Human amniotic epithelium (HAE) as a possible source of stem cells (SC)

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Abstract

There have been major recent advances in the field of developmental biology due to the investigation on stem cells (SC). Stem cells are characterized by their capacity of auto-renewal and differentiation to different cellular phenotypes. Based on the developmental stage, they can be classified into two different types: embryonic SCs and adult SCs. It has been widely reported that several problems need to be resolved before their possible clinical applications. As a result, fetal membranes have been suggested as an alternative source of SCs. In the human amniotic epithelium, the presence of markers of pluripotent SC's has been reported, and its capacity as a feeder layer for expansion of different SC types. Also, fetal membranes are a discarded product after delivery, and thus there are not any ethical issues related to its use. In conclusion, the human amniotic epithelium can be a strong candidate for regenerative medicine. (Gac Med Mex. 2015;151:61-8)

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Introduction

Research on embryonic stem cells (ESCs) has importantly contributed to the recent advance in the fields of developmental and cell biology. Also, due to their wide differentiation capability, this type of cells have raised interest on and kept the promise of their possible use in cell-replacement therapies. However, there are serious problems that remain to be solved before translating ESCs use from basic to clinical research. An example of such a problem is their probability to form tumors when these cells are grafted in an undifferentiated stage[1], as well as the ethical issues their derivation entails[2]. Likewise, the use of adult stem cells (ASCs), obtained from tissues of adult organisms, e.g. bone marrow, brain, or muscles, has been suggested. Although these cells have been studied, their extraction is difficult and their obtained number is insufficient for use in cell-replacement therapies. Additionally, ASCs show limited proliferation and plasticity compared with ESCs[3], which reveals the need of alternative SC sources, such as the human amniotic epithelial cells (hAECs)[4,5], since they show a series of unique features[6]. Therefore, this paper focuses on hAECs as a possible source of SCs and hAEC potential use in this interesting research area.

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Table 1. Stem cells classification based on their differentiation potential

Classification	Differentiation potential
Totipotent	Capability to form all lineages of the organism; in mammals, only the zygote is totipotent
Pluripotent	Capability to form all the embryo lineages, including germ cells; for example, ESCs
Multipotent	Potential to form cells restricted to an embryonic layer in particular; for example, neural SC, which will differentiate into neurons, astrocytes and oligodendrocytes
Unipotent	Cells that form a single specific cell type; for example, epithelial SC

Modified from Jaenisch et al.[68]

SCs

SCs are characterized by their dedifferentiation and potential to auto-renew and differentiate into a variety of specialized cell types. SCs are present in several tissues during embryonic stages as well as in adult animals, and can be classified according to their differentiation potential (Table 1).

On the other hand, the classification based on their developmental stage at isolation assort SCs as ESCs or ASCs.

ESCs

In 1981, the Martin Evans and Gail Martin groups isolated pluripotent cell lines from the blastocysts of mouse pre-implantation for the first time, and named them embryonic stem cells (ESCs)[7,8]. Derivation of these cells was achieved growing the inner cell mass of the blastocyst on a mouse embryonic fibroblast (MEF) feeder layer of 14 days of gestational age. The fibroblasts secreted soluble factors that allowed for cells of the inner cell mass to be preserved *in vitro* at a pluripotent stage, thus allowing the obtainment of these cell lines. Mouse-derived ESCs are currently among the most widely studied SCs and are characterized by presenting large nuclei and small cytoplasms, growing in compact colonies, having high telomerase activity (telomerase is associated with the capability for cell proliferation, protection and chromosome stabilization)[9,] and showing a normal caryotype

(i.e., only a small proportion present chromosomal abnormalities). Furthermore, these cells can be preserved *in vitro* without an apparent loss of their self-renewal and differentiation capabilities. ESCs express molecular markers such as the tissue-unspecific alkaline phosphatase (an enzyme also present in germ line cells), transcriptional regulatory factors that maintain the cells in an undifferentiated state (octamer-binding transcription factor 4 [Oct-4], sex-determining region Y (SRY)-box 2 [Sox2] and Nanog) and transmembrane proteins (stage-specific embryonic antigen [SSEA-1], tumor rejection antigen [TRA-1-60]). This way, preserved in conditions that favor their pluripotent status, these cells do not present morphological or molecular characteristics of differentiated cells.

