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Investigating the expression of pluripotency-related genes in human amniotic fluid cells: A semi-quantitative comparison between different subpopulations, from primary to cultured amniocytes

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ABSTRACT

Various classifications have been proposed for human amniotic subpopulations, including classification of spindle-shaped (SS) and round-shaped (RS) cells, as well as the more referred triple-category of epithelioid (E-type) cells, amniotic fluid-specific (AF-type) cells and fibroblastoid (F-type) cells. The present study aims to investigate these amniotic subpopulations regarding the expression of some stem cell markers, including *OCT4*, *NANOG*, *SOX2*, *C-KIT* (*CD117*), *C-MYC*, *KLF4*, and *THY1* (*CD90*). Flow cytometry was performed to characterize the isolated clonal subpopulations for a hematopoietic and a mesenchymal marker using PE-CD31 and FITC-CD90, respectively. A semi-quantitative RT-PCR analysis was carried out on the isolates in the second half of their lifespan when the cells were at the stationary phase of the growth curve. Characterization of isolated cells demonstrated that all clones including both epithelioid and fibroblastoid cells, had mesenchymal, not hematopoietic, lineage. RT-PCR analysis also revealed a higher expression of the target genes in epithelioid cells. Furthermore, the expression pattern of the genes and their correlations were remarkably different between primary- and long term-cultured amniocytes. Taken together, our results showed that the primary-cultured cells express the stemness genes equally, whereas the long term-cultured amniocytes exhibited a highly variable manner in the expression pattern of the genes. Diverse derivation site of amniocytes and individual genetic background can potentially explain the observed variation in the expression level of the target genes. These can also explain why amniocytes differ in many respects observed in our study, including survival rate, plastic adhesion, and growth characteristics.

1. Introduction

Amniotic fluid contains a heterogeneous population of amniocytes, from totally differentiated and lineage-committed progenitor cells to pluripotent and highly multipotent stem cells [1,2], and even some maternal cells [3]. Depending on gestational age, amniocytes have various morphologies, activities, and features. So far amniocytes have been differently categorized based on morphology and growth characteristics. Some authors have grouped these cells into adherent and non-adherent cells using conventional culture methods [4], while others have further classified adherent amniocytes into two or three main groups, including classification of spindle-shaped (SS) and round-shaped (RS) cells [5], as well as the triple-category of epithelioid (E-type), fibroblastoid (F-type) and amniotic fluid-specific (AF-type) cells [6,7]. Many studies have revealed that amniotic fluid stem cells (AFSCs) are a special population of amniocytes with unique characteristics. AFSCs have numerous features making them distinctive from other sources of stem cells, including pluripotent cells such as embryonic stem cells (ESCs), multipotent cells like adult stem cells (bone marrow- or adipose tissue-derived), and even those derived from extra-embryonic tissues [8–16]. Therefore, many authors have suggested an intermediate state for AFSCs, meaning that their cellular

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phenotype is between ESCs and mesenchymal stem cells (MSCs). On the other hand, AFSCs express both markers and transcription regulators of pluripotency and mesenchymal commitment [6,14,15]. Moreover, these cells exhibit higher proliferative and clonogenic potentials compared with other fetal stem cells, including neonatal bone marrow and preterm umbilical cord blood [17]. Unlike MSCs which have a higher tendency for mesodermal differentiation, AFSCs are able to differentiate into multiple lineages including endodermal (epithelial lung lineages) [18], mesodermal (chondrocytes) [19] and ectodermal (neural precursors) cells [20].

For the first time in 1990s, two studies by Torricelli et al. [21] (who reported hematopoietic progenitors in amniotic fluid) and Streubel et al. [22] (who proved the presence of non-hematopoietic precursors in amniotic fluid) disclosed that the amniotic fluid contains cells with proliferation and differentiation potentials. However, the conclusive proof of existing stem cells in amniotic fluid was provided by Prusa et al. when they succeeded to isolate OCT4-positive stem cells from human amniotic fluid [23]. OCT4 is a transcription factor expressed by human embryonic stem cells (hESCs) and embryonal carcinoma cells as a critical factor in maintenance of self-renewal and differentiation potential [24]. In addition to OCT4, there are some other renowned markers for stemness which are expressed in AFSCs. For example, in 2007, a subpopulation of AFSCs expressing C-KIT (or CD117) was isolated by an immunoselection approach using magnetic microspheres. These cells are characterized by the expression of the stem cell factor (SCF) receptor, i.e. C-KIT, a surface antigen of the type III tyrosine kinase [25]. It has been found that C-KIT$^+$ AFSCs can be readily expanded in culture as stable lines and would be able to differentiate into the cells representative of all three germ layers [6]. In this regard, Moschidou and colleagues revealed that C-KIT$^+$ AFSCs have 82% transcriptome identity with ESCs, in addition to capability of forming embryoid bodies *in vitro* [26]. They also displayed expression of pluripotency markers in these cells, including OCT4, NANOG, SOX2, C-MYC and KFL4 [26]. Nonetheless, they cannot be rigorously considered as pluripotent stem cells, because of lacking the pluripotency's distinguishing features, i.e. tumorigenesis and generating germline-competent chimeras *in vivo* [6].

In this study, we isolated 14 clones from human amniotic fluid which are classified according to morphology into three main categories. The present paper aims to make a comparison between isolated cell lines based on their morphology and expression of some well-known stem cell markers, including *OCT4*, *NANOG*, *SOX2*, *C-KIT* (*CD117*), *KLF4*, *C-MYC* and *THY1* (*CD90*). We also investigated the expression of stem cell markers in 5 primary-cultured samples when amniocytes first appeared in the vessels without going through subculturing process.

2. Materials and methods

2.1. Culture of human amniotic fluid cells

Amniotic fluid samples used in this study were taken from pregnant women with their informed consent according to the guidelines of Yazd Reproductive Sciences Institute. These samples were donated by Genetics laboratory, PND (prenatal diagnosis) section, where fetuses are routinely screened for genetic abnormalities. Due to the risk of miscarriage (approximately 0.25%- 0.50%) and ethical issues surrounding amniocentesis, it is only authorized to use this procedure for women who are candidates because of high risk pregnancy. Indeed, genetically normal samples were used in the study. At the beginning, a total of 16 mL human amniotic fluid was collected by ultrasound in the process of amniocentesis from fetuses at the second trimester, ranging from 16th to 20th weeks of pregnancy. The obtained amniotic fluid was centrifuged at 400 *g* for 15 min, supernatant was discarded and the cell pellets were seeded in two glass Leighton tubes containing AmnioMAX II complete medium supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid), 1% penicillin/streptomycin or PenStrep (all from Gibco) and incubated at 37 °C in a dry incubator without CO_2. If one of the Leighton tubes was sufficient to conclude the PND test, the other one would have been used for investigating stem cell markers in the primary culture (5 samples).

