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PRACE ORYGINALNE
położnictwo

Gene expression of extracellular matrix components in human amniotic cells – a pilot study

Ekspresja genów składników macierzy pozakomórkowej w komórkach owodni łożyska ludzkiego – badanie pilotażowe

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Abstract

Objectives: *Identification of microenvironmental components, which significantly affect the fate of amniotic stem cells, is one of the essential conditions for their in vitro preparation for clinical applications. Control of their gene expression seems to be crucial for in vitro reconstitution of the natural amniotic microenvironment and modulation of the stem cell phenotype both, in vitro and in vivo. The aim of the study was to analyze multi-gene expressions for ECM components in human amnion cells (hACs) as compared to differentiated cells of a mesenchymal and ectodermal origin.*

Material and methods: *hACs were isolated from normal human placentas at term. SSEA-4+ cells were visualized in vitro by immunofluorescence microscopy and counted by flow cytometry. The expressions of 84 genes were assayed by the RT-PCR Array method and analyzed by Web-based PCR Array Data Analysis Software. These expressions were compared among hACs, fibroblasts, and keratinocytes.*

Results: *Almost 86% of the primarily cultured hACs exhibited expression of the SSEA-4 surface marker. In these cells, the expression of 7 genes for ECM structural components, 4 for adhesion proteins, 3 for integrin subunits, and 2 for metalloproteinases was strong: over 100-fold up- or down-regulated as compared to fibroblasts. These differences in gene expressions were less distinct when hAC were compared with keratinocytes.*

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Conclusions: *hACs exhibit highly different expression of several ECM-associated genes as compared to differentiated cells, especially of mesenchymal origin. The products of these genes, via an autoregulatory mechanism, might determine the nature of ECM-stem cell interactions, which are crucial for their stemness and potential differentiation abilities.*

Key words: **human amnion cells** / **SSEA-4** / **gene expression** / **extracellular matrix** / / **basement membrane** /

Streszczenie

Cel pracy: *Identyfikacja składników ECM istotnych dla losów owodniowych komórek macierzystych i zastosowanie ich in vitro jest jednym z kluczowych warunków odpowiedniego przygotowania tych komórek dla celowanych aplikacji klinicznych. Kontrola ekspresji genów może mieć kluczowe znaczenie dla odtworzenia warunków naturalnego mikrośrodowiska owodni in vitro oraz dla sterowania fenotypem komórek macierzystych. Celem pracy była ocena ekspresji genów składników ECM w komórkach izolowanych z owodni łożyska ludzkiego (hAC), w porównaniu z ich ekspresją w komórkach zróżnicowanych pochodzenia mezenchymatycznego i ektodermalnego.*

Materiał i metody: *hAC izolowano z łożysk ludzkich uzyskanych po prawidłowych ciążach zakończonych terminowo cesarskim cięciem. Liczbę komórek wykazujących ekspresję antygenu SSEA-4 określano przy użyciu cytometru przepływowego. Do wizualiacji komórek SSEA-4+ w hodowli zastosowano metodę mikroskopii immunofluorescencyjnej. Badano ekspresję 84 genów metodą mikromacierzy RT-PCR i analizowano uzyskane wyniki przy użyciu oprogramowania Web-based PCR Array Data Analysis Software. Ekspresję badanych genów w hAC wyrażono jako krotność ekspresji tych samych genów oznaczonej w fibroblastach i keratynocytach.*

Wyniki: *Około 86% hAC w hodowli pierwotnej stanowiły komórki posiadające marker SSEA-4. W komórkach tych ekspresja 7 genów składników strukturalnych ECM, 4 genów białek związanych z adhezją, 3 genów podjednostek integrynowych i 2 genów metaloproteinaz, wykazywała ponad 100-krotną różnicę w stosunku do fibroblastów. Znaczne różnice w ekspresji dotyczyły większej liczby genów niż to miało miejsce w hAC porównanymi z keratynocytami.*

Wnioski: *hAC wykazują wysoce zróżnicowaną ekspresję niektórych genów składników ECM w stosunku do komórek zróżnicowanych, szczególnie pochodzenia mezenchymatycznego. Składniki te, pośrednio, poprzez mechanizm autoregulacyjny mogą decydować o charakterze oddziaływań ECM-komórki macierzyste, decydujących o fenotypie komórki macierzystej i potencjalnych możliwościach różnicowania.*

Słowa kluczowe: **komórki ludzkiej owodni** / **SSEA-4** / **ekspresja genów** / / **macierz pozakomórkowa** / **błona podstawna** /

Abbreviations:

ECM – extracellular matrix; ESC – embryonic stem cells; hACs – human amniotic cells; hAECs – human amniotic epithelial cells; hAM-MSCs – human amniotic mesenchymal stromal cells; hESCs – human embryonic stem cells; MMP – metaloproteinases; MSCs – mesenchymal stem cells; SSEA-4 – Stage-Specific Embryonic Antigen 4.

Skróty:

ECM – macierz pozakomórkowa; ESC – embrionalne komórki macierzyste; hAC – ludzkie komórki owodni; hAC – ludzkie komórki nabłonka owodni; hAM-MSC – ludzkie mezenchymalne komórki zrębowe owodni; hESC – ludzkie embrionalne komórki macierzyste; MMP – metaloproteinazy; MSC – mezenchymalne komórki macierzyste; SSEA-4 – *ang.* Stage-Specific Embryonic Antigen 4.