With procedures similar to those used to derive mouse ESCs, the group of Thompson generated non-human primate (Rhesus monkey)[10] and, later, human ESCs. In 1988, they reported the isolation and culture of five human embryonic stem cell (hESC) lines obtained from embryos of less than a gestational week coming from *in vitro* fertilization clinics and donated by parents for this specific purpose. As a result, when cultured *in vitro*, rounded with well-defined edges, compact and flat colonies, with cells presenting a prominent nucleus and a high nucleus-cytoplasm ratio and that maintained an unlimited proliferation capability, were obtained [11].

Once an ESC line is obtained, its pluripotent state must be verified by means of a series of laboratory tests. In cells obtained from mouse, its pluripotency can be demonstrated by injecting ESCs into blastocysts of the same species and observing their contribution to every tissue of the animal, spermatozoids and oocytes included[12]. Thus, chimeric organisms with a functional germ line are obtained[13]. Pluripotent state has also been demonstrated by means of tetraploid complementation, through the production of 4n blastocysts by cell fusion and ESCs introduction to the blastocyst. Since 4n cells are not able to produce somatic cells, the resulting organisms are entirely formed by ESC derivatives[14]. A third form to test the ESC pluripotent capability is by forming differentiated cells *in vitro*, either directly or indirectly. The first method consists in removing the signals that allow for self-renewal; consequently, cells differentiate spontaneously. In the second method, the differentiation protocols allow cell exposure to specific culture conditions and growth factors to induce them into a particular cell lineage[15].

In the case of the hESCs, ethical issues prevent the production of chimeric animals or tetraploid comple-

mentation. Therefore, the highest standard of evidence is the formation of teratomas (tumors with ectoderm, endoderm and mesoderm cell lineages) by injecting the hESC into immunodeficient mice[16], and the demonstration that cells are able to differentiate *in vitro* into tissues of the three embryonic layers. The recognition of the ESC identity has been achieved by using the above mentioned tests together with molecular markers (Oct-4, Sox-2, Nanog and alkaline phosphatase) and surface antigens exclusive to this cell type (SSEA-3, SSEA-4, TRA-1-60 and TRA-1-18). However, research with hESC entails ethical and political controversies to be solved before its inclusion in clinical research[2].

Other types of pluripotent cells, such as epiblast SC and induced pluripotent stem cells (iPSC), have also been described. However, due to space limitations, we will not review them in detail. We refer the reader to the works published by Chenoweth et al.[17], Stadtfeld et al.[18], Teoh et al.[19] and Wu et al.[20].

ASCs

SCs from adult tissue are embedded in tissue and organ differentiated cells, and their main role is to maintain and repair their niches. These niches represent a specialized tissue microenvironment where SCs establish complex and reciprocal interactions with supporting cells and the extracellular matrix through adherent unions mediated by cadherins, catenins, integrins and selectins and their ligands, adhesion molecules, growth factor and chemokines receptors, etc.[21]. ASCs have also a role in the secretion of various soluble factors functioning as non-adhesion-mediated cell interaction mechanisms (Wnt/frizzled, Notch/Delta-Jagged) that contribute to their restricted mobility and adoption of either a quiescent or an active state within their niche [22,23].

These SCs are usually multipotent, since they have certain compromise to form cells of the same cell type of the tissue embedding them. This cell type has been identified in several organs, such as brain, bone marrow, skeletal muscle, skin, intestine, etc. Though, the ASCs number is very limited, they are difficult to find and isolate, and their differentiation range is restricted to the cell type of their niche, as previously mentioned[24,25].

Due to the above-mentioned disadvantages associated to the obtainment and use of both, hESC and ASC, alternative sources like the human extra-embryonic tissues have been examined.