Between days 8–11 of seeding, when the cell clusters could be observed by inverted microscope, non-adherent cells and cell debris were discarded, and replaced with fresh medium. Following passages 1–3, when diagnostic procedure had been accomplished, the amniocytes were used for research purposes. At this time, cells were detached from Leighton tubes by means of PBS (phosphate-buffered saline) and 0.25% trypsin-EDTA and cultured in T25 flasks using a modified medium composed of 2:1 (v/v) DMEM:AmnioMAX II. DMEM was supplemented with 4 mM L-glutamine, 10 mM HEPES, 15% FBS (fetal bovine serum) and 1% PenStrep (all from Gibco). The flasks were incubated at 37 °C under 5% humidified CO_2.

2.2. Flow cytometry analysis

One of the most common surface markers of mesenchymal stem cells, CD90, was used to characterize the cell subpopulations phenotypically. Expression of the hematopoietic marker, CD31, was also analyzed as a negative marker for amniocytes to evaluate cellular heterogeneity. For this purpose, at least 1×10^5 cells for each assay were collected by centrifugation at the same time when cells were investigated for RT-PCR analysis. The cell pellet was washed twice in PBS containing 0.2% FBS. Cells were then incubated with the following antibodies in a solution containing PBS supplemented with 1% bovine serum albumin (BSA). The incubation was performed with phycoerythrin (PE)-conjugated anti-human CD31 (from Immunostep) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 (from Exbio). Expressions were analyzed on a BD FACSCalibur and the graphics were generated in FlowJo (v 10.1, Tree Star, Inc.) software.

2.3. RT-PCR

Determining when RT-PCR analysis will be performed depends on the doubling time (DT) of the cells during the growth curve. That means when the cells proliferate stably, the growth curve is in the stationary phase and there is a higher possibility of having homogeneous population in culture. The clones were semi-quantitatively analyzed by RT-PCR for a seven-gene panel of stem cell markers including *OCT4*, *NANOG*, *SOX2*, *C-KIT* (*CD117*), *C-MYC*, *KLF4* and *THY1* (*CD90*). To do so, total RNA was isolated from three morphologically homogenous cultures representing each type of amniocytes using the Qiagen RNeasy™ Mini Kit according to the manufacturer's instructions. Then 50 ng of extracted RNAs were used for reverse transcription process in which cDNA synthesis was performed by means of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's manual. Finally, cDNA samples were investigated doubly by thermocycler in 35 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 1 min, besides an initial denaturation step at 95 °C for 2 min and final extension step of 72 °C for 7 min. Human specific primers were designed for intron spanning regions, except for *SOX2* because of its intron-less structure. All primers were already tested by using the cDNA samples of human ESCs and human adipose tissue-derived MSCs. Table 1 lists primer sequences and the size of amplified products. Primers used to amplify *SOX2*, *NANOG*, *KLF4* and *C-MYC* were taken from Zhao et al. paper [27].

2.4. Semi-quantitation of RT-PCR products and statistical analysis

As cDNA samples were equally normalized (50 ng), the mean intensity value of double bands on gel electrophoresis could be used to compare the expression levels of targets in different samples. The semi-quantitative measurement of PCR products was performed using the

Table 1
List of human-specific primer sequences used in RT-PCR.

Genes	Primer sequence	Product size
OCT4	Forward: GATGTGGTCCGAGTGTGGTT Reverse: AGAGTGGTGACGGAGACAGG	245 bp
NANOG	Forward: TTTGGAAGCTGCTGGGGAAG Reverse: GATGGGAGGAGGGGAGAGGA	194 bp
SOX2	Forward: GCCGAGTGGAAACTTTTGTCG Reverse: GCAGCGTGTACTTATCCTTCTT	154 bp
KLF4	Forward: GCGCTGCTCCCATCTTTCT Reverse: GGGGGAAGTCGCTTCATGT	125 bp
C-MYC	Forward: TGGTCGCCCTCCTATGTTG Reverse: CCGGGTCGCAGATGAAACTC	151 bp
C-KIT	Forward: CCAACACCGGCAAATACACG Reverse: TTGATCATGATGCCCGCCTT	250 bp
THY1	Forward: TCAGCATCGCTCTCCTGCTA Reverse: TGCTGGTATTCTCATGGCGG	120 bp
18S rRNA	Forward: AGAAACGGCTACCACATCCA Reverse: CCCTCCAATGGATCCTCGTT	150 bp

digital image analysis software ImageJ [28]. For this purpose, agarose gel electrophoresis image of bands representative of each PCR amplicon were compared to the corresponding band of 18S rRNA in the same sample as calibrator. The *t*-test analysis was used to make a comparison between expression levels of genes in epithelioid and fibroblastoid isolates. Pearson's and Spearman's coefficients were also used to determine possible correlations between the levels of gene expressions and ranked variables regarding samples, such as fetal age at the time of amniocentesis, maternal age, type of isolated cells as well as their lifespan. The data about each sample are listed in Table 2.

3. Results

3.1. There are different subpopulations in primary- and long term-cultured human amniocytes

Based on previous studies, the adherent cells in amniotic fluid have three entirely distinct morphologies including epithelioid, amniotic fluid-specific, and fibroblastoid; which are called E-type, AF-type and F-type, respectively. Our findings have confirmed these typical cellular morphologies with clonogenic capability in primary-cultured human amniocytes (Fig. 1). As it is obvious in Fig. 2, there were three cellular groups in cultures, including epithelioid, short- and long-spindle fibroblastoid cells. Shape discrimination between these two fibroblastic cells requires years of observation and skill, but based on what we have found from precisely recorded data of 68 amniotic fluid samples, frequency and growth properties of individual cell types were significantly different among them (data not shown). Although here we call fibroblastic cells short- and long-spindle fibroblastoid cells, but based on frequency and growth properties they must be AF-type and F-type cells, respectively, as referred in the literature.

Follow-up of clonal cell lines showed that individual cell types preserve their morphologies through long term cultures. However, some E-type cell lines lose their typical morphology in a phenomenon named epithelial to mesenchymal transition or EMT. It is believed that maintenance of epithelial cells in culture may result in morphological change into a fibroblastic-like shape, though we have not used such lines for molecular analysis. Generally, E-type cells are large polygonal cells, with cuboidal to columnar morphology and appear in culture as cobblestone-shape clusters (Fig. 2a). E-type colonies have distinct boundaries with smooth margins that grow actively in marginal regions (Fig. 1a). AF-type cells, the most frequent amniocytes, are short-spindle fibroblastic cells without needle-shape projections (Fig. 2b) that establish colonies with nearly soft margins (Fig. 1b). F-type cells that are fibroblastic as well, but with long-spindle morphology and needle-shape projections (Fig. 2c), constitute colonies with ruffled, jagged margin and indistinct boundaries (Fig. 1c).

