathway through RNA-seq[6]. In agreement, a previous study reported the
335 differential expression of mitogen-activated protein kinase and transforming growth factor beta
336 pathways, as well as proinflammatory cytokines between both regions[17].
337
338 Although the evidence showed that the subpopulations from the amnion differ in their
339 morphology and physiological properties, we previously demonstrated that the expression and
340 presence of the core of the pluripotency factors does not change in HAEC derived from placental
341 and reflected regions[6]. In this context, in addition to the relatively limited number of cells from
342 the umbilical region, subsequent studies should focus on isolated HAEC from the placental and
343 reflected amnion independently, considering the physiological implications, because the
344 different subpopulations would not respond equally to specific events, such as prolonged
345 pregnancy or inflammatory processes during labor, although there are no specific positive cells
346 to the main pluripotency markers (**Figure 7**).
347
348 **FIGURE AND TABLE LEGENDS:**
349 **Figure 1: Anatomical regions from the amniotic membrane at term.** Umbilical amniotic
350 membrane (yellow), placental amniotic membrane (white), and reflected amniotic membrane
351 (black).
352

Figure 2: Mechanical dissection of the amniotic membrane. (**A**) Umbilical region, (**B**) placental region, and (**C**) reflected region.

Figure 3: Representative primary culture of HAEC derived from the amniotic membrane. Culture 48 h after isolation without (**A**) and with (**B**) a change of media. Scale bars = 50 µm.

Figure 4: Representative micrographs of negative results of primary culture of HAEC. (**A**) Excess of erythrocytes. (**B**) Bacterial contamination. (**C**) HAEC not adhered after 48 h of isolation. (**D**) Primary culture composed of cells with fibroblast morphology. Scale bars = 200 µm.

Figure 5: Morphology of HAEC from different anatomical regions in vitro. Representative micrographs of confluent HAEC from reflected (upper panel), placental (middle panel), and umbilical (lower panel) regions cultured though P0−P2. Scale bars 200 = µm.

Figure 6: Expression of E-cadherin and KI-67 in HAEC. Representative epifluorescence microscopy images of E-cadherin^{+} and KI-67^{+} HAEC from reflected (left panel) and placental (right panel) amnion cultured through P0−P2. Scale bars = 100 µm.

Figure 7: HAEC from different anatomical regions display a pluripotency marker panel. Representative confocal microscopy images of double immunofluorescence amnion for NANOG with TRA-1-60, OCT4 with E-cadherin, and SOX2 with SSEA-4 in HAEC (P1) from reflected (upper panel) and placental (lower panel) regions. Scale bars = 100 µm.

Table 1: Number of HAEC isolated per region from amniotic membranes. (NP = not processed).

Supplementary Figure 1: TUNEL staining in HAEC (passage 2) from reflected region and in positive control cells (HL-60 cells treated with camptothecin). Scale bars = 50 µm.

DISCUSSION:

We implemented a new protocol to isolate HAEC from term membranes. It differs from previous reports in that each membrane was divided into its three anatomical regions prior to isolation to analyze cells from each one.

One of the most critical steps in the protocol is the washing of the membrane to remove all blood clots, because they can interfere with the activity of trypsin when separating the epithelial cells. Failure to carry out this step properly can lead to obtaining a primary culture with excessive erythrocytes and few adherent epithelial cells. If no attachment is observed in the first 24−48 h after seeding the cells, we recommend discarding the culture. Another critical point is the incubation time for the enzymatic digestion. If the incubation period is too long, cell viability may decrease and/or cell cultures with mesenchymal instead of epithelial morphology may be obtained (**Figure 4**). Also, if membrane digestion is not done with the proper agitation, the probability of obtaining a low number of cells per membrane increases.

In this protocol, we can maintain populations of HAEC in vitro without losing their epithelial

phenotype, as demonstrated by the presence of specific epithelial cell marker E-cadherin for most cells along the passages (**Figure 6**). However, in our experience the HAEC can be only be expanded for three passages (P1−P3). The limited number of passages should be taken into consideration for experiments that require long periods of culture or multiple passages. In addition, it has been reported that after P3 the cells begin to change their morphology and reduce the expression of E-cadherin, so the prolonged culture could induce epithelial-mesenchymal transition (EMT)[18]. Recently, it was demonstrated that progesterone prevents EMT in ovine amniotic epithelial cells[19,20], so it would be interesting to analyze the effect of different concentrations of progesterone in the HAEC culture medium to avoid the mesenchymal phenotype after the third passage.

In all previous reports where HAEC were isolated, the amnion has been assumed to be a uniform tissue despite the possible heterogeneity of epithelial populations that comprise the whole membrane. In contrast, this protocol divides the membrane into its three anatomical areas to separately isolate and culture HAEC considering the previous morphological and gene expression reports that suggest functional differences. It is also unnecessary to purify through cell sorting to obtain only epithelial populations, as demonstrated by detection of the E-cadherin marker during passages until P2.