Introduction

Components of the extracellular matrix (ECM), creating human amniotic cells (hACs) niches, are the key factors influencing their phenotype [1]. In the niche, interactions between cells, as well as between cells and soluble (growth factors and cytokines) [2] and insoluble (structural proteins) [3] factors, take place. Human amniotic epithelial cells (hAECs) are located at the boundary between two different microenvironments: amniotic fluid and basement membrane. hAECs are exposed to the influence of amniotic fluid cytokines such as TNFα and IL-1β, which act in a pro-apoptotic way, activate metalloproteinases (MMP), and are responsible for the elevation of the prostaglandin PGE2 level, which in turn activates MMP. In consequence, these actions may result in ECM remodeling and fetal membrane rupture [4]. In the early stages of pregnancy, hyaluronic acid effectively binds the cytokines, thus protecting the amnion membrane from their activity.

hAEC basement membrane is one of the thickest membranes synthetized in the human body. It is composed of, among others, collagen type III, IV and V, fibronectin, nidogen, and laminins [5]. *In vitro* studies, carried out on human embryonic stem cells

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(hESCs), have proven several basement membrane components to be able to modify the phenotype of amniotic stem cells [6], e.g. laminin 5 increases cell proliferation and laminin 10 supports the stemness of pluripotent cells [7, 8]. These effects result from an interaction of the laminin ligands with integrin receptor α6β1 [7].

Other important cells of the human amnion are mesenchymal stromal cells (hAM-MSCs), which are located in the mesenchymal tissue underlying the epithelial basement membrane. These cells are a documented source of numerous ECM components, which can influence the hAC phenotype. The result of the action of the cellular and extracellular factors is a set of unique features, specific to amniotic cells, such as the lack of the ability to malignant transformation [9], or immunomodulating properties [10, 11].

The purpose of the study was to compare ECM-associated gene expression in cells isolated from a human amnion, and in differentiated cells of an ectodermal (keratinocytes) and mesenchymal (fibroblasts) origin. We aimed to identify ECM components which might be crucial for supporting the hAC phenotype. The expression of 84 human ECM-associated genes, including genes for adhesion molecules, in hAC has been evaluated. Identification of ECM components which are vital for the fate of stem cells and their future application in *in vitro* cultures may be crucial for their adequate preparation and use in regenerative and transplantation medicine [12].

Material and methods

Human placentas

Term placentas from 3 healthy donor primagravidas, aged 33 years, were obtained with an informed consent from the Perinatology and Gynecology Ward of the Central Clinical Hospital, Medical University of Silesia, Katowice. The placentas were taken after uncomplicated elective cesarean deliveries performed due to breech presentation of the fetus, placenta previa or uterine fibroids, at 38-39 weeks of gestation. Then, they were promptly transferred to the laboratory in phosphate buffered saline (PBS; Sigma, USA) containing antibiotic-antimycotic solution (Penicillin 0.1 Units/ml, Streptomycin Sulphate 0.1 mg/ml, Amphotericin B 0.25 µg/ml; PAA, Austria) and EDTA (5 mM EDTA; Invitrogen, USA), and rinsed several times to remove blood.

Isolation and culture of cells from human amniotic membranes

The amniotic membrane was manually separated from the chorion, washed in PBS, and cut into pieces (~20x20 mm). The fragments were then subjected to enzymatic digestion, at 37°C; 7 min. with 2.4 U/ml dispase solution (Roche, Japan), 2 × 40 min. with 0.05% trypsin-0.54 mM EDTA solution (PAA), and 60 min. with 0.75 mg/ml collagenase solution (Roche, Germany). Cells which were recovered after digestion were collected by centrifugation (300g, 4°C) and suspended in a complete medium. Fractions collected after each digestion step were combined to constitute a mixture of the cells. The resulting hAC cell suspensions were depleted of red blood cells by incubation with Lysing Buffer (Becton Dickinson, USA). After centrifugation and filtration through a 100 µm cell strainer, the cells were placed into culture flasks (1×10^5 cells/cm^2). Each cell culture was maintained in: Alpha MEM (Alpha Modification of Minimum Essential Medium) (Sigma-Aldrich, USA) supplemented by 10% FBS, 1% antibiotic-antimycotic solution, and 1% L-glutamine (all from PAA, Austria) at 37°C with water saturated atmosphere and 5% CO_2. The cells were grown for 16 hours under these conditions in an incubator (Sanyo, Japan).

Culture of differentiated cells

Human fibroblasts and keratinocytes (Lonza, Switzerland) isolated from adult individuals were cultured at the Department of Molecular Biology and Genetics, MUS, Katowice (Poland).

In vitro immunodetection of stem cell markers

After 16 hours of culture, the amniotic cells were collected, centrifuged, suspended in PBS supplemented with 10% FBS and 5 mM EDTA, and incubated (30 min., 4°C, in dark) with anti-human primary anti-SSEA-4 Mouse IgG3 antibody and Mouse IgG3 (559806) (both from BD Pharmingen, USA) isotype control. The antibody was conjugated with FITC and used in accordance with the manufacturer's protocol (20 µl solution per 100 µl cell suspension containing 1×10^6 cells). Finally, the cells were fixed in 2% paraformaldehyde, and mounted in Vectashield Hard-Set Mounting Medium – with DAPI (Vector, USA) in order to prevent photobleaching. Immunocytochemical reactions were analyzed in fluorescence microscope (Nikon Eclipse Ti-U, Japan) equipped with a camera (Nikon Digital Sight DS-SMC, Japan) using NIS-Elements AR 3.00 (Nikon Instruments Inc.).

Flow cytometry

In order to complete flow cytometric analysis, the cells obtained from the amniotic membrane were harvested sequentially on the 16th hour of culture management. Immunostaining was performed for 30 min. at 4°C in the dark, using PerCP anti-human/mouse SSEA-4 mouse IgG_3 (FAB1435C, R&D Systems, USA). In addition, matched isotype control, namely PerCP Mouse IgG3 (IC007C, R&D Systems, USA), was used. Each time, at least 10 000 events were analyzed by flow cytometry (FACS Aria, Becton Dickinson, USA) with the BD FACSDiVa software.

RT-PCR Array

RNA isolation

RNA was prepared from hACs containing 86% of SSEA-4$^+$ cells, as well as from fibroblast and keratinocyte samples (3 mln per sample) using the RNeasy Mini Kit (Qiagen, USA) protocol. Total RNA quality and quantities were determined by measuring the absorbance spectra on a UV/Vis spectrophotometer, the NanoDrop 2000 (ThermoScientific, USA). Higher quality, undegraded RNA was processed further for RT-PCR Array experiments.