3.2. The primary-cultured human amniocytes uniformly express pluripotency markers

The first set of analyses examined the expression of key transcriptional factors associated with pluripotency in 5 samples of amniocytes at their primary cultures when they had not been gone through sequential sub-culturing. To obtain a better understanding of the stem cell state in amniocytes, we have focused on the ESC core regulatory circuit, i.e. OCT4, NANOG, and SOX2, and some other well-known transcription factors associated with stemness, including C-KIT (CD117), C-MYC, KLF4 and THY1 (CD90). Regarding RT-PCR analysis, nearly all investigated stem cell markers were positive in these primary-cultured amniocytes (Fig. 3). In this regard, the amounts of standard deviation (SD) demonstrated that *OCT4* (in fibroblastoid isolates) and *THY1*, have the lowest variability across investigated markers whereas *SOX2* and *NANOG* exhibited the highest SD, meaning that they are expressed in a variable manner in primary-cultured amniocytes. As depicted in the heatmap of Fig. 3, *KLF4* and *C-KIT* demonstrate remarkably higher expression compared with other genes examined. Given the fact that two cases (C-1 and C-2) exhibited a detectable trace of *SOX2*, the pluripotency regulatory circuit, i.e. *OCT4*, *NANOG* and *SOX2*, was positive in all the primary-cultured amniocytes. Interestingly, Pearson's correlation analysis showed a high degree of association between some of the target genes in these cells, so that Pearson's coefficient was greater than 0.9 or 90% in the correlated genes (Fig. 4). The results

Table 2
Summary of general information of 14 amniotic fluid samples used for isolation of cell lines in long-term culture. Each isolate is outlined as reception code plus number of passages they have been sub-cultured at the time of investigation.

	Amniotic isolates	Cell type		Lifespan (passages)	Maternal age (years)	Gestational age (weeks)	Karyotype
1	1011-P5	**Fibroblastoid**	**LS**	12	34	18 w	46,XX
2	1020-P6			10	25	17 w	46,XX
3	939-P18			23	36	16 w	46,XY
4	1024-P5		**SS**	6	23	16 w	46,XX
5	1027-P5			6	31	17 w	46,XX
6	1023-P5			9	35	17 w	46,XY
7	1016-P10			12	42	16 w	46,XY
8	1044-P3	**Epithelioid**		4	36	17 w	46,XX
9	1041-P3			4	21	17 w	46,XX
10	1043-P5			6	24	16 w	46,XY
11	1039-P3			4	39	17 w	46,XX
12	1046-P3			4	32	17 w	46,XY
13	1007-P7			9	29	20 w	46,XX
14	972-P7			8	40	20 w	46,XY

Abbreviations: LS: long spindle, SS: short spindle.

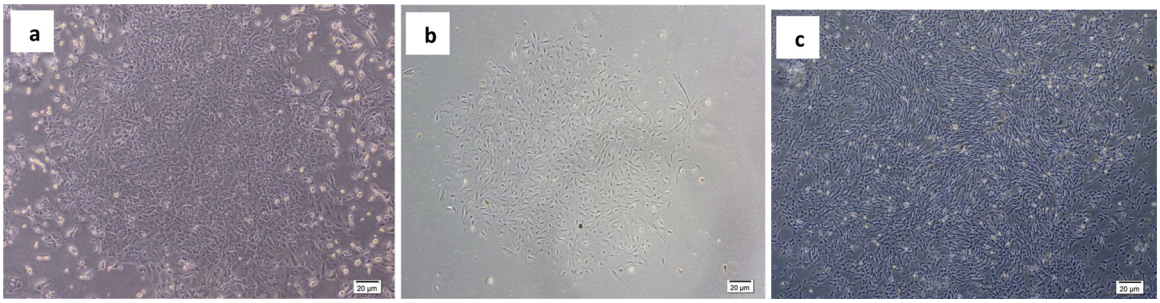

Fig. 1. Three kinds of amniotic clones with totally different morphologies in primary cultures: (a) epithelioid colonies (E-type) can grow from marginal regions; (b) an amniotic fluid colony (AF-type) with central proliferating pattern; (c) fibroblastoid colonies (F-type) show a universally growing pattern.

have shown that except for *NANOG* and *SOX2*, all other genes were highly correlated, including *OCT4*, *KLF4*, *C-MYC*, *C-KIT* and *THY1*.

3.3. Long term-cultured individual isolates exhibit substantial variability in expression of pluripotency markers

3.3.1. Growth characteristics and possible correlations with maternal and fetal ages

Based on previous studies, the adherent cells in amniotic fluid contain three clonogenic subpopulations with distinct morphologies (Fig. 1). Long term follow-up of the colonies in culture demonstrated that each subpopulation has individual characteristics. For example, in a larger study (data have not been shown here), we calculated the lifespan of amniocytes, i.e. the mean number of passages undergone by a cell line, across 68 amniotic fluid samples. Statistical analysis of data by *t*-test demonstrated significantly different lifespans among cell types, so that F-type cells had longer lifespan (on average 15 passages) compared with E-type cells (4.6 on average) and AF-type cells (6.6 on average) [29].

In addition, the results obtained in present study were examined for possible associations between gene expression and other qualitative information on specimens, such as fetal age at the time of amniocentesis, maternal age, type of isolated cells and their lifespan. Except for the last one, all the others are ranked variables, so Spearman's coefficient was used to measure the strength and direction of possible associations among them. As expected, Spearman's coefficient analysis indicated a correlation between the type of cells isolated and their lifespan (*p*-value: 0.007). However, no correlation was found between other qualitative variables, namely fetal and maternal age. Furthermore, the analysis showed that there is no correlation between the ranked variables with expression level of target genes.

3.3.2. Phenotypic analysis of surface antigens demonstrated mesenchymal, and not hematopoietic origination of subpopulations

Two well-known markers of hematopoietic and mesenchymal stem cells were used to characterize the isolated cells by flow cytometry, including CD31 and CD90, respectively. The results pertaining to the former revealed that all cell lines are negative for CD31 (more than 95% of cells in each line), confirming the non-hematopoietic origination of the cells (Fig. 5). In contrast to CD31, analysis of the latter resulted in completely different outcomes, that means regardless of cell type, a very high percentage of the cells (more than 90%) in each sample expressed the mesenchymal marker of CD90 on their surface (Fig. 5). For this reason, all isolated cells from individual samples could be considered as homogeneous cell lines. Moreover, depending on the plots depicted in Fig. 5, it seems that a higher percentage of the cells in both fibroblastoid subpopulations exhibit the enhanced levels of CD90 rather than epithelioid ones. Accordingly, we have considered all fibroblastoid isolates as a single subpopulation and made a comparison with epithelioid cells as another distinct subpopulation. The median fluorescent intensity or MFI was used to compare expression levels of CD90 between the two morphological groups. The *t*-test analysis of data in terms of MFI demonstrated that fibroblastoid isolates (LS and SS in total) expressed significantly (*p*-value: 0.0022) higher levels of CD90 (MFI: 84.50), compared to epithelioid cell lines (MFI: 50.30).