It has been shown that isolated HAEC from each of the membrane regions have a similar expression of pluripotency core, being a latent reservoir of cells with stemness. In this context, these cells can be used in vitro to study molecular mechanisms, such as the dynamic localization of pluripotency-related transcription factors or their ability to remain quiescent despite expressing cancer-associated genes, regardless of their anatomical region of origin. However, future research must consider molecular differences reported (global expression genes, metabolic activity, signaling pathways) between each region, which would have repercussions for their application in regenerative medicine. We previously reported a different expression of genes involved in PI3K/AKT and focal adhesion signaling pathways between the different amniotic regions by RNA-seq[6]. PI3K/AKT regulates self-renewal and maintains pluripotency in HPSC, while activation of focal adhesion kinase promotes their differentiation[21,22]. Thus, we propose to characterize the role of both pathways in HAEC isolated from different anatomical regions during lineage-specific differentiation protocols. In addition, several studies demonstrate the differences between both regions regarding cellular components, molecular function, and biological processes. For example, the region-specific gene expression and protein distribution of aquaporins, which are involved in the transport process of intramembranous absorption, have been reported[23]. Another study compared the miRNome by microarrays, showing region-specific expression of miR-145 and miR-143, the latter being able to posttranscriptionally regulate the prostaglandin-endoperoxidase synthase 2 under specific conditions such as labor at term[16]. Moreover, the placental region presents higher mitochondrial respiration but lower reactive oxygen species detection as compared with reflected tissue[15]. Dissecting the amnion in reflected and placental regions is encouraged to isolate HAEC and analyze their peculiar properties separately, such as the release of growth factors and cytokines, their low immunogenicity, production of extracellular matrix, the effect of their conditioned medium in coculture with other cell types, and novel functions such as their capacity to maintain HPSC lines as a feeder layer[24].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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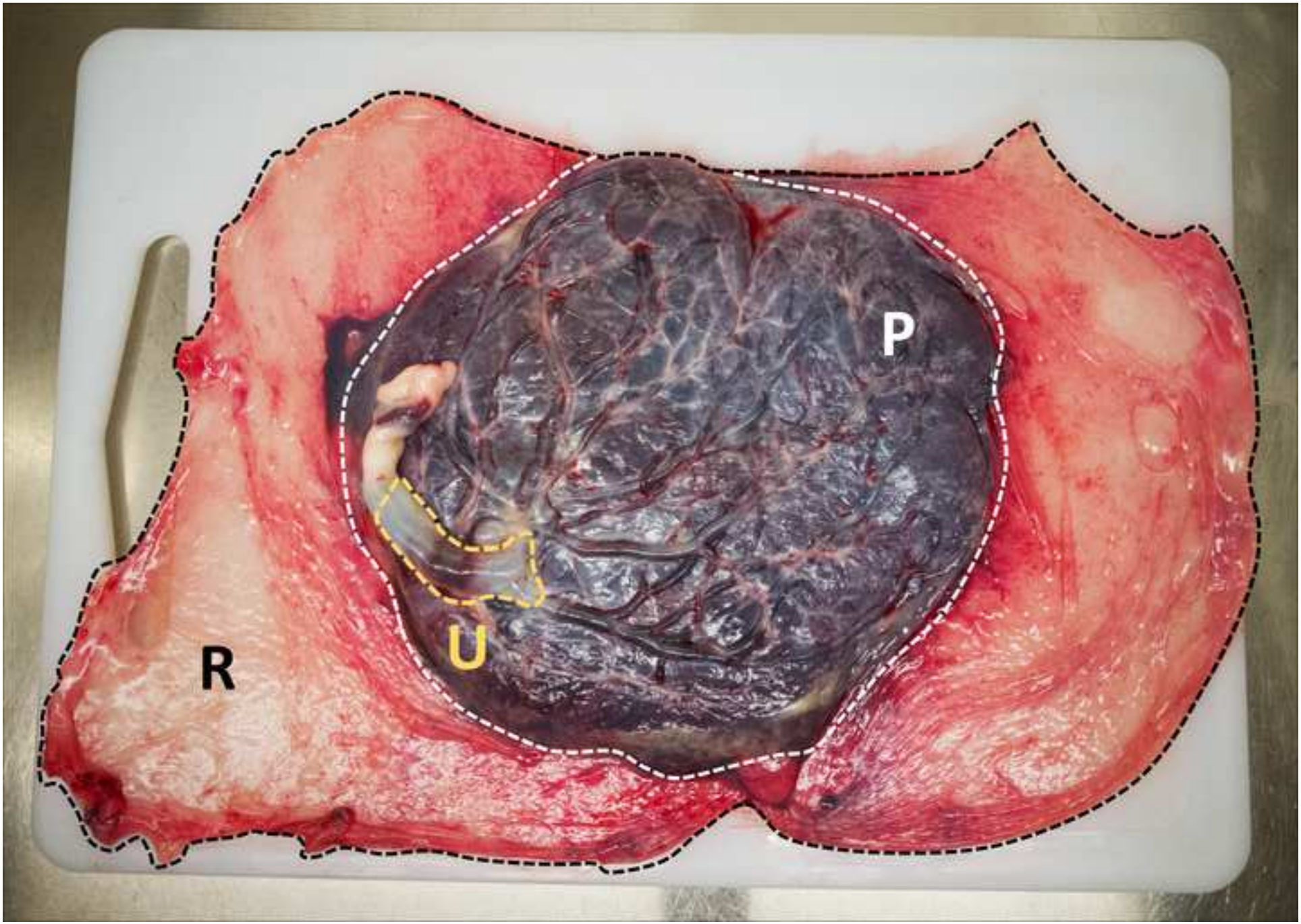