Human extra-embryonic tissues

The extra-embryonic tissues comprise the placenta, umbilical cord and fetal membranes. A term placenta weighs between 400 and 600 g[26], and has a discoid shape, with a 15-20 cm diameter and 2-3 cm thickness. It has two sides: the first known as chorionic plate has a fetal origin, faces the amniotic cavity, and is formed by the amnion and the chorion; the other side, is named basal plate, is formed by the decidua (basal and parietal), and has a maternal origin[27,28]. The placenta acts as a mother-fetus interphase, involved in gas, nutrients and waste products exchange. It also works as an endocrine organ producing hormones and growth factors that regulate both fetal growth and maternal physiology[29]. The umbilical cord consists of a connective mucous tissue known as Warthon´s jelly that extends along the amniotic epithelium and covers the umbilical vessels. Its main function is to prevent vessel compression, torsion or flexion, to allow the bi-directional blood flow between maternal and fetal circulation that the vessels provide[30]. Finally, fetal membranes are formed by two attached membranes, the amnion and the chorion (Fig. 1).

Human fetal membranes

The amniotic membrane is a translucent biological membrane without nerves, muscles or lymphatic vessels. The amnion thickness ranges from 0.02 to 0.5 mm and consists of three histological layers: epithelial layer, basal layer and avascular mesenchymal tissue. The inner layer, adjacent to the amniotic fluid, is formed by a homogeneous mantle of cuboidal cells firmly attached to the basal membrane, which, in turn, is attached to an acellular layer composed by collagen, types I, II and V. A stromal cell lining composed of fibroblasts and scarce macrophages embedded in a reticular-fiber mesh covers this layer externally. The most external, intermediate or spongy layer considerably varies in thickness, and is rich in elastin, hydrated proteoglycans (especially perlecan), glycoproteins and type III collagen fibers that separate amnion from chorion. The chorion is the thickest histological structure among the fetal membranes and holds more cellularity than the amnion. The final part of the chorion is interlocked with the uterine decidua.

Particularly, hAECs display microvilli on their apical surface and perform an intra- and trans-cellular secretory function. Additionally, it has been described a large irregular nucleus with intra-cytoplasmic organelles
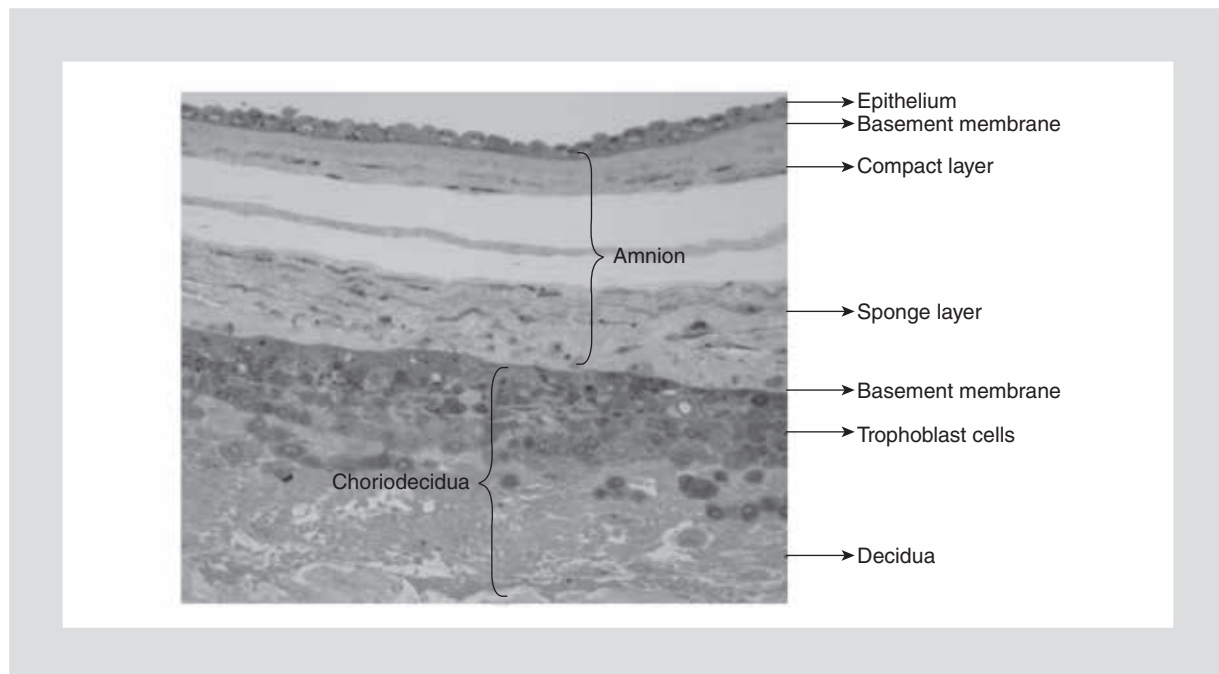
Figure 1. *Semifine cross-section (1 µm) of a 40-gestational-week human fetal membrane obtained from elective cesarean, included in EPON and stained with 0.5% toliudine blue. The image shows the two fetal-membrane constitutive regions: amnion and chorion (20X)* (modified from the Guadalupe García Gómez Masters Degree thesis, UNAM, 2007).