3.3.3. OCT4, NANOG and SOX2, hyper variably expressed genes in human amniocytes

RT-PCR analyses were carried out on 14 isolates (in two groups of 7 cell lines, epithelioid and fibroblastoid) at the second half of their lifespan. Our findings showed that the triple markers of pluripotency regulatory circuit, *OCT4*, *NANOG* and *SOX2*, were simultaneously expressed in half of 14 isolates, including 4 epithelioid lines and 3 fibroblastoid lines (1 long-spindle and 2 short-spindle). However, considering single genes showed that *OCT4* was positive in 10, *NANOG* in 11 and *SOX2* in 9 out of 14 isolates, though the last one was expressed at just detectable levels in most samples (Fig. 6, upper panel). Taken together, our results indicate that, regardless of the cell type, there is a substantial inter-patient variability in expression of the triple markers, from totally negative (sample 1020) to highly positive (sample 1046).

As depicted in the heatmap of Fig. 6, considering the cell lines individually demonstrated higher expression of the triple genes in the

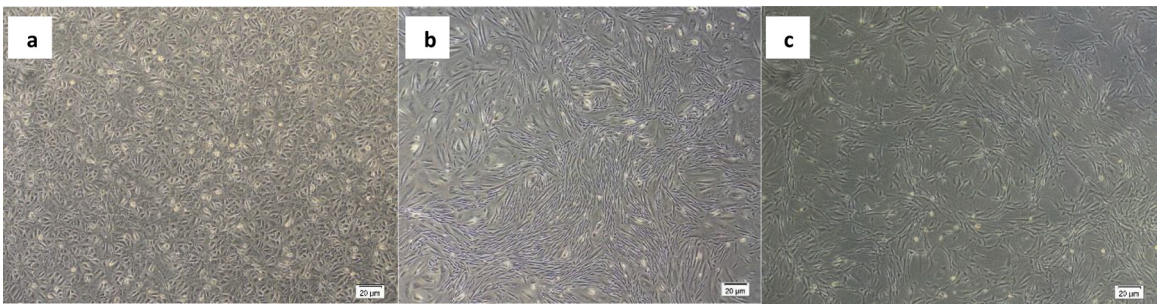

Fig. 2. Human amniotic fluid cells are classified according to their morphology into three main categories: (a) epithelioid (E-type) amniocytes with large polygonal, from cuboidal to columnar, morphology; (b) amniotic fluid-specific (AF-type) amniocytes are short-spindle fibroblastic cells without needle-shape projections; (c) fibroblastoid (F-type) amniocytes that are fibroblastic cells, with long-spindle morphology and needle-shape projections.

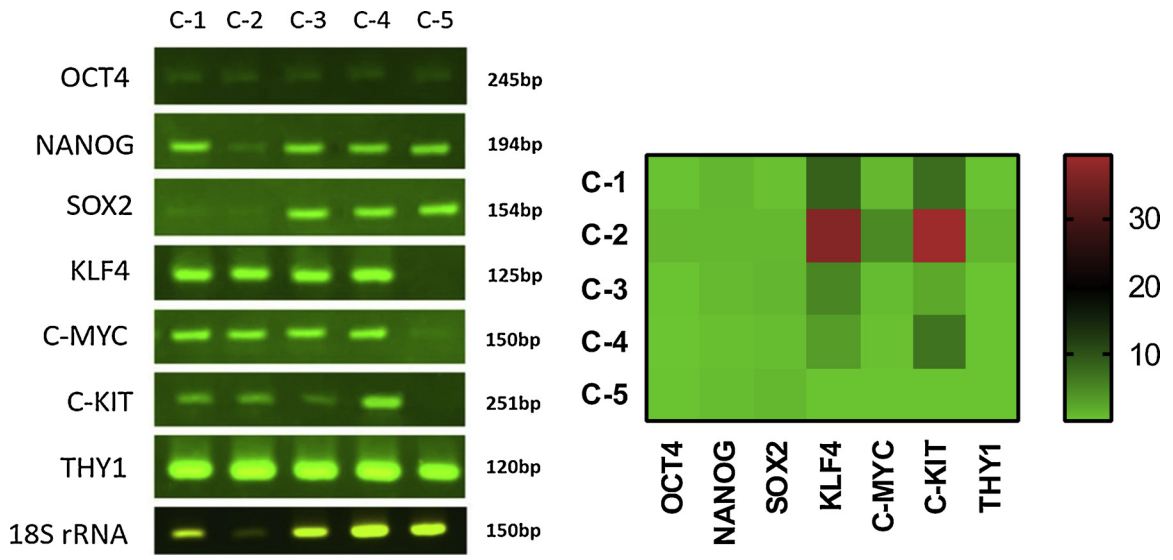

Fig. 3. Expression of specific pluripotency markers as detected by RT-PCR in human amniocytes at the first passage (cases 1–5). Left panel: gel electrophoresis images of stemness genes. Right panel: the heatmap of semi-quantitative results obtained from ImageJ software according to the area under the plot.

epithelioid area of the plot. In addition, the plot clearly exhibits high diversity in the expressions of *NANOG* and *SOX2* while *OCT4* expression was less variable across different isolates, particularly in fibroblastoid ones. This can also be seen in Fig. 7 where extremely variable expression of *NANOG* and *SOX2* with high numeric values of SD resulted in non-significant difference between epithelioid and fibroblastoid (LS and SS) cells. On the contrary, *OCT4* is significantly expressed at different levels between epithelioid and fibroblastoid isolates (with the *p*-value of 0.02) because of fairly lower differential rate of expression and thus minor value of SD compared with *NANOG* and *SOX2* (Fig. 7). This finding was confirmed by Pearson's coefficient correlation where *OCT4* expression was significantly correlated with the cell type at the significance level of 0.04. Furthermore, investigating possible relationships between these pluripotency markers revealed their correlations, so that as depicted in Fig. 8b, *OCT4* was directly correlated with *NANOG* and *SOX2* (p-value < 0.05), while there was not a significant correlation between *NANOG* and *SOX2* (Fig. 8).