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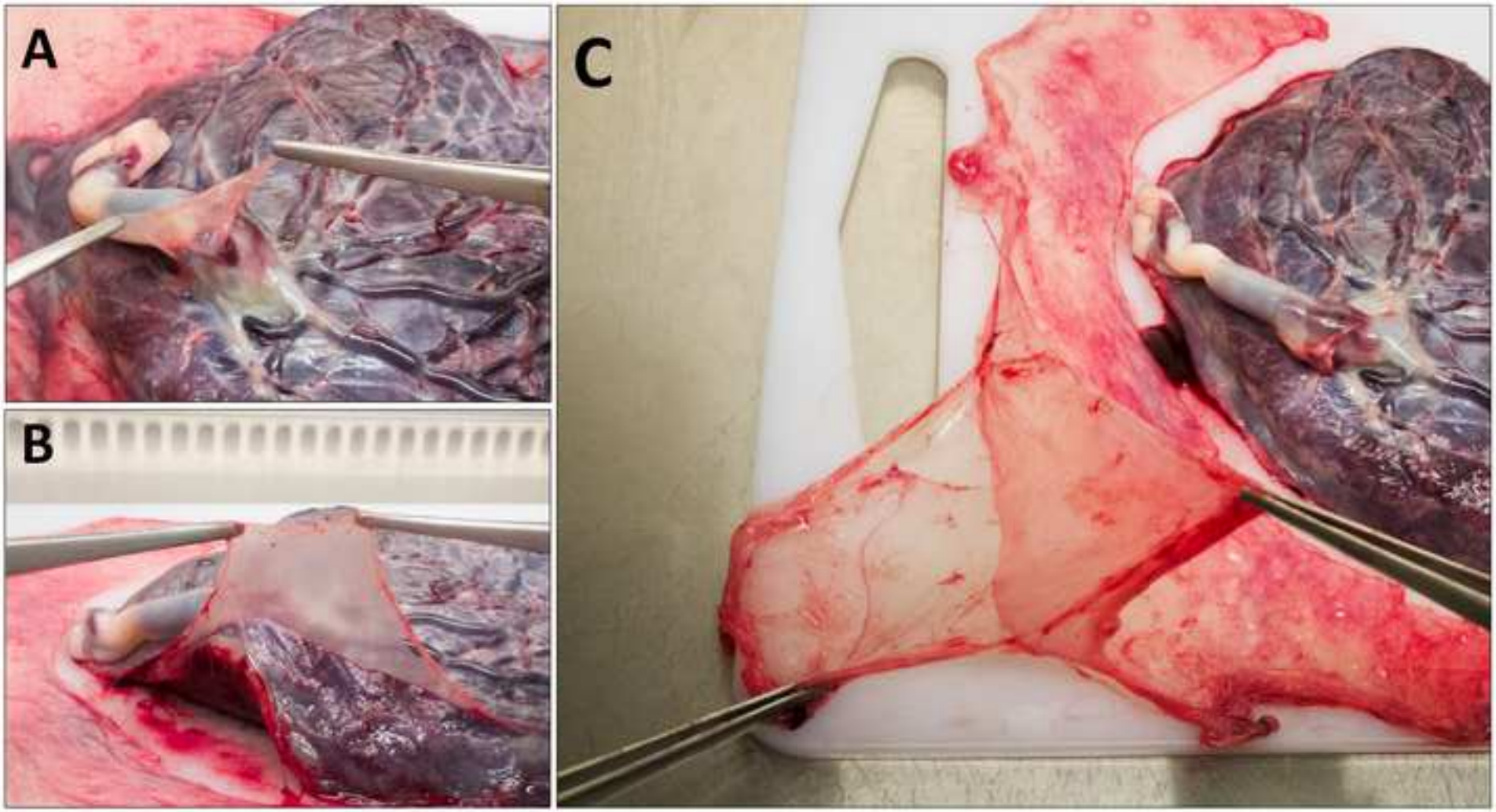

Figure 3

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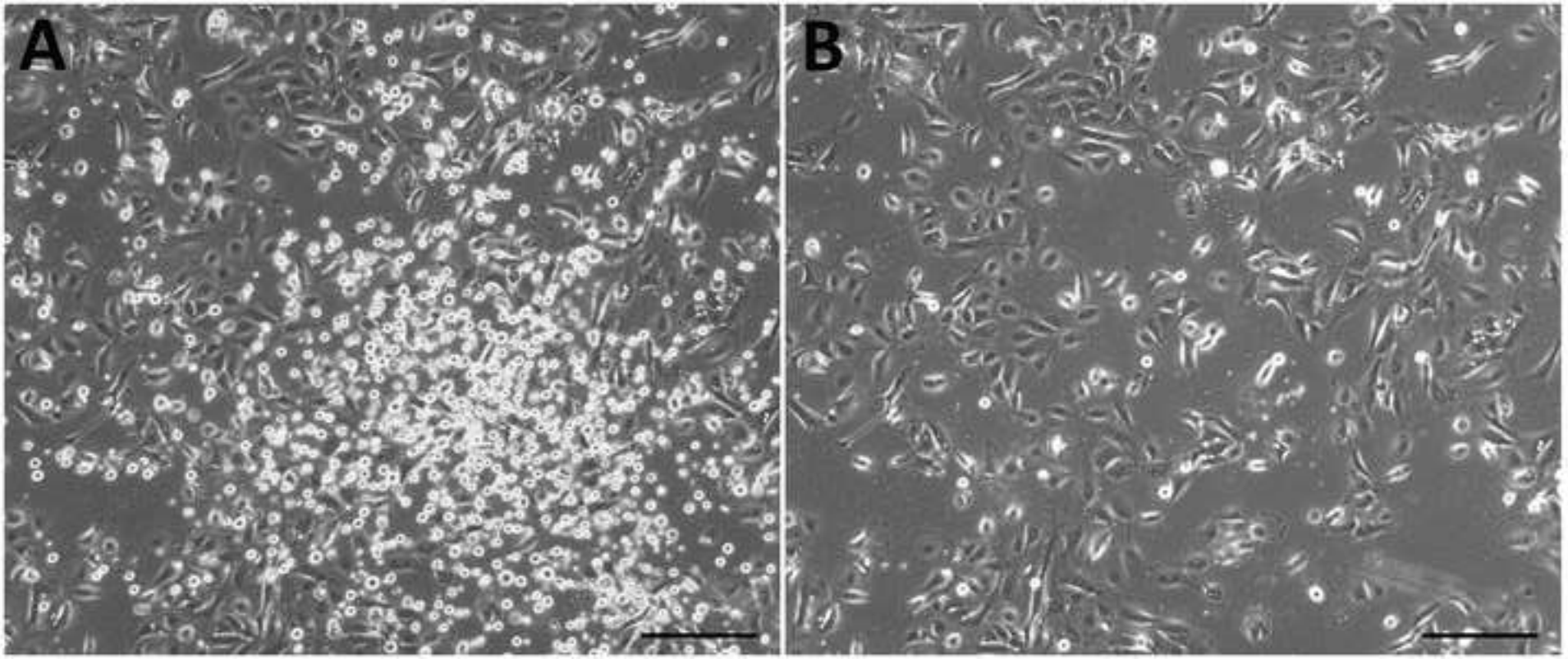