and pinocytic vesicles. One of the hAECs basic functions is to provide the developing embryo with an environment to be suspended, where the embryo will be able grow free of the pressure exerted by structures surrounding its body and protected against desiccation. In addition, multiple metabolic functions, such as water and soluble material transportation and production of factors without a specific biologic function, including vasoactive peptides, growth factors and cytokines, have been described[31]. Interestingly, presence of SCs among hAECs has been suggested, as described later.

Embryonic development of human amniotic epithelium

Unlike other structures forming the placenta, the amnion is generated around the 8th day after fertilization, before gastrulation. In this period, the blastocyst is implanted in the endometrial stroma and the inner cell mass is differed into two layers: one composed by cuboidal cells (hypoblast) and other of cylindrical cells (epiblast). The three embryonic cell layers that make up the embryo originate in the epiblast, whereas the hypoblast generates the extra-embryonic endoderm[32]. Within the epiblast, a small cavity (the amniotic cavity)

appears, and the adjacent cells (amnioblasts) form the amniotic epithelium. The development of the amnion from the epiblast is very important, since this tissue has been suggested to contain a SC population[5,33].

In vitro derivation and preservation of hAECs

Different hAEC *in vitro*-derivation protocols have been reported. These cells are usually obtained from 38-to-39-gestational-week term placentas by means of elective cesarean section, after informed consent is obtained. It is important pointing out that women must not be in labor or have premature membrane rupture, chorioamnioitis or chromosomal abnormalities for tissue donation. hAEC-isolation protocols consist in mechanically detaching the amniotic membrane from the chorion, washing it with a buffering solution, then incubating it at 37 °C with trypsin at different times and different concentrations[5,34-40] or using dispase 1.2 U/ml and scraping the tissue to remove the cells[35]. Once the hAECs are obtained, they are cultured at different densities per cm^2 ranging from 6 x 10^4 to 1 x 10^5, in supplemented Dulbecco's Modified Eagle's Medium (DMEM) and in the presence of epidermal growth factor (EGF) at a 10 ng/ml concentration[37,41,42]. It is worth

mentioning that the composition of this medium is very similar to the one used in ESC[43,44].

Finally, derivation of hAEC in xenocontaminant-free medium (free of animal derivatives) has been reported, thinking on its possible application in the clinic. This way, modified reagents have been employed, which include Trypzean as digestion solution (it does not contain contaminants traditionally found in bovine or porcine trypsin) and EpiLife medium (serum-free basal medium)[45].

Molecular and cell-surface markers found in hAECs

When stained with hematoxylin-eosine *in vitro,* hAECs present an homogeneous appearance[37,46], but express a heterogeneous phenotype when membrane markers are determined, with different antigenic expression degrees. These findings would indicate the presence of cell subpopulations, probably SCs in conjunction with more differentiated cells. This way, the cells express a large variety of surface antigens (Table 2).

Interestingly, they express ESC-associated molecular markers such as Oct-4, Sox-2, Nanog, reduced expression 1 (Rex-1), fibroblast growth factor-4 (FGF-4), cryptic family 1 (CFC-1), developmental pluripotency-associated protein 3 (DPPA-3) and c-kit[5,35,37,47]. These transcription factors play an important role to maintain the pluripotent state of the cells, through complex networks between them[48,49]. In addition, *in vitro* cultured hAECs have been reported to be tissue non-specific alkaline phosphatase-positive (ESC marker)[35]. Finally, hAECs have been shown to express low or moderate levels of the polymorphic antigens HLA A-B-C (class Ia), lack HLADR (class II)[41] and express HLA-G[50], data that can explain the low immunogenic of the human amniotic membrane and the absence of rejection when it is transplanted[51,52].