3.3.4. *KLF4 and C-MYC were expressed differently between amniotic subpopulations*

The results obtained from expression levels of classical pluripotency markers, *KLF4* and *C-MYC*, showed that 8 out of 14 isolates express both markers coincidently (Fig. 6). However, considering the *KLF4*-positive clones (10 isolates) and their expression level showed that the epithelioid lines (6 isolates) express *KLF4* more than fibroblastoid ones (4 isolates). It seems *KLF4*-positive amniocytes, regardless of their types, have revealed two levels of expression concerning *KLF4*, i.e. high and low level-expressing isolates while *C-MYC* was expressed in a more variable mood. In this regard, our findings have shown that fibroblastoid clones express *C-MYC* at a higher level compared to epithelioid ones, though the difference was statistically non-significant (Fig. 7). As depicted in Fig. 8, the relationship of *KLF4* and *C-MYC* with other stem cell markers revealed a totally different outcome. Although the results of Pearson's coefficient displayed no correlation for *KLF4*, but *C-MYC* was directly correlated with *C-KIT* and *THY1*, at the 0.05 and 0.01 significance level, respectively.

3.3.5. *C-KIT is expressed by epithelioid clones twice the number of fibroblastoid ones*

From a qualitative point of view, nearly all epithelioid (6 out of 7), but only 3 fibroblastoid isolates were *C-KIT*-positive while the semi-quantitative results revealed non-significant difference between expression level of *C-KIT* in these cell types (Fig. 7). As mentioned earlier, examining the Pearson coefficient among the target genes confirmed

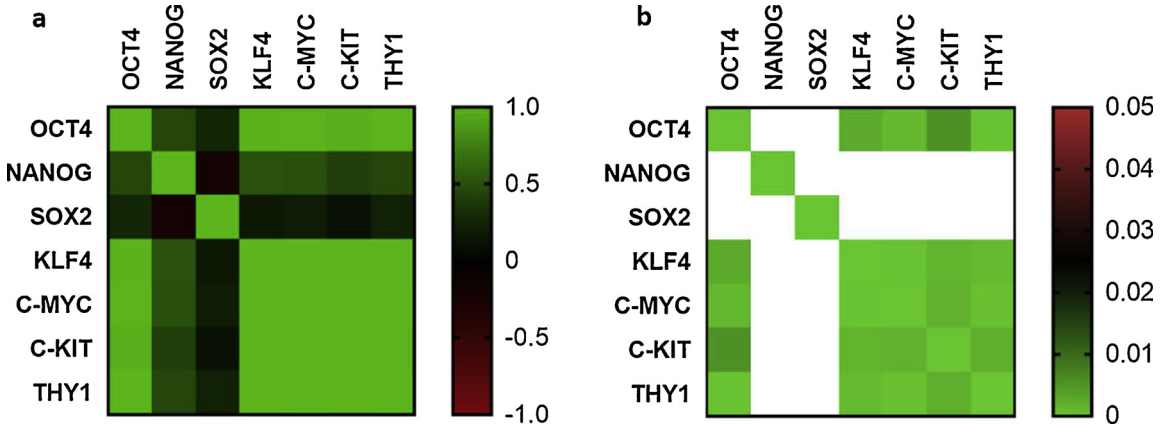

Fig. 4. The heatmaps representing the relationships between semi-quantitative variables of gene expressions in primary-cultured human amniocytes based on Pearson's coefficient. (a) The plot illustrates correlation strength between the genes, so that Pearson's coefficients of +1 and −1 imply perfect correlations between two genes, directly and indirectly, respectively. A value of 0 implies that there is no correlation between the variables. (b) The plot demonstrates significant *p*-values of the correlation in the range of 0 to 0.05. The non-significant *p*-values are excluded (white cells).

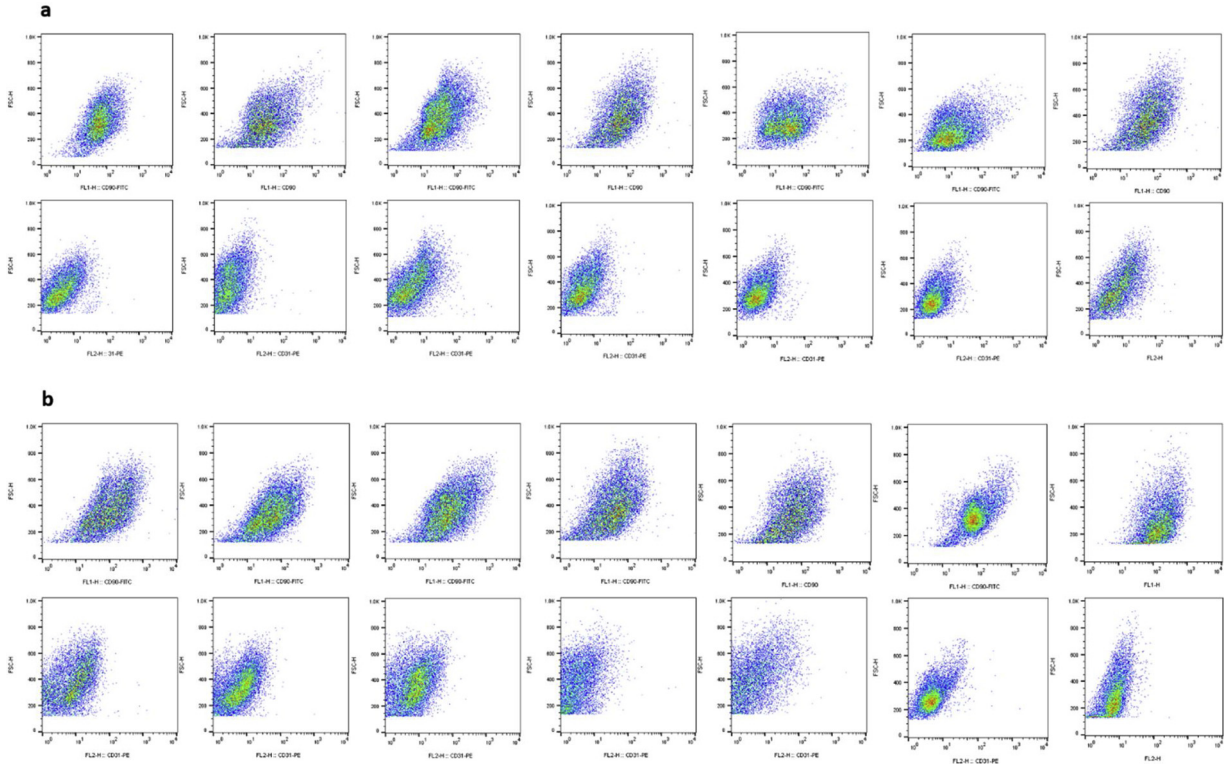

Fig. 5. Flow cytometry plots represent CD90 and CD31 expression in adherent human amniocytes according to subpopulations. The y-axis represents forward scatter (FSC-H), where increasing the signal indicates increased cell size. The x-axis indicates CD90 (in upper row) and CD31 (in lower row) fluorescence. (a) Flow cytometry analysis of epithelioid (E-type) amniocytes. (b) Flow cytometry analysis of fibroblastoid (long-spindle and short-spindle) amniocytes.

the correlations of *C-KIT* with *C-MYC* and *THY1* expressions, both at the level of 0.05 (Fig. 8).