Figure 4

Click here to access/download;Figure;Figure 4 new.psd

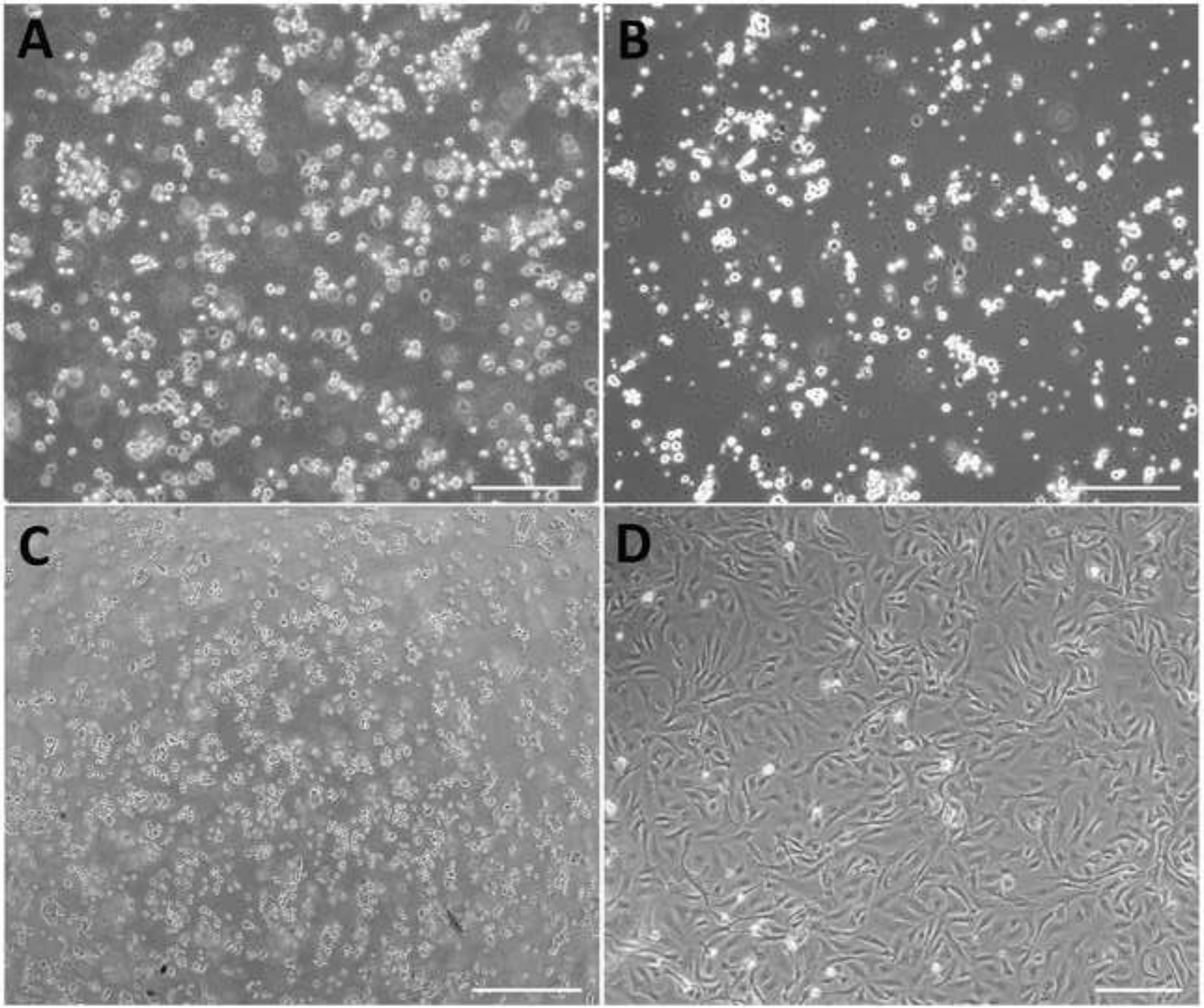


Figure 5