Additionally, when cultured *in vitro,* hAECs express markers specific to each embryonic layer. Regarding the ectoderm, nestin, microtubule-associated protein (MAP-2), intermediate neurofilament protein, glial fibrillary acidic protein (GFAP) and neuron-specific enolase have been identified[37]. Endoderm markers are: globin transcription factor (GATA) binding protein 4 (GATA-4), hepatocyte nuclear factor 3b (HNF-3b), a-fetoprotein and glucose transporter (GLUT-2). Finally, mesoderm markers are: atrial natriuretic peptide, heavy and light chain myosin, actin, etc., all of them associated with the cardiomyocitic lineage[37]. Together, these data

suggest a pluripotent state as well as a wide heterogeneity of the hAECs.

Pluripotency tests in hAECs

As previously mentioned, there is a series of laboratory tests to demonstrate the pluripotent state of the SCs. In the case of hAECs, Tamagawa et al., in 2004, were the first to describe their pluripotent features, by creating xenogenic chimeras *in vitro* from cells isolated from human amnion with mouse embryonic cells, and demonstrating the contribution of hAECs to the

Table 2. Expression of surface antigens present in the hAECs

Reference	Antigen in hAEC
Miki et al.[37],	CCR-4, CD117 (c-kit), CD24, CD324
Raisanen et al.[69]	(e-cadherin), CD338 (ABCG-2), CD49f, CD54 (ICAM-1), CD9, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81
Ilancheran et al.[5]	CD90, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81
Minas et al.[70]	GCTM-2, SSEA-4
Parolini et al.[71]	CD10, DC105, CD117 (c-kit), CD13, CD140b, CD166 (ALCAM), CD29, CD324 (E-cadherin), CD44, CD49e, CD73, CD90, GTM-2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81
Banas et al.[72]	c-met, CD24, CD29, CD324 (E-cadherin), CD338 (ABCG-2), CD9, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81
Stadler et al.[73]	CD54 (ICAM-1)
Bilic et al.[35]	CD166 (ALCAM), CD44, CD49d, CD73, SSEA-3, SSEA-4
Fatimah et al.[74]	CD44, CD73, CD9
Murphy et al.[45]	CD1b, CD10, CD26, CD29, CD34, CD45, CD46, CD55, CD58, CD29, CD63, CD73, CD77, CD81, CD90, CD91, CD95, CD98, CD104, CD108, CD109, CD142, CD147, CD151, CD164, CD166, CD227, EGFR, fll-R, HLA-ABC, HLA-DQ
Pratama et al.[41]	E-cadherin, CD49f, CK7, EpCAM, vimentin, CD44, CD105, CD90, CD146, CD29, HLA-ABC, CD40
Maguire et al.[75]	SSEA-1, SSEA-4, TRA-1-60, TRA-1-81

CCR-4: chemokine receptor type 4; CD: cluster of differentiation; GCTM-2: germ cell tumor marker; CK7: cytokeratine 7; EGFR: epidermal growth factor receptor; HLA: human leukocyte antigen.

Table 3. hAEC *in vitro* differentiation into specialized cells of the three embryonic layers

| Reference | Lineage | | |
	Endoderm	Mesoderm	Ectoderm
Miki et al.[37]	Pancreatic cells	Cardiomyocytes	Neural cells
Ilancheran et al.[5]	Pancreatic cells Hepatic cells	Myocytes Osteocytes Adipocytes Cardiomyocytes	Neural cells
Kim et al.[36]	–	Adipocytes Osteocytes Condrocytes	Neural cells
Bilic et al.[35]	–	Osteocytes Myocytes Adipocytes	–
Murphy et al.[45]	Pulmonary epithelium cells	Osteocytes Adipocytes	Neural cells Glial cells
Pratama et al.[41]	Alveolar epithelium cells Pancreatic cells Hepatic cells	Osteocytes Condrocytes	–
Marongiu et al.[76]	Hepatic cells	–	–
Fant et al.[77]	–	Cardiomyocytes	–

formation of all three germ layers (endoderm, ectoderm and mesoderm) through the formation of embryoid bodies[53].