3.3.6. THY1 is consistently expressed in all clones

The results showed that, irrespective of the cell type, *THY1* was expressed in all clones. Compared to other target genes *THY1* displayed the lowest variability over the clones and there was no significant difference between the cell types. Regarding the relationship with other markers, *THY1* showed direct correlations with *C-MYC* and *C-KIT*.

4. Discussion

4.1. Growth characteristics and morphological properties of human amniocytes

Amniotic fluid is composed of an extremely heterogeneous cell population and the biochemical, morphological and growth characteristics are widely used for classification of amniocytes [30]. In this regard, our investigation showed that all three mentioned cell types are clonogenic and have some distinguishable characteristics during long term cultures. Furthermore, trauma caused by amniocentesis will lead to some maternal blood interference in the samples, however, the incapability of these cells to adhere to the culture surface and proliferate under such conditions reduces their possible cross contamination [7]. Compelling evidence to characterize each cell type needs antibody-based approaches, whereas our findings depend simply on morphologic features and growth properties, which is the main drawback of our study. Usually immunostaining of cell populations against keratins and vimentin is used for characterization of amniocytes. These proteins are members of intermediate filament superfamily widely used as markers of epithelial and stromal cells, respectively [31]. For the first time, Hoehn et al. divided the amniotic fluid cells into three main subtypes based on clonal characteristics and cellular morphology, including E-type, AF-type, and F-type [32,33]. As both AF-type and F-type cells have fibroblastic morphology, it is difficult to distinguish them using morphological criteria, therefore co-expression of keratins and vimentin are used as indicator of AF-type cells, in other words they exhibit markers of epithelial and stromal cells simultaneously [34]. Although we have used the triple classification in our descriptive study, but only epithelioid and fibroblastoid (LS and SS) morphological groups were considered for statistical analysis, including 7 isolates in each set. Our observations in a larger study (68 samples) revealed that the frequency and lifespan of each subpopulation are significantly different [29].

As early mentioned, Roubelakis and coworkers categorized adherent, clonogenic amniocytes into “round”- shaped (RS) epithelial cells and “spindle”-shaped (SS) fibroblastic cells [5]. Their analysis revealed that SS-amniocytes exhibited significantly higher proliferative capacity (with the lifespan of 45 passages) compared with RS-amniocytes (with the lifespan up to passage 4–7). Their results are nearly consistent with our data in which fibroblastoid cells were long-lived as compared with epithelioid ones. They have also shown that primary-cultured amniocytes contained a mixture of SS and RS cells, nonetheless, after a couple of passages the cultures were overtaken by the RS cells. Conversely, this was not consistent with our observations, as most cultures were also heterogeneous mixtures of SS and RS cells at higher passages. For example, our investigation displayed that from more than 70 amniocentesis samples, only 7 morphologically homogenous epithelioid isolates were achieved.

4.2. RT-PCR analysis of stemness genes in amniotic subpopulations

Comparing qualitative results of RT-PCR with cell growth characteristics demonstrated that there was an inverse relationship between the number of isolates expressing pluripotency markers in each subpopulation and their lifespan. It means that although lower number of fibroblastoid clones expressed stemness genes but the mean value of their lifespan was greater than that of the epithelioid group. However,

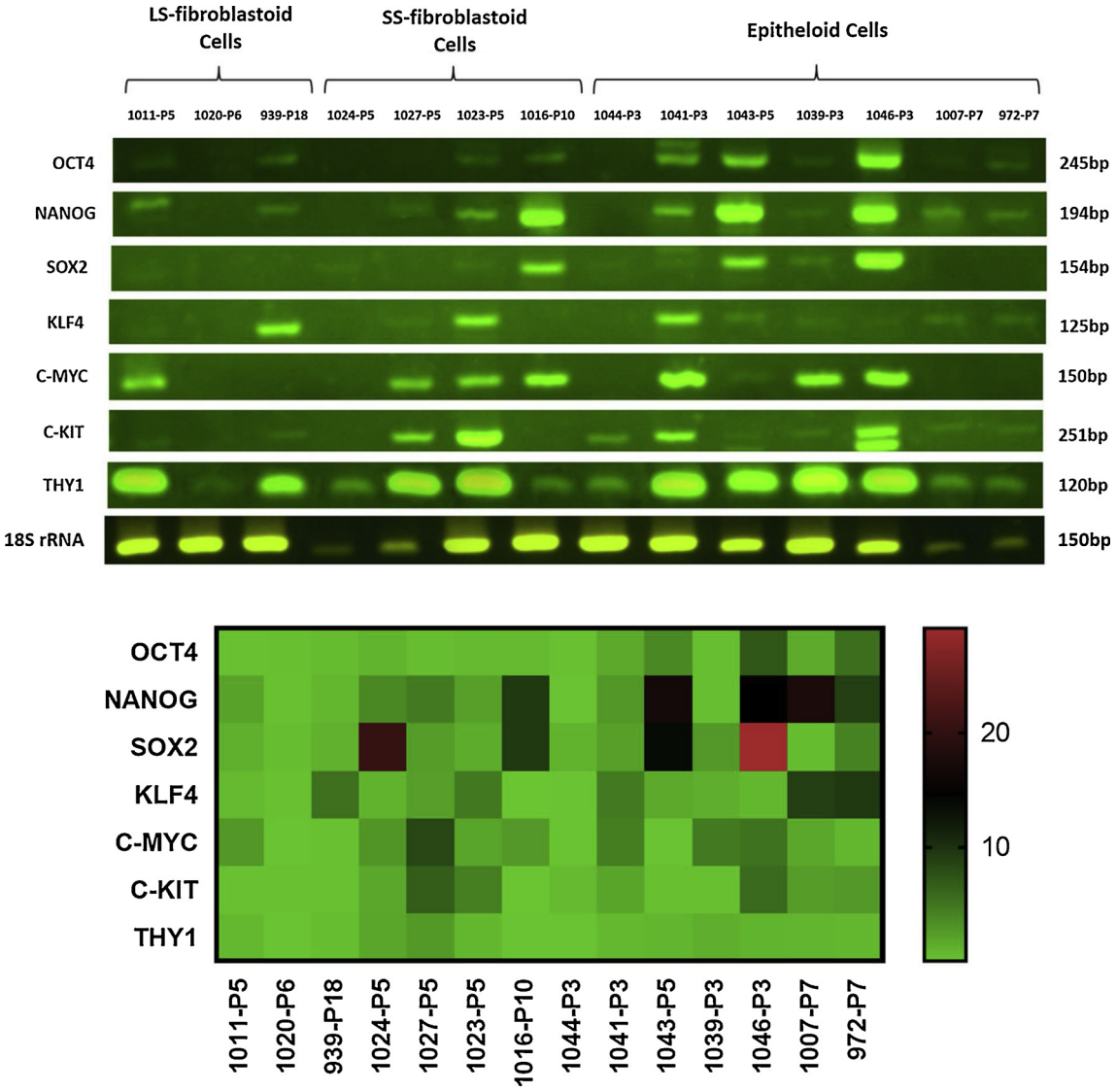

Fig. 6. Expression of the stemness genes in long term-cultured human amniocytes as detected by RT-PCR at the second half of their lifespan. **Upper panel**: The image shows gel electrophoresis of RT-PCR products in each isolate based on their cell type. **Lower panel**: The heatmap represents relative expression level of each gene across individual isolates normalized to the quantity of 18S rRNA transcript in each sample. LS- and SS-fibroblastoid stand for long- and short-spindle fibroblastic-like cells, respectively.