Click here to access/download;Figure;Figure 5.psd

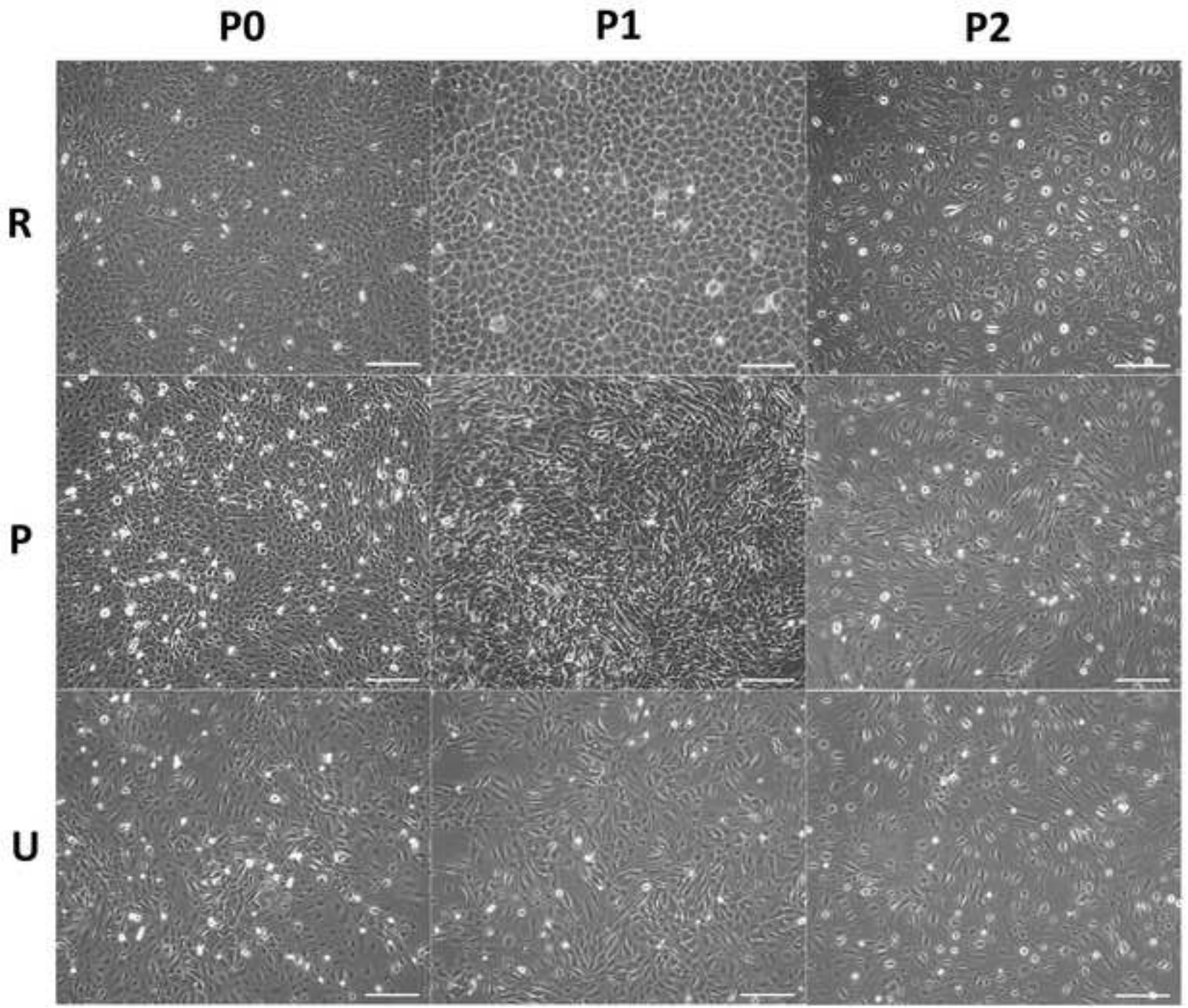


Figure 6

