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# Expression and co-expression of surface markers of pluripotency on human amniotic cells cultured in different growth media

## Ekspresja i koekspresja powierzchniowych markerów pluripotencjalności na komórkach owodni łożyska ludzkiego w warunkach hodowli w różnych podłożach

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## Abstract

***Objectives:*** *Despite constant advances in the field of biology and medical application of human embryonic stem cells, the molecular mechanism of pluripotency remains largely unknown. So far, definitions of pluripotent stem cells (SC) have been based on a limited number of antigenic markers and have not allowed for unambiguous determination of the homogeneity of each subpopulation. Moreover, the use of some crucial pluripotency markers such as SSEA-3 and SSEA-4 has recently been questioned due to the possibility that the pattern of surface glycans may be changed depending on the content of the cell culture medium.*

***Aim:*** *Quantitative analysis of amniotic SC subpopulations cultured in different media, based on the following pluripotency surface markers: SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 expression and co-expression.*

***Material and methods:*** *Immunofluorescence and fluorescence microscopy were used to identify and localize SC within a normal human placenta at term. The number of SSEA-4$^+$, SSEA-3$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ cells and cells with co-expression of the above mentioned markers, cultured in media containing different protein supplements of animal origin, was counted by flow cytometry.*

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***Results and conclusions:*** *Cells with characteristics of embryonic SC were identified in the amniotic epithelium and the chorion, but not in the decidua basalis. Amniotic epithelium contained various types of SC, with SSEA-4⁺ as the most numerous. Disproportion in the number of SSEA-4⁺, SSEA-3⁺, TRA-1-60⁺ and TRA-1-81⁺ cells and cells characterized by co-expression of these antigens, as well as lack of quantitative differences between SC subpopulations cultured in different media, was observed. In conclusion, the amniotic epithelium is composed of SC at different stages of the development but human amnion might become an alternative source of SSEA-4⁺ embryonic-like SC. The composition of the evaluated media, characterized by different content of animal-derived proteins, does not influence the number of cells identified within the SC subpopulations.*

Key words: **human placenta** / **amnion** / **pluripotent stem cells** / / **markers of pluripotency** / **co-expression** / **SSEA-3** / **SSEA-4** / **TRA-1-60** / / **TRA-1-81** / **culture media** /

## Streszczenie

***Wstęp:*** *Mimo ciągle rosnącej wiedzy o biologii i zastosowaniach medycznych ludzkich embrionalnych komórek macierzystych, molekularny mechanizm utrzymujący ich pluripotencjalność pozostaje nieznany. Dotychczas stosowane definicje komórek pluripotencjalnych opierają się na oznaczeniach ograniczonej liczby markerów antygenowych i nie pozwalają na jednoznaczne określenie homogenności danej subpopulacji. Ponadto, zastosowanie niektórych kluczowych markerów pluripotencji, np. SSEA-3 i SSEA-4, jest obecnie kwestionowane, ponieważ skład powierzchniowych glikanów przypuszczalnie może się zmieniać w zależności od ich zawartości w podłożu hodowlanym