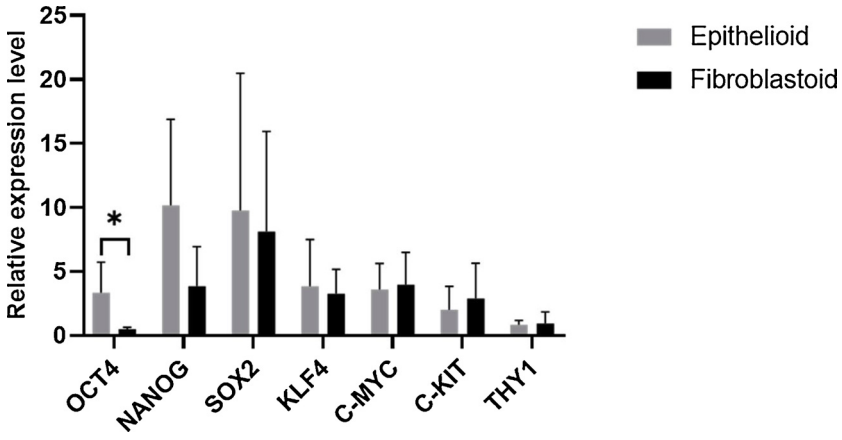

Fig. 7. Relative expressions of stem cell markers. The semi-quantitative results are illustrated as the average expressions in epithelioid and fibroblastoid isolates with SD. To normalize the expression levels of individual genes, the area under each plot was divided to the corresponding amount of 18S rRNA in the same isolate. *OCT4* is the only gene differentially expressed in epithelioid and fibroblastoid isolates (with the *p*-value of 0.02).

the semi-quantitative analysis of data revealed the non-significant difference of the genes between epithelioid and fibroblastoid lines (except for *OCT4*). This is either as a result of the high variations in gene expressions and consequently high numeric value of SD or due to comparable expression levels between the two cell types. So we cannot reach a definitive conclusion about the expression level of stem cell

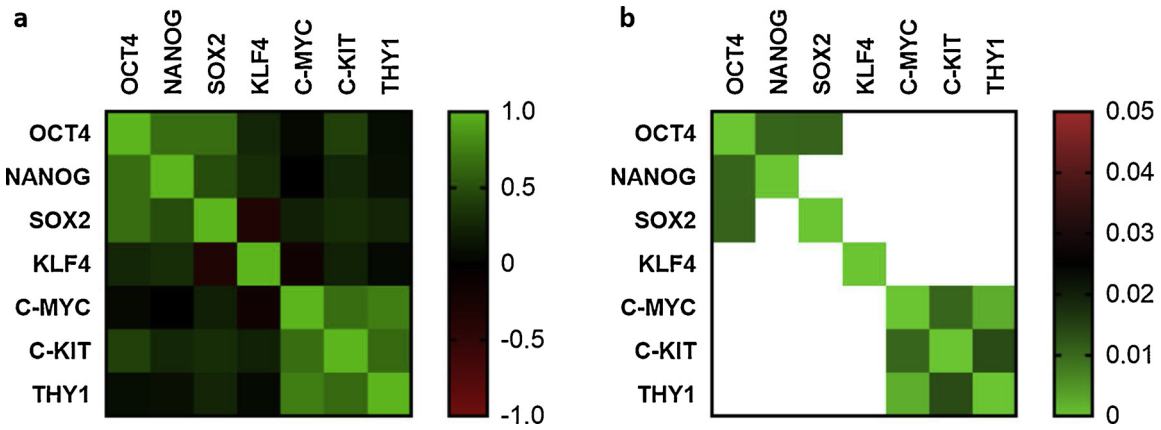

Fig. 8. The heatmaps representing the relationships between semi-quantitative variables of gene expression in long term-cultured human amniocytes based on Pearson's coefficient. (a) The plot illustrates correlation strength between the genes, so that Pearson's coefficient of +1 and −1 implies perfect correlation between two genes, directly and indirectly, respectively. A value of 0 implies that there is no correlation between the variables. (b) The plot demonstrates significant *p*-values of the correlation in the range of 0 to 0.05. The non-significant *p*-values are excluded (white cells).

markers over the cell types, but obviously epithelioid isolates display higher frequency of the target genes, particularly the triple markers of pluripotency regulatory circuit, *OCT4*, *NANOG* and *SOX2*. The efficient maintenance of pluripotency is based on the cooperation of OCT4, NANOG and SOX2 proteins to transcriptionally regulate key stemness genes [35]. Interestingly, this is in a good agreement with our findings where missing the triple genes results in complete lack of other stemness genes (Fig. 6).

Some studies categorized amniocytes into two groups depending on the size, 8 μm and 15 μm cells. It has been reported that smaller cells are OCT4, NANOG, SOX2 and C-MYC positive, also express surface markers of pluripotency including SSEA3, SSEA4, TRA-1-60 and TRA-1-81, while the larger cells are fibroblastoid and do not express the triple factors of *OCT4*, *NANOG* and *SOX2* [15]. This is fairly consistent with our findings but the results reported by Roubelakis and colleagues have shown that, compared to epithelioid cells, fibroblastoid cells exhibit higher expression of *OCT4*, *NANOG* and *SOX2* [5], while in our results epithelioid cells expressed higher levels of ESC markers. Li and coworkers [36] have reported the lack of OCT4, NANOG and SOX2 in long term-cultured human amniocytes, whereas our clones expressed the genes differently, from total lack to highly-expressed ones. Remarkably, Li and colleagues isolated a subpopulation of amniocytes expressing *OCT4* and *C-KIT*, and came up with this conclusion that human amniocytes may contain pluripotent stem cells or represent a precursor state that is prone to a rapid conversion into the pluripotent state [36]. However, another study demonstrated that clones isolated from amniotic fluid did not express OCT4 and SOX2 at both transcript and protein levels [37].