RT-PCR Arrays

We used the PCR Arrays sets of optimized real-time PCR primer assays on 96-well plates. Each set contained appropriate RNA quality controls and five housekeeping genes (Table I). cDNA was generated and amplified from 2 µg total RNA isolated from each sample using the applied RT^2 First Strand Kit (SABiosciences, USA), following the manufacturer's protocol. We mixed our cDNA template with the appropriate ready-to-use PCR master mix (RT^2 qPCR SYBR Master Mix Kit, SABiosciences, USA), aliquot equal volumes to each well of the same PCR Array, and then ran the real-time PCR cycling program (LightCycler 480, Roche Diagnostics).

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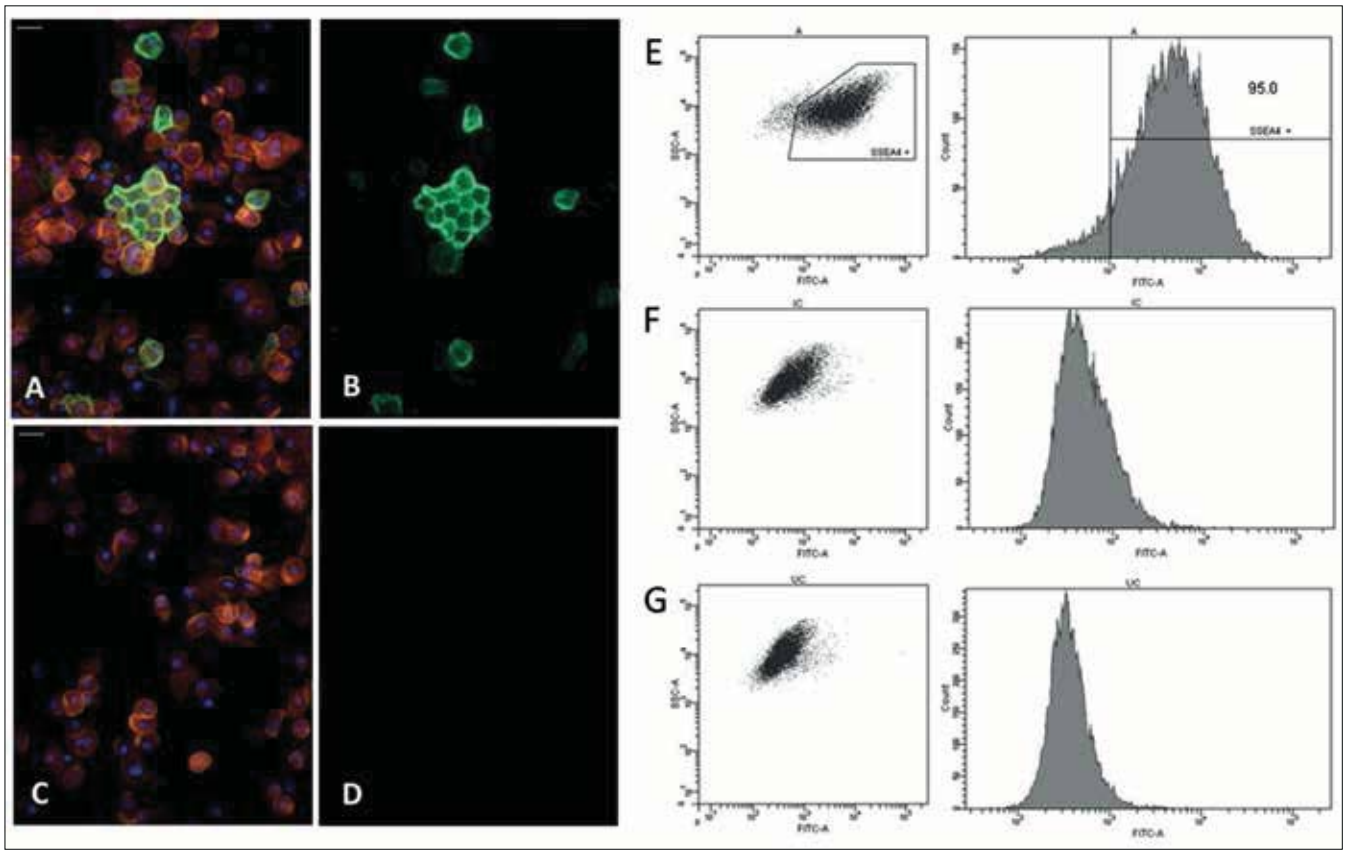

Figure 1. Immunodetection of SSEA-4+ cells (A and B) and adequate isotype controls (C and D) in primary culture of amniotic cells. On the right side, the flow cytometric analysis of amnion-derived cells is shown. 95% of presented cells exhibit SSEA-4 expression (E). Isotype control (F) and a population of unlabeled cells (G) is also shown. FITC-conjugated anty-SSEA4 antibody – Green; F-actin stained with Rhodamine phalloidin - Red-orange; cell nuclei stained with DAPI - Blue. Scale = 20 μm.