Click here to access/download;Figure;Figure 6.psd

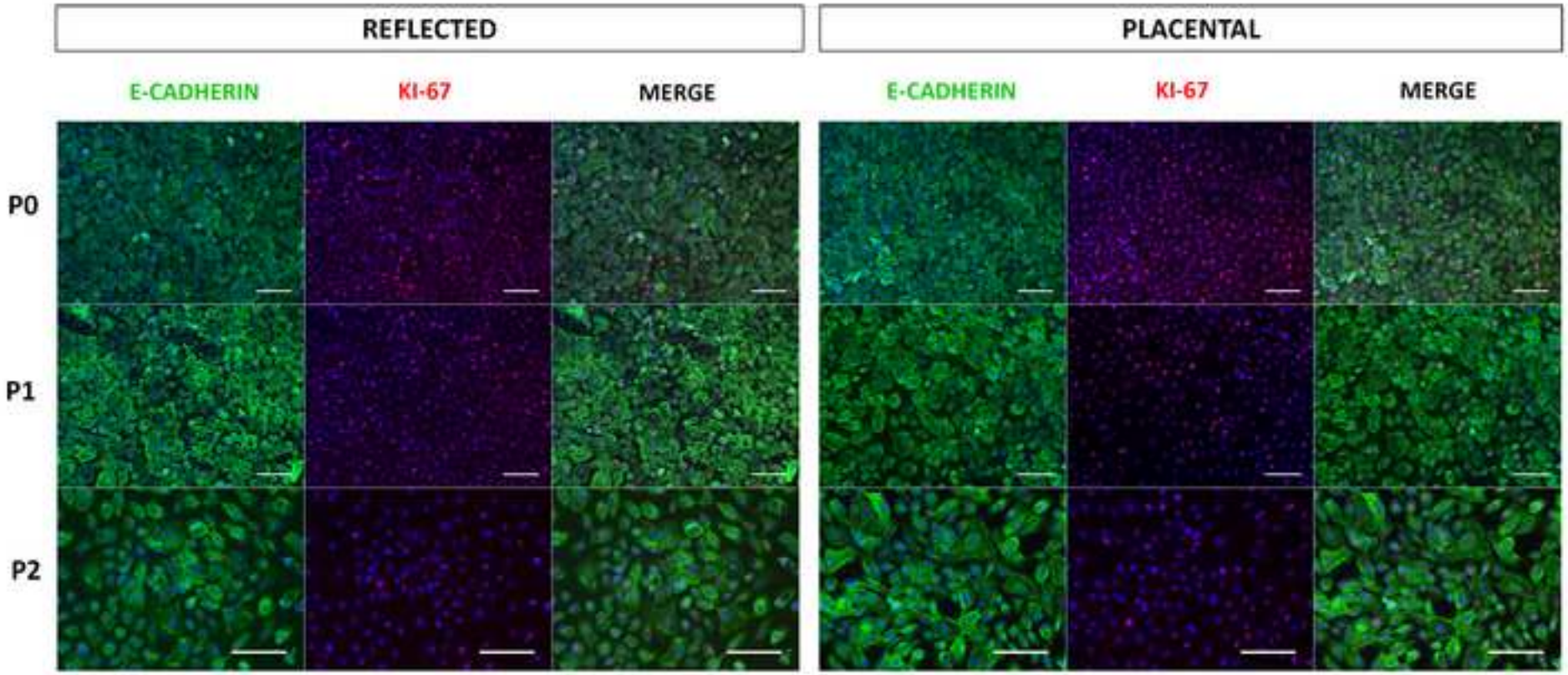
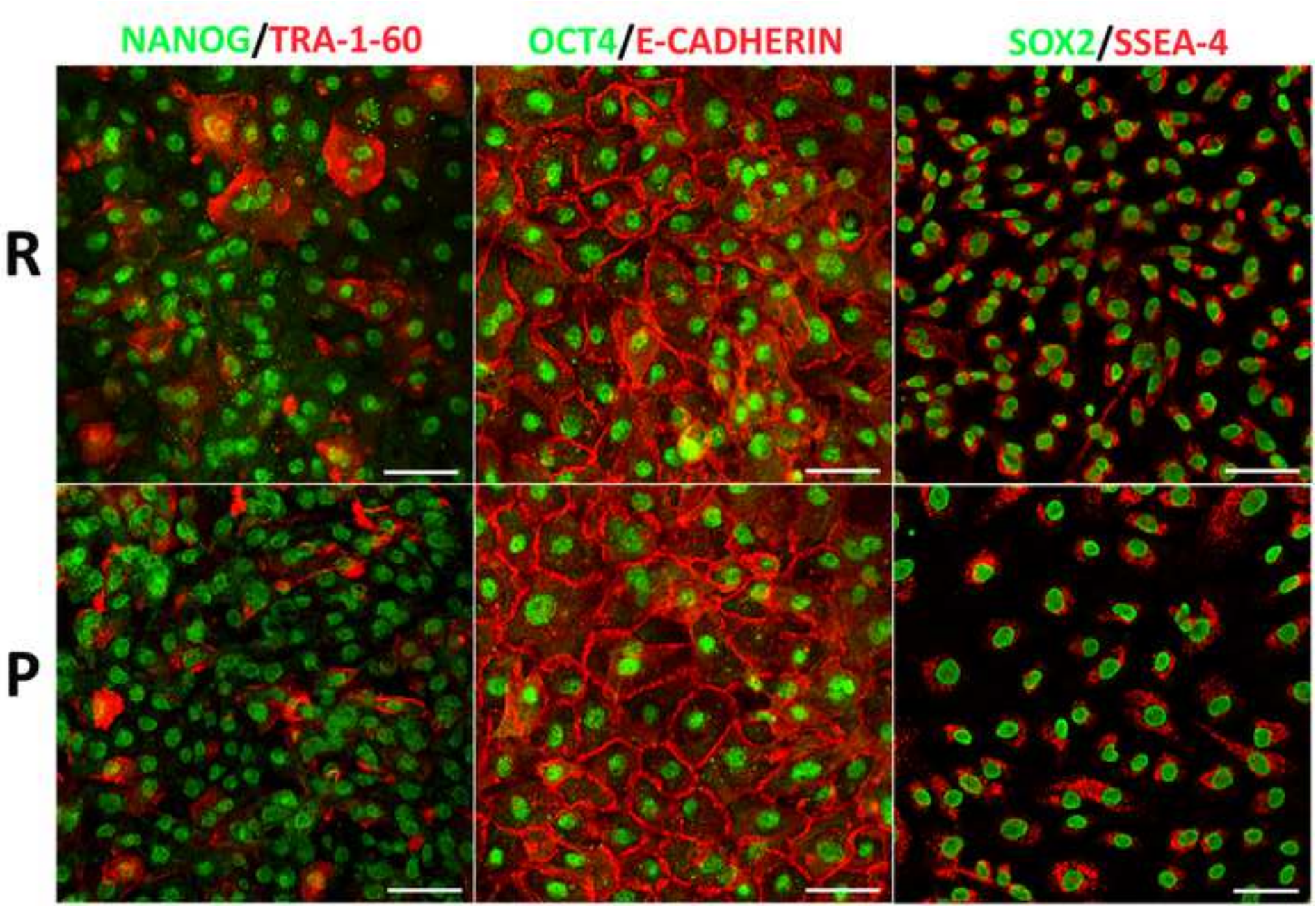