Besides fetal tissues, human amniocytes might be derived from fetal membranes, including epithelial and stromal cells released by amnion layer into the amniotic fluid [6,30]. Human amniotic epithelial cells (hAECs) isolated from amnion are round-shaped epithelial-like cells that probably have a common origin with amniotic fluid-derived epithelioid cells. It has been theorized that the hAECs may preserve molecular characteristics of pluripotency in epiblast stem cells originated from epiblast layer. Since the amnion is separated from the epiblast during early development of embryo, epiblast stem cells may escape from distinctive signals that make epiblast differentiate into the germ layers, ectoderm, mesoderm, and endoderm [38]. In this regard, high expression of *OCT4*, *NANOG* and *SOX2* detected in our epithelioid isolates is sensible. Although it is very difficult to achieve an accurate conclusion, but there are some evidences that need to be addressed more, such as molecular profile of pluripotency observed in both hAECs and amniotic fluid-derived epithelioid cells, as well as their similarities in size, morphology and growth characteristics. For example, it has been revealed that in a culture condition without epidermal growth factor (EGF), hAECs gradually turn into giant multinucleated cells, a situation reminiscent of trophoblastic differentiation in hESCs that has also been realized within epithelioid cultures in our work [39].

C-MYC not only plays an important role in proliferation of MSCs and their expansion *in vitro*, but also is involved in cell cycle progression, differentiation and apoptosis [40]. For the first time, Wilson and colleagues indicated that the stem cells' homeostasis between self-renewal and differentiation is preserved by c-Myc [41]. Remarkably, most of isolates expressing high levels of *C-MYC* were the short-lived ones with lifespan of 4 passages (Table 2 and Fig. 6). It means that higher expression of *C-MYC* stimulates differentiation and consequently attenuates proliferation of the cell lines.

For the first time, in 2007, De Coppi and colleagues described a new stem cell fraction in human amniotic fluid named CD117 (C-KIT)-positive amniotic fluid stem cells [25]. Since then, many research groups have isolated the same cells similarly [9,12,16]. It is believed these cells are broadly multipotent with intermediate state between ESCs and adult MSCs, in other words, the cells are able to differentiate into mesodermal, endodermal and ectodermal lineages [15]. Arnhold and coworkers showed that C-KIT-positive AFSCs have a fibroblastic morphology, whereas C-KIT-negative amniocytes exhibit epithelial morphology [9]. However, some authors have reported that C-KIT-positive AFSCs display different morphological features in culture conditions, ranging from epithelioid (oval or polygonal) to fibroblastoid shapes [6] which is in agreement with our results. Roubelakis and colleagues reported that both epithelioid and fibroblastoid amniocytes similarly expressed *C-KIT* at very low/undetectable levels, as was previously demonstrated by some other authors [42,43]. This was inconsistent with our findings because high expression of *C-KIT* was detected in some isolates.

4.3. Flow cytometry analysis of surface antigens

Flow cytometry analysis of all clones was carried out to characterize the subpopulations and their lineage commitment concerning renowned mesenchymal and hematopoietic markers, CD90 and CD31, respectively. Flow cytometry analysis of CD90 and CD31 showed that all cell lines are basically a homogeneous population of non-hematopoietic mesenchymal cells. Moreover, RT-PCR analysis has also revealed a universal expression pattern of *THY1* in all subpopulations which could potentially be a sign of mesenchymal origination in our isolates. There are many reports displaying high *THY1* expression in almost all sources of MSCs, especially in undifferentiated state. It has also been demonstrated that lineage commitment of MSCs *in vitro* leads

to decreased *THY1* expression [44]. It is similarly reported that fibroblastoid cells express higher level of CD90 than epithelioid cells [5]. These results were in good agreement with our findings in which, based on the MFI, the expression level of CD90 were greater in fibroblastoid isolates compared with epithelioid cells.

4.4. Correlation of stemness genes in primary- and long term-cultured amniocytes

As the results show, the target genes exhibited heterogeneity and variability in both cell types. Although some authors have reported various conditions of the culture or different gestational ages as the effective factors responsible for this variation [43], but it is still unclear exactly which factors account for such substantial differences. Diverse derivation site of amniocytes and individual genetic background can potentially explain the observed variation in the expression level of the target genes. It has been suggested that amniocytes maintain an individual expression profile during long term culture [43], whereas naïve amniocytes in primary culture express the markers in the fairly similar level. It is reflected in the assessment of correlation among the target genes between primary- and long term-cultured amniocytes. Such differences have been also confirmed by other studies, for instance, making a comparison between primary and culture-expanded BM-MSCs disclosed a considerable number of differentially expressed genes [45].

Many studies have shown a remarkable reduction in the expression of key pluripotency markers, *OCT4*, *NANOG*, and *SOX2*, throughout the long-term culture of different human MSCs [46–48]. Although the explanation for such differences is challenging, it seems that the core pluripotency markers, *OCT4*, *NANOG*, and *SOX2*, are expressed only in specific cells that contain a small percentage of amniocytes in primary cultures, whereas other target genes, *C-KIT*, *C-MYC*, *KLF4*, and *THY1*, are expressed in a large proportion of naïve amniocytes, so their expression levels are similar in different samples. As amniocytes expanded during long term cultures, only multipotent stem cells expressing the core pluripotency markers can proliferate by sequential subculture. Results from other studies have also shown that such cells express stemness markers in a hypervariable manner [43,49]. Generally, the present study suggests that the partial correlation between *OCT4*, *NANOG*, and *SOX2* is probably due to the potential of long term-cultured amniocytes to express the triple genes in most of the samples.

5. Conclusion

Characterization of isolated cells demonstrated that all cell lines, both epithelioid and fibroblastoid, have mesenchymal, not hematopoietic lineage. RT-PCR analysis of the stemness genes revealed a higher level of expression in the epithelioid isolates. This reinforces the hypothesis that the epithelioid cells are derived from the hAECs and the possibility of their origination from epiblast. Taken together, our results indicate that amniotic isolates express the core pluripotency markers, *OCT4*, *NANOG*, and *SOX2*, in highly variable manner, but similarly exhibit *KLF4*, *C-MYC*, and *C-KIT*. Furthermore, *THY1* showed the least variability over the all isolates. These results made that possible to draw a comparison with the expression level of target genes in the primary-cultured amniocytes. It is concluded that correlation between the genes can profoundly change during long term culture. Such apparent variability observed in the clones may be due to the existence of cells from different origins which can explain why amniocytes differ in many respects, including survival rate, plastic adhesion, growth characteristics and responses to environmental factors. It is worth noting that the heterogeneous nature of human amniocytes is a potential contributor to different results observed in independent studies regarding the isolation of clones with different characteristics. Therefore, further characterization of the isolated cells is of great importance.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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