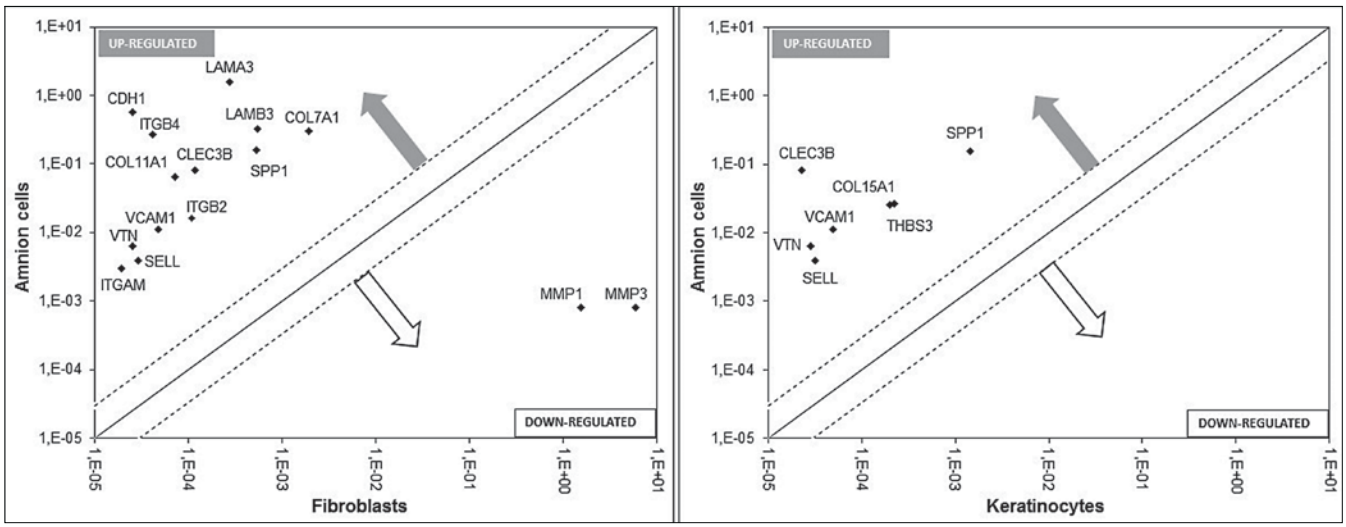

Figure 2. Strongly up- and down-regulated ECM & adhesion genes in amniotic cells revealed by PCR Arrays. Total RNA from normal human hAEC, as well as fibroblasts and keratinocytes were characterized in technical triplicates, and the relative expression levels (< or > 100x) for each gene are plotted against each other in the Scatter Plot (outside the dashed lines). 7 genes encoding the ECM structural components, 4 genes encoding adhesion proteins, 3 genes for integrin subunits and 2 genes for metalloproteinases are up-regulated, while genes encoding matrix metallopeptidases MMP1 and MMP3 are down-regulated in hAEC in relation to fibroblasts (left plot). 3 genes encoding adhesive molecules, 2 genes encoding structural proteins and 2 genes for other ECM-associated proteins are up-regulated in hAEC in relation to keratinocytes (right plot).

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Table I. Expression of housekeeping genes in amniotic cells in relation to differentiated cells. Almost all genes are down- or up-regulated by less than three-fold (statistically insignificant).

House keeping genes	Full name	Over/under-expression of gene in amniotic cells related to differentiated cells	
		vs. fibroblasts	vs. keratinocytes
B2M	Beta-2-microglobulin	-1,19	1,33
HPRT1	Hypoxanthine phosphoribosyltransferase 1	**-3,60**	-2,23
RPL13A	Ribosomal protein, large, 13A	1,90	-1,02
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2,31	1,33
ACTB	Actin, beta	-1,02	1,29

The PCR array of 84 genes was performed using a commercially available set of plates (RT^2 Profiler PCR Array PAHS-013: Human Extracellular Matrix and Adhesion Molecules PCR Array), following the manufacturer's protocol. Template cDNAs prepared from total RNA of hACs were characterized in technical triplicates. The relative expression levels of 84 genes in hACs and two different types of differentiated cells, were compared. The detection threshold was set according to the manufacturer's recommendation, as at least 3-fold in hACs in comparison to differentiated cells. The results were analyzed using Web-based PCR Array Data Analysis Software (http://sabiosciences.com/pcrarraydataanalysis.php).

Results

Characteristics of the isolated hACs: *in vitro* quantification

Cell isolation from the amnion was followed by immunocytochemical detection of pluripotent stem cell marker - SSEA-4, and flow cytometric qualitative and quantitative analysis of the SSEA-4^+ subpopulation in the primary culture. Our analysis showed that SSEA-4^+ cells were numerous among the isolated amniotic cells, constituting 85.6% (±4.6, n=3) of the entire population (Figure 1).

Gene expression

The undertaken screening showed that most of the studied genes exhibit an over 3-fold expression difference as compared to fibroblasts and keratinocytes, allowing us to identify the genes whose expression is extremely distinct, more than 100-fold, as compared to differentiated cells (Figure 2). It might suggest a special role of these genes in the regulation of pluripotent amnion cell stemness.

Genes for adhesion proteins, and markers of proliferation and differentiation

In hACs, we observed elevated expression of the genes for adhesion proteins, as compared to differentiated cells, characterizing either pluripotent cells or indicating a potential for differentiation towards cells representing germinal layers. Among 27 genes from that group, 16 exhibited over 3-fold higher expression as compared to fibroblasts, and 17 as compared to keratinocytes. It may suggest their significant role in the maintenance of the phenotype of poorly differentiated cell (Tables II and III). Higher expression of catenin B1, ICAM1 and KAL1 was observed in hACs as compared to fibroblasts, but unchanged or decreased as compared to keratinocytes. In hACs, the expression of only 4 genes was over 3-fold lower than in fibroblasts, and of 5 genes was lower than in keratinocytes. In turn, it suggests a potential role of these genes in cell differentiation towards mesenchymal and ectodermal lineage, respectively. Lower expression of markers for neurogenesis (NCAM1 and tenascin) distinguishes fibroblasts from keratinocytes, in which it remains at the level comparable with hACs.

We observed higher levels of CLEC3B, catenin D1, HAS1 PECAM1, selectin E, L and P, SPARC, SPG7, SPP1, thrombospondin THBS1 and THBS3, as well as the VCAM1 gene expression in hACs as compared to differentiated cells. In hACs, some of the proteins involved in the adhesion and differentiation (CLEC3B, SELL, SPP1 and VCAM1) exhibited over 100-fold higher expression as compared to differentiated cells.

Genes for ECM structural components

The expression of genes for insoluble components of the basement membrane/ECM, especially laminins (LAMA3, LAMB3), collagens (COLL11A1, COL7A1), and cadherin (CDH1), as well as integrins (ITGAM, ITGB2 and ITGB4), exceeded many times their expression in fibroblasts, much more than in keratinocytes (Table IV).