Figure 7



Supp Fig 1

Click here to access/download;Figure;Suppl Figure 1.psd

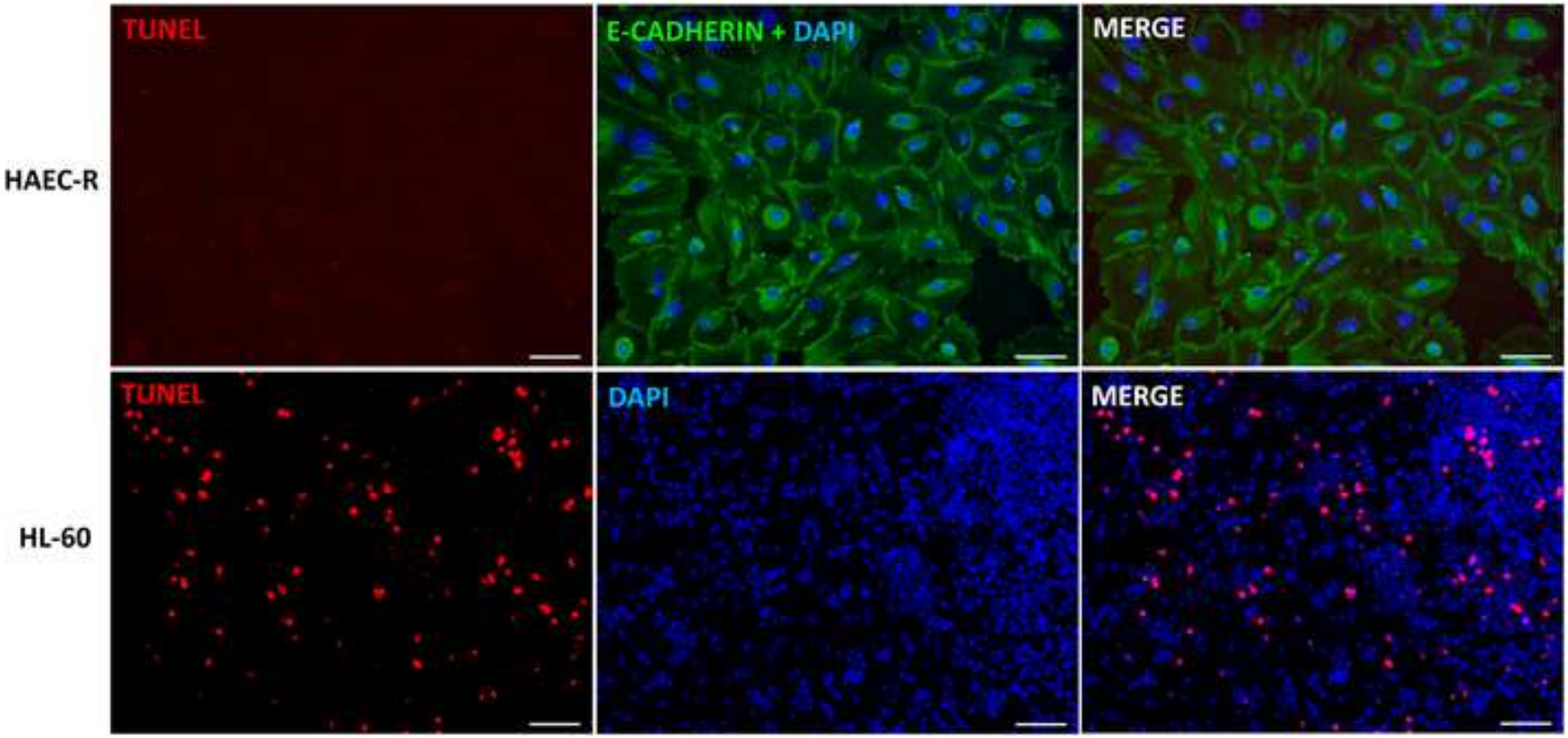


Table 1

# MEMBRANE	REFLECTED	PLACENTAL	UMBILICAL	COMPLETE MEMBRANE
1	75×10^6	130×10^6	0.2×10^6	205.2×10^6
2	38.5×10^6	52×10^6	0.53×10^6	91.03×10^6
3	59×10^6	53×10^6	0.2×10^6	112.2×10^6
4	42×10^6	27×10^6	0.36×10^6	69.36×10^6
5	44.8×10^6	22.3×10^6	NP	76.1×10^6
6	100×10^6	140×10^6	1×10^6	241×10^6
7	72×10^6	78×10^6	NP	150×10^6
AVERAGE	61.6×10^6	71.8×10^6	0.45×10^6	133.8×10^6

Name of Material/ Equipment	Company	Catalog Number
Culture reagents		
2-Mercaptoethanol	Thermo Fisher Scientific/Gibco	21985023
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15
Antibiotic-Antimycotic	Thermo Fisher Scientific/Gibco	15240062
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific/Gibco	12430054
EDTA	Thermo Fisher Scientific/Ambion	AM9260G
Embryonic stem-cell FBS, qualified	Thermo Fisher Scientific/Gibco	10439024
Non-Essential Amino Acids	Thermo Fisher Scientific/Gibco	11140050
Paraformaldehyde	any brand	
Phosphate-Buffered Saline	Thermo Fisher Scientific/Gibco	10010023
Saline solution (sodium chloride 0.9%)	any brand	
Sodium Pyruvate	Thermo Fisher Scientific/Gibco	11360070
Trypsin/EDTA 0.05%	Thermo Fisher Scientific/Gibco	25300054
Disposable material		
100 µm Cell Strainer	Corning/Falcon	352360
100 mm TC-Treated Culture Dish	Corning	430167
24-well Clear TC-treated Multiple Well Plates	Corning/Costar	3526
6-well Clear TC-treated Multiple Well Plates	Corning/Costar	3516
Non-Pyrogenic Sterile Centrifuge Tube	any brand	
Non-Pyrogenic sterile tips of 1,000 µl, 200 µl and 10 µl.		
Sterile cotton gauzes		
Sterile serological pipettes of 5, 10 and 25 mL	any brand	
Sterile surgical gloves	any brand	
Equipment		
Biological safety cabinet		
Centrifuge		
Micropipettes		
Motorized Pipet Filler/Dispenser		
Sterile beakers of 500 mL		
Sterile plastic cutting board		
Sterile scalpels, scissors, forceps, clamps		

Sterile stainless steel container		
Sterile tray		
Tube Rotator	MaCSmix	
Antibodies and Kits		
Anti-E-cadherin	BD Biosciences	610181
Anti-KI67	Santa Cruz	23900
Anti-NANOG	Peprotech	500-P236
Anti-OCT4	Abcam	ab19857
Anti-SOX2	Millipore	AB5603
Anti-SSEA-4	Cell Signaling	4755
Anti-TRA-1-60	Cell Signaling	4746
Goat Anti-Mouse Alexa Fluor 488	Thermo Fisher Scientific	A-11029
Goat Anti-Rabbit Alexa Fluor 568	Thermo Fisher Scientific	A-11036
Tunel Assay Kit	Abcam	66110

	Comments/Description
55 mM	
100X	
Supplemented with high glucose and HEPES	
0.5 M	
100X	
1X	
100 mM	
with conical bottom	

Antibody ID

RRID:AB_3975

RRID:AB_627859)

RRID:AB_1268274

RRID:AB_44517

RRID:AB_2286686

RRID:AB_1264259

RRID:AB_2119059

RRID:AB_2534088

RRID:AB_10563566

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Title of Article: In vitro culture of epithelial cells from different anatomical regions of the human amniotic membrane

Author(s): D. Avila-González, G. García-López, N.E. Díaz-Martínez, H. Flores-Herrera, A. Molina-Hernández, W. Portillo, N.F. Díaz

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CORRESPONDING AUTHOR

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Institution:	Instituto Nacional de Perinatología
Title:	M.D., Ph. D.
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August 28, 2019

Dr. Vineeta Bajaj,
Review Editor
JoVE

Dear Dr. Bajaj enclosed you will find the revised version of our manuscript “In vitro culture of epithelial cells from different anatomical regions of the human amniotic membrane” JoVE60551. The major changes in this new manuscript were highlighted in color turquoise.

We thank the reviewers for their constructive comments that helped us to improve our manuscript.

We hope that the new version is suitable for publication in JoVE.

Sincerely,

Daniela Avila, Ph.D.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: As suggested by the editorial reviewer, this new version of the manuscript was revised by a scientific English native speaker.

2. Please provide an email address for each author.

Response: In this new version of the manuscript, we add the email address for each author.

3. Please reword ethics statement to show that the experiment in your lab was performed with all approval from your institution.

Response: As suggested by editorial reviewer, in this new version of the manuscript ethics statement was reword. The sentence was rewritten: This protocol was approved by the ethical committee of Instituto Nacional de Perinatología in Mexico City (Registry number 212250-21041). All procedures performed in these studies were in accordance with the ethical standards of the Instituto Nacional de Perinatología, Helsinki declaration and the guidelines set forth in the Ministry of Health's Official Mexican Standard (page 2, lines 90-93).

4. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Response: As suggested by the editorial reviewer, we made the changes requested on the format of our manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: As suggested, we made the changes requested in the text of our manuscript.

6. The Protocol should contain only action items that direct the reader to do something.

Response: As suggested, we made the changes requested in the text of our manuscript.

7. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: In this new version of our manuscript, we following the recommendation.

8. 1.2: How is this done?

Response: As suggested, we clarify in the new version of the manuscript the instructions to prepare media HAEC (page 2, lines 102-105).

9. 2.1: Do you place it on ice, do you have any solution where the placenta is placed?

Response: As suggested, we clarify in the new version of the manuscript the collection and transport of the placenta (page 2, lines 116-126).

10. 2.3. 2.4: How do you visually identify the different layers of the placenta. Please include this as a note. How much tissue is collected?

Response: As suggested, we add a note in this section how to identify each region of the tissue (page 3, lines 135-138).

11. 3: Do you perform all these steps at room temperature? Do you perform this in sterile condition?

Response: In this new version of our manuscript, we add a note (page 3, lines 164-165) in this section to include these specifications.

12. 5,6,7: For each step please explain how is this done.

Response: In this new version of our manuscript, we following the recommendation.

13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

Response: In this new version of our manuscript, we following the recommendation.

14. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

Response: In this new version of our manuscript, we following the recommendation.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that

should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next.

Response: In this new version of our manuscript, we following the recommendation.

16. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

Response: figure 1 shown the anatomic regions for the dissection of the amnion described in the protocol; the rest of the figures were discussed in the Representative Results.

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Response: We do not include any previously published figures.

18. Please do not abbreviate the journal titles in the references section.

Response: As editorial reviewer suggested, we changed the journal title in the references section.

Reviewer 1

Major Concerns:

- Authors claim that the potential differences they identified among the 3 cell types could have implications on the cells' physiological properties. Have the authors done any functional assays to determine these potential differences? It would be beneficial to discuss what assay could be performed to determine any potential functional differences.

Response: Previously, we demonstrated differential genes expression between the different amniotic regions included components of focal adhesion and PI3K-Akt signaling pathways (García-López et al., Exp Cell Res, 2019). Interestingly, it has demonstrated that these pathways are involved in the differentiation process during early development as

well as pluripotent stem cells. We add in the discussion of our manuscript, the suggestion of use lineage-specific differentiation protocols to determine the possible role that these pathways could have in the functional differences between the different amniotic regions (page 8, lines 386-391).

Minor Concerns:

- Line 51: "at around of 8 days", "of" should be removed.
- Line 62: "… to obtain cells positive to" should be " to obtain cells positive for".

Response: as the reviewer suggested, we did the change in the phrases (page 1, line 54 and 65.

Reviewer 2

1) Amniotic membrane is the innermost layers of placenta in direct contact with amniotic fluid. Even after the mechanical separation of Amnion from Chorion, Amniotic epithelial cell lay on a basal membrane and just below there is the mesenchymal layer containing a rich extracellular matrix with disperse amniotic mesenchymal cells. In several protocols, there is a step in which the mesenchymal layer is mechanically peeled off from amniotic epithelial cell layer with forceps, working under stereomicroscope. How can the authors be sure that the trypsin digestion is sufficient to get rid of mesenchymal cells? How much is the percentage of contaminant mesenchymal cells, if any?

Response: Trypsin cleaves peptide bonds; therefore, it used for dissociation of the cell-cell adhesions in epithelial tissues. As the reviewer mentioned, amniotic mesenchymal cells (AMC) are surrounded by extracellular matrix (ECM). In the literature, it reported that trypsin is not enough to break the cell-ECM attachments. Thus, it is necessary treatment with one or more types of collagenase (I-IV) and DNAse I to get the separation of mesenchymal cells from ECM. Recently, in a protocol where only used trypsin to isolate AEC, they reported that the percentage of contamination with AMC is less than 1% (Motedayyen et al., BMC Res Notes 2017). These data corroborated with our immunofluorescence results, where almost all isolate cells from amnion are positive for the epithelial marker E-cadherin (Figure 6).

2) Amniotic epithelial cell literature is full of evidences that demonstrate that these cells undergo epithelial-mesenchymal transition (EMT) after few (generally three) cultural passages. This process completely change the phenotype and also the biological properties of these cells, therefore representing an issue for their use in regenerative medicine. The authors stated that the amniotic epithelial cells use should be limited at passage three to avoid the occurrence of EMT. However, it is demonstrated that EMT process starts from the first passage. In this regard, recently it was demonstrated that progesterone inhibits EMT in amniotic epithelial cells, thus preventing the spontaneous lost of the epithelial markers (Canciello et. al 2017 - https://doi.org/10.1038/s41598-017-03908-1; Canciello et. al 2018 - DOI: 10.1007/978-1-4939-8600-2_7). Therefore, in the section entitled: "Limitations of the methods" the authors should discuss about the possibility to avoid the EMT also by using Progesterone in culture medium. Moreover, in the same section authors state that the proposed isolation protocol avoids the occurring of EMT but it is not well explained the precise way they obtained this inhibition. For this reason, it would be nice to observe the expression of mesenchymal markers (such as Vimentin, α-SMA or N-Cadherin) to properly assess that the isolation protocol does only allow the isolation of epithelial cells. Furthermore, it would be nice to evaluate that amniotic epithelial cells, from passage 0 to passage 3, do not increase the expression of mesenchymal markers, thus indicating that EMT is indeed inhibited.

Response: It has demonstrated that loss of E-cadherin is an indicator of EMT, so in our cultures we carried out immunocytochemistry to detect the presence of E-cadherin, suggesting that the cellular phenotype was epithelial throughout the three passages. However, we add in the section "Limitations of the methods" the suggestion of analyzing the effect of progesterone in HAEC to avoid the EMT after the third cellular passage (page 8, lines 365-369).