The capability of hAECs to form clonal colonies (a property of ESCs) in low-density cultures (30-50 cells per cm^2) has also been reported[5,41]. However, and in contrast to hESCc, these cells are not capable of forming teratomas: when being inoculated at a 5×10^5 cell-density in testicles of immunodeficient mouse, no formation of tumors was reported after 10 weeks[5]. Miki et al. injected hAEC at 1×10^6 into the liver and rear legs of mice with severe combined immunodeficiency (SCID); the animals were examined at 7 months looking for any evidence of tumor growth, but the authors did not report any finding[37]. These data demonstrate that, when being injected to immunodeficient mice, hAECs are unable to form teratomas, a property that would be very useful in the clinical setting, since one of the biggest problems with the use of hESCs is the possible development of teratomas when being transplanted in undifferentiated state.

In spite of the loss of teratoma formation, hAECs have been differentiated *in vitro* into endoderm, mesoderm and ectoderm derivatives (Table 3). The techniques used to perform the phenotype determination involved polymerase chain reaction (PCR) and immunocytochemistry.

hAECs as a feeder layer of pluripotent cells

hAECs, as opposed to hESCs, do not require a second cell type as a feeder layer for their growth. They adhere to plastic and to flasks covered with basement membrane or other materials in conventional media, as previously mentioned. However, they have been proposed as a substitute niche for the preservation of SCs of different origins[54]. This way, limbal epithelial cells[55], epiblast-derived SCs (EpiSC)[56], iPSC[57,58] and spermatogonial stem cells (SGSC)[59] have been shown to be successfully cultured using hAEC as culture substrate. In the particular case of SGSCs, the expression of Nanog and Oct-4 is higher in cells cultured in hAEC than in those cultured in MEF or with other cell type[60]. Furthermore, Lai's group used hAEC to maintain the undifferentiated state of murine ESC, and reported that the ESCs expressed 3-5 times more FGF, Oct-4, Nanog, Sox-2 and Rex-1, than cells cultured in MEF, using the reverse transcription technique followed by real-time polymerase chain reaction (RT-PCR). The authors suggested that these effects might be caused by production of leukemia inhibitory factor (LIF)[61]. On the other hand, Liu et al. reported having obtained human iPSC on hAEC, an effect mediated by micro RNA 145 when the levels of Sox-2 were increased in the reprogrammed cells[57].

Anti-inflammatory, anti-microbial and low-immune-response properties of amniotic cells

Amnion-derived cells have been considered an ideal tissue for allogeneic transplantation, based on their anti-inflammatory and low immune response effects. hAECs have been reported to express several anti-angiogenic markers and anti-inflammatory proteins including the interleukin 1 receptor-agonist, metalloproteases 1, 2, 3, 4 and 5 inhibitors and interleukin 10[62]. In addition, they suppress the tumor growth factor b isoforms and its type II receptor, which triggers collagen deposit in the site of lesion and fibrosis[63].

Beta defensins have also been described to be a group of antimicrobial peptides secreted by epithelial cells of the mucosae and the amnion, which are integral part of the innate immune system[64].

With regard to low immune response, no rejection signs have been reported when the amniotic membrane has been grafted in healthy volunteers without underlying diseases[65]. On the other hand, amnion cells express the non-polymorphic antigen HLA-G, but they lack polymorphic antigens HLA-A (class A) and HLA-DR (class B)[66]. This characteristic confers them an immune advantage by decreasing the risk of rejection when being transplanted.

The human amniotic membrane is an inexpensive biological source of matrix for cell culture. Additionally, expression of several growth factors and cytokines (EGF, keratinocyte growth factor [KGF], hepatocyte growth factor [HGF], basic fibroblastic growth factor [bFGF], TGF-α, transforming growth factor-β [TGF-β], bone morphogenetic growth protein-4 [BMP-4], etc.) has been described[67].

Conclusions

The information here presented suggests that hAECs have similar characteristics to pluripotent SCs, which makes them excellent candidates to be used for cell-replacement therapies. In addition, hAEC derivation does not entail ethical issues, these cells do not form teratomas when transplanted at an undifferentiated state, do not present immune response and have anti-inflammatory and antimicrobial properties. Nevertheless, further investigation to enable their characterization is required since hAECs form heterogeneous populations, and the reported protocols for their isolation are diverse.

Acknowledgements

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