Laminins. The studied subunits of laminins exhibited elevated expression in amnion cells, as compared to differentiated cells, with the exception of LAMA1 whose expression remained unchanged. Particularly high up-regulation of LAMA3 and LAMB3 expression (over 5700× and 600×, respectively) was observed in amnion cells as compared to fibroblasts. Significantly smaller difference in the expression of these subunits was observed among hACs and keratinocytes.

Collagens. Differences in collagen expression between hACs and keratinocytes were more distinct than in relation to fibroblasts. An up-regulation in the expression of most collagens, with the exception of only one collagen, was observed. A particularly high up-regulation was noted for COL11A1 and COL7A1 as compared to fibroblasts (almost 900× and 160×, respectively), and for COL15A1 (125×) as compared to keratinocytes.

Other structural proteins. The expression of genes for fibronectin, e-cadherin and vitronectin was elevated in hACs as compared to differentiated cells. Extremely high difference in the expression between amnion cells and fibroblasts was noted for the CDH1 gene as compared to fibroblasts, and vitronectin (VTN) as compared to both differentiated cell types.

Table II. Expression of genes of adhesive molecules in amniotic cells in relation to differentiated cells. Genes encoding Selectin L, thrombospondin 3 and VCAM1 are strongly up-regulated in amniotic cells (shaded boxes). Thrombospondines and tenascin-C has been found to have both adhesive and anti-adhesive properties for cells. A, This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample. ESC, embryonic stem cells; HA, hyaluronic acid.

Gene	Full name	Function	Up/down-regulation of genes in amniotic cells by at least three-fold	
			vs. fibroblasts	vs. keratinocytes
CD44	CD44 molecule (Indian blood group)	Cell migration, receptor for HA	**-17,70[A]**	**-18,85[A]**
CNTN1	Contactin 1	Formation of axon connections	-1,74	**-10,90[A]**
CTGF	Connective tissue growth factor	Related to platelet-derived growth factor, cell proliferation and differentiation	-1,01	**4,47**
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	Regulation of cadherins' adhesion properties, cell differentiation	1,91	1,18
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	Cell-cell interactions, component of adhesion junctions, signal transduction	**6,00**	1,87
CTNND1	Catenin (cadherin-associated protein), delta 1	Cell-to-cell adhesion, signal transduction	**36,61**	**15,96**
ICAM1	Intercellular adhesion molecule 1	Typically expressed on endothelial and immune cells	**3,67[A]**	**-3,80[A]**
NCAM1	Neural cell adhesion molecule 1	Cell-to-cell and cell-ECM interactions during cell differentiation.	**-15,38[A]**	1,08[A]
PECAM1	Platelet/endothelial cell adhesion molecule	Leukocyte migration, angiogenesis, integrin activation	**74,23**	**43,31**
SELE	Selectin E	Cell proliferation and migration	**34,15**	**11,16**
SELL	Selectin L	Mostly expressed on endothelial cells, recruitment of immune cells	**136,62**	**122,22**
SELP	Selectin P (granule membrane protein 140kDa, antigen CD62)	Induction of hematopoiesis, expressed mostly on endothelial cells	**63,00**	**13,99**
SGCE	Sarcoglycan, epsilon	Linking the actin cytoskeleton to the ECM; marker of differentiation in ESC	1,67	**8,28**
THBS1	Thrombospondin 1	Cell-to-cell and cell-ECM interactions	**4,40**	**26,54**
THBS2	Thrombospondin 2		**-11,82**	**-6,65**
THBS3	Thrombospondin 3		**99,32[A]**	**116,43[A]**
TNC	Tenascin C	Differentiation of hematoendothelial cells, organogenesis	**-10,72**	1,67
VCAM1	Vascular cell adhesion molecule 1	Cell-cell adhesion adhesion, miogenesis	**240,07**	**227,54**
VCAN	Versican	Proliferation, migration and angiogenesis, tissue morphogenesis	-1,28	**3,31**

Genes for integrins

In hACs, we observed a higher expression of most investigated genes for integrin subunits, particularly ITGAM and ITGB2, as compared to differentiated cells (Table V). ITGB4 exhibited extremely high expression (6400×) as compared to fibroblasts. Expression of ITGA4 was lower in hACs than in fibroblasts and keratinocytes, whereas the expression of ITGA5 and ITGB1 was comparable among these cells.

Genes for metalloproteinases, MMP inhibitors, and ADAMST proteases

Very strong down-regulation of metalloproteinase MMP1 and MMP3 genes, and lower expression of MMP2, MMP10 and MMP12 genes, as compared to fibroblasts, was noted in the amnion cells. In turn, the expression of MMP8, MMP9, MMP11, MMP13 and MMP15, and TIMP inhibitor genes, as well as ADAMST protease genes, was several times higher in hACs than in differentiated cells of both types (Table VI).

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Table III. Expression of genes of ECM-associated proteins in amniotic cells in relation to differentiated cells. Genes CLEC3B and SPP1 are strongly up-regulated in amniotic cells (shaded boxes). A, This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample. HA, hyaluronic acid.

Gene	Full name	Function	Up/down-regulation of genes in amniotic cells by at least three-fold	
			vs. fibroblasts	vs. keratinocytes
CLEC3B	C-type lectin domain family 3, member B	Tissue remodeling	**679,03[A]**	**3524,82[A]**
ECM1	Extracellular matrix protein 1	Not fully elucidated role in skin differentiation, endochondral bone formation and angiogenesis	-1,97	**3,31**
HAS1	Hyaluronan synthase 1	Synthesis of proangiogenic HA	**31,43**	**11,74**
KAL1	Kallmann syndrome 1 sequence	Cell migration	**23,27**	**-3,41[A]**
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	Counteradhesion and antiproliferation, antiangiogenic properties	**10,49**	**31,78**
SPG7	Spastic paraplegia 7 (pure and complicated autosomal recessive)	Mitochondrial metalloprotease; marker of neurogenesis	**10,51**	**6,70**
SPP1	Secreted phosphoprotein 1	Marker of pluripotency, hyaluronan binding	**297,62**	**107,39**
TGFBI	Transforming growth factor, beta-induced, 68kDa	Regulation of cell proliferation, differentiation and migration	-2,47	-1,11

Discussion

In our study, we determined the expression of 84 genes which are important for creation of an extracellular microenvironment in the hAC population containing 86% SSEA-4$^+$ cells. We compared that expression between hACs and differentiated cells representing two germ layers: ectoderm and mesenchyme. Among the tested gene groups, there were genes responsible for the synthesis of ECM proteins and their receptors, metalloproteinases and their inhibitors, cell pro- and anti-adhesion factors, as well as markers for proliferation and differentiation. Expression of these genes characterizes the potential of amniotic cells in terms of proliferation capacity, ability to differentiate into the cells of germ layers, and secretion of the components of their microenvironment. These factors can potentially influence the amniotic cell phenotype in an autocrine way, e.g. through interactions with integrin receptors. In this way, hACs would be able to regulate synthesis and degradation of ECM components, as well as cell adhesion, themselves.

We confirmed our earlier observations [13], and the results of other authors [14, 15], that cells expressing a marker of pluripotency - SSEA-4, are the most numerous among hAC. Previously, we also found that other classical markers of pluripotency (SSEA-3, TRA-1-60 and TRA-1-81) are expressed only on several percent of cells. Pluripotent and epithelial nature of the isolated hACs was reflected by a very high expression of the E-cadherin gene (CDH1), as compared to fibroblasts [16]. E-cadherin has a double role, taking part in epithelialization and pluripotency maintenance, which has been confirmed in various studies on hESCs [17]. TGFβ is an important factor regulating cell pluripotency. Inhibition of TGFβ signaling pathway in hESCs leads to differentiation towards muscle cells [18].

In our study, TGFβ expression in the primary hAC culture did not differ from its expression in differentiated cells. However, it is known that an epithelial-mesenchymal transition due to autocrine secretion of TGFβ takes place during culture of the hAEC. Activation of TGFβ-dependent signaling pathways also inhibits cell proliferation [19]. As a consequence, an increase in MMP9 and a decrease in CDH1 expression level was observed [20].

High expression of the following markers: ectodermal neuronal cell (SPG7), mesenchymal muscle cell (CLEC3B), or endodermal hepatocyte (SGCE), might be a reflection of cell population heterogeneity and/or their great differentiation potential. Expression of CLEC3B, a gene for tetranectin, is a marker of miogenesis [21]. High expression of SPG7 in the cultured mesenchymal stem cells isolated from umbilical blood was a sign of their differentiation towards nerve cells [22]. The SGCE protein is a common but late hepatocyte (endoderm), as well as a marker of osteogenic cell (mesenchyme) differentiation. Its expression occurs also on neuronal stem cells (ectoderm) and is maintained in dopaminergic neurons [23]. The SGCE gene expression in hACs, which is comparable to fibroblasts but several times higher than in keratinocytes, suggests some similarity of hACs to mesenchymal cells. Also, elevated expression of the hyaluronan synthase (HAS1) gene may reflect a potential for mesenchymal differentiation, for example towards blood [24], and cardiac cells [25]. These results seem to be especially interesting in the context of the previous studies, in which the absence of hyaluronan in amnion epithelium and its particularly high concentration in sub-endothelial layers increasing with gestational age was determined [26].

Table IV. Expression of genes for structural proteins being potential ligands for integrin receptors in amniotic cells in relation to differentiated cells. Genes for all investigated laminin subunits, excluding LAMA1, most of collagens and fibronectin are up-regulated in amniotic cells as compared to differentiated cells by at least three-fold (bold letters). Strong up-regulation of genes (>100x) is shown as shaded boxes. A, This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample.

Gene	Full name	Up/down-regulation of genes in amniotic cells	
		vs. fibroblasts	vs. keratinocytes
COL1A1	Collagen I, α1	**3,81**	**20,87**
COL4A2	Collagen IV, α2	**24,94**	**5,39**
COL5A1	Collagen V, α1	**6,61**	**31,49**
COL6A1	Collagen VI, α1	-1,50	**3,15**
COL6A2	Collagen VI, α2	-2,22	**3,74**
COL7A1	Collagen VII, α1	**158,02**	**78,79**
COL8A1	Collagen VIII, α1	**37,63**	**39,76**
COL11A1	Collagen XI, α1	**895,99[A]**	**9,87**
COL12A1	Collagen XII, α1	**3,05**	**12,70**
COL14A1	Collagen XIV, α1	**-7,50[A]**	**33,28**
COL15A1	Collagen XV, α1	2,70[A]	**125,08**
COL16A1	Collagen XVI, α1	1,17	**3,92**
LAMA1	Laminin α1	-1,18[A]	-1,29[A]
LAMA2	Laminin α2	**10,91**	**25,16**
LAMA3	Laminin α3	**5728,73[A]**	**7,03**
LAMB1	Laminin β1	**5,00**	**5,13**
LAMB3	Laminin β3	**602,16**	**5,72**
LAMC1	Laminin γ1	**8,17**	**6,87**
CDH1	Cadherin 1 (E-cadherin)	**22599,45**	1,99
FN1	Fibronectin 1	**11,99**	**23,64**
VTN	Vitronectin	**247,97**	**220,81**

Furthermore, in hACs, a high expression of adhesion protein VCAM-1, being also a marker of hESCs differentiation into cardio-vascular system cells [27, 28], should be noted. This expression, which may be a feature of epithelial cells, exceeded the expression assayed in differentiated cells over 200-fold. ICAM-1 (Intercellular Adhesion Molecule-1) is another adhesion molecule important for intercellular interactions, especially among endothelial cells [29]. Endothelial progenitor cells do not exhibit ICAM expression [30]. In *in vitro* studies on mesenchymal amnion cells, very low ICAM-1 expression and lack of VCAM-1 expression after 2nd passage were shown [31]. Our results indicate that ICAM-1 expression is only 3.7-fold higher in hACs as compared to fibroblasts, and 3.8-fold lower as compared to keratinocytes. It is also known that the VCAM-1 protein is an endothelial ligand for integrin α4β1. VCAM-1 interacts with ITGA4 (α4), what in mice results in interactions between the myocardium and the epicardium during the development of the heart [27]. In the cells isolated from the amnion, the level of this integrin subunit expression was significantly lower than in fibroblasts and keratinocytes, what may indicate a limited activity of this signal pathway.

Generally, it is believed, that hAM-MSCs show angiogenic properties but resist differentiation into mature endothelial cells, whereas hAECs are characterized by high anti-angiogenic potential [32, 33]. hAECs secret a number of compounds which are inhibitors of angiogenesis. These are, among others: trombospondin 1 (THBS1), endostatin, and tissue metalloproteinase inhibitors (TIMP-1,-2,-3, and -4) [34]. Our isolated population of amnion cells was characterized by various properties of production of pro- and anti-angiogenic factors. High expression of PECAM-1 gene, higher in hACs than in both types of differentiated cells, might confirm their angiogenic potential. The CD31 protein, a product of the expression of this gene, is a marker used in histopathology for the evaluation of angiogenesis in developing tumors [35, 36]. On the other hand, we found lower expression of anti-adhesive tenascin-C and higher expression of the SPARC gene. This glycoprotein acts as a factor of counter-adhesion and anti-proliferation. It supports the thesis on the complexity of relationships between pro- and anti-adhesion factors, which usually have many other functions in cells [37, 38, 39].

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Table V. Expression of genes of integrins in amniotic cells in relation to differentiated cells. Genes encoding ITGAM, ITGB2 and ITGB4 are strongly up-regulated in amniotic cells relative to fibroblasts. Most of other genes for integrins are up-regulated in hAEC as compared to differentiated cells by at least three-fold. A, This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample.

Gene	Full name	Up/down-regulation of genes in amniotic cells	
		vs. fibroblasts	vs. keratinocytes
ITGA1	Integrin, α1	**-4,29[A]**	-1,60[A]
ITGA2	Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor)	**16,04**	**3,02**
ITGA3	Integrin, α3 (antigen CD49C, α3 subunit of VLA-3 receptor)	**3,23**	**-3,85**
ITGA4	Integrin, α4 (antigen CD49D, α4 subunit of VLA-4 receptor)	**-24,24[A]**	**-10,27[A]**
ITGA5	Integrin, α5 (fibronectin receptor, α polypeptide)	-1,47	-1,49
ITGA6	Integrin, α6	**15,21[A]**	-1,21[A]
ITGA7	Integrin, α7	**15,53**	**57,81**
ITGA8	Integrin, α8	**-3,13[A]**	**24,31**
ITGAL	Integrin, αL (antigen CD11A (p180), lymphocyte function-associated antigen 1; α polypeptide)	**44,14**	**22,84**
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	**152,99**	**16,72**
ITGAV	Integrin, αV; (vitronectin receptor, α polypeptide, antigen CD51)	**12,85**	**7,53**
ITGB1	Integrin, β1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)	-1,47	-1,44
ITGB2	Integrin, β2 (complement component 3 receptor 3 and 4 subunit)	**147,78**	**19,84[A]**
ITGB3	Integrin, β3 (platelet glycoprotein IIIa, antigen CD61)	**4,02[A]**	**4,86[A]**
ITGB4	Integrin, β4	**6400,64[A]**	**6,60**
ITGB5	Integrin, β5	**5,65**	**19,93**

Structural insoluble ECM proteins play a particular role in the shaping of the hAC phenotype. These proteins (i.e. laminins, collagens, fibronectin and vitronectin) are localized mainly in the basement membrane. They are ligands for integrins, heterodimeric receptors initiating signal transduction inside the cell, thus regulating cell adhesion, migration and proliferation, as well as pluripotency or differentiation of stem cells [40]. Laminins bind integrins directly on the cell surface. The laminin α1 and α4 subunits were not detected in human amniotic tissue [41]. It has been proven that in hESCs, laminin α1 cooperates with integrin receptor α6β1, what impacts epithelial-mesenchymal transduction [42]. It can be supposed that low expression of this subunit in cells isolated from a human amnion is associated with supporting their epithelial character. One of the laminins which maintains pluripotency state and induces proliferation is laminin 332. Our analysis showed elevated α3 and β3 subunits gene expression in hACs as compared to fibroblasts, and α3 gene expression as compared to keratinocytes. These results might confirm the importance of laminin 332 in maintaining the phenotype of undifferentiated cells. However, the expression of the γ2 subunit was not determined in our study.

Particular collagen types exhibit specific localization in the amniotic membrane. Basement membrane of hAECs contains collagen type III, IV, V, VII and XVII. Deeper layers of the amniotic membrane contain collagen type I, III, IV, V and VI in various configurations [43]. We have proven that hACs are able to synthetize also other collagens, such as type VIII, XI, XII, XIV, XV and XVI. In most cases, gene expression for these collagens exceeds its level found in differentiated cells several times. Especially high expression (>100-fold) was noted in the cases of COL11A1 and COL7A1 (comparing to fibroblasts), and COL15A1 (comparing to keratinocytes). Four out of 12 collagen genes, namely COL6A1, COL6A2, COL15A1 and COL16A1, exhibited similar expression level in hACs and fibroblasts. The significance of an elevated expression of various collagen types for the characteristics of amniotic cells consists in the fact that,

Table VI. Expression of genes of metalloproteinases (MMP), ADAM metallopeptidase with thrombospondin type 1 motif (ADAMT) and MMP inhibitors (TIMP) in amniotic cells in relation to differentiated cells. Genes encoding MMP1 and MMP3 are strongly down-regulated in amniotic cells relative to fibroblasts. Some other MMP are down- or up-regulated by at least three-fold (bold letters), and all ADAMT and TIMP are up-regulated in hAEC as compared to differentiated cells. A, This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample; B, This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05). This fold-change result may also have greater variations; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

Genes	Full name	Up/down-regulation of genes in amniotic cells by	
		vs. fibroblasts	vs. keratinocytes
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	**-1941,11[A]**	**-8,04[A]**
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	**-7,55**	2,85
MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	**-7328,66[A]**	**-12,91[A]**
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	**4,21**	**-3,31[A]**
MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	**10,27**	**30,77**
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	**4,70[A]**	**5,75[A]**
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	**-4,92[A]**	-2,10[A]
MMP11	Matrix metallopeptidase 11 (stromelysin 3)	**3,42[A]**	**4,00[A]**
MMP12	Matrix metallopeptidase 12 (macrophage elastase)	**-20,53[A]**	2,45[B]
MMP13	Matrix metallopeptidase 13 (collagenase 3)	**18,37[B]**	**23,62[B]**
MMP14	Matrix metallopeptidase 14 (membrane-inserted)	1,10[A]	**3,22[A]**
MMP15	Matrix metallopeptidase 15 (membrane-inserted)	**72,87**	**12,79**
MMP16	Matrix metallopeptidase 16 (membrane-inserted)	-2,22[A]	**6,28[A]**
TIMP1	TIMP metallopeptidase inhibitor 1	**7,13**	**44,53**
TIMP2	TIMP metallopeptidase inhibitor 2	**5,93**	**44,22**
TIMP3	TIMP metallopeptidase inhibitor 3	**51,53**	**27,22**
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	**3,41**	2,96
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	**29,94**	**27,16**
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	**25,35**	**25,93**

except for their structural role in amnion ECM, they play a role in maintaining hESC pluripotency [6], differentiation towards hematopoietic progenitor cells [44], or corneal epithelial cells [45].

Integrins, which are cellular receptors for ECM components, show a wide substrate specificity depending on subunit combination. Integrins α4β1, α4β7, α5β1, αVβ6, αVβ3 and αIIβ3 are receptors for fibronectin [46], which on one hand acts as a trophic factor for hESC-derived cardiomyocytes [47] and chondrocytes [48], and on the other hand is able to support the pluripotency state of hESCs *in vitro* [6]. Migration activity of stem cells is the result of, among others, a cooperation between ligands and α5 and αV integrin subunits. Nevertheless, in our study, the first one exhibited an expression level comparable with differentiated cells. In contrast, αV subunit was expressed at a several-fold higher level. It suggests an important role of αV-dependent signaling in hACs, all the more that β5 subunit expression was also elevated. Integrin αVβ5 is a receptor for another strongly expressed glycoprotein – vitronectin, which has the ability to maintain an undifferentiated state of hESCs [49]. E-cadherin shows similar properties [50], and in our study it presented an extremely high expression as compared to fibroblasts.

Structural components of ECM and ECM-deposited soluble factors such as growth factors, cytokines and chemokines are metabolized by matrix metalloproteinases (MMP), disintegrin and metalloproteinases (ADAM), and ADAM with thrombospondin motifs (ADAMTS), which are members of the metzincin superfamily. Besides the low level of MMP-1 and MMP-3 metalloproteinases expression specific for fibroblasts and keratinocytes *in vitro* [51], hACs exhibited high expression for MMP-8, MMP-9, MMP-11, MMP-13 and MMP-15. Particular attention should be paid to MMP-9 (collagenase IV), which during labor contributes to ECM degradation of the fetal membranes, thereby facilitating their rupture and detachment of the placenta from the maternal uterus. Its precursor form (proMMP-9) is activated in amnion cells by autocrine or paracrine action of TNFα and IL-1β [52]. MMP function in various pathological

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conditions of both, neoplastic and non-neoplastic diseases by digesting different substrates under control of tissue inhibitors of metalloproteinases (TIMP). We noted notably higher expression of TIMP genes in hACs as compared to differentiated cells, what may result in low tumorigenicity of amnion cells [5].

ADAMTS proteins, in addition to proteolytic properties, confer cell adhesion properties. The function of ADAMTS1 and ADAMTS8 proteases is to modify proteoglycans and hyaluronan, while ADAMTS13 is responsible for inhibition of the activity of the von Willebrand factor (VWF) [53]. An increase in the expression of ADAMTS1 during ESC differentiation has been shown [54]. Small differences in ADAMTS1 expression between hACs and differentiated cells may indicate a low differentiation ability of hACs and their activity in proteoglycan degradation similar to mature cells. ADAMTS8 seems to play a more important role in the rearrangement of amnion ECM. Its prominent expression in hACs, as compared to differentiated cells, might be one of the mechanisms protecting amnion cells from malignant transformation, since ADAMTS8 is a suppressor gene [55]. A product of expression of this gene acts as an antagonist for EGFR-MEK-ERK signal pathway, and exhibits lytic action on actin fibers preventing metastasis.

Conclusions

The results of our study indicate that hACs, comprising 86% of the SSEA-4$^+$ cells, exhibit the features of epithelial ectodermal cells more than mesenchymal fibroblasts. On the other hand, they may actively participate in the production of insoluble components of the basement membrane and ECM, as well as integrin subunits responsible for signal transduction from ECM to the cells. Expression of some genes, insoluble components of the basement membrane/ECM, i.e. laminins (LAMA3, LAMB3), collagens (COLL11A1, COL7A1) and cadherin (CDH1), as well as integrins (ITGAM, ITGB2, ITGB4), exceeds their expression in fibroblasts several fold, significantly more than in keratinocytes. Some adhesion proteins and factors participating in cell differentiation (CLEC3B, SELL, SPP1 and VCAM1) exhibit distinctly higher expression in hACs as compared to both, fibroblasts and keratinocytes. The expression of the above mentioned genes seems to be particularly important for hACs to maintain their own properties as low-differentiated cells. Extremely high expression of some genes, and also elevated expression of many other ECM-associated genes, might be the decisive factor in the composition of amniotic fluid/basement membrane/ECM. Indirectly, this expression might influence the capability of autoregulatory mechanism of ECM-stem cells relationships, influencing stem cell phenotype and potential ability of these cells to differentiate into various germinal layers. Control of ECM-associated gene expression might also be crucial for the reconstitution of the natural microenvironment for amnion cells *in vitro* and for the management of stem cell phenotype both, in culture and clinical applications *in vivo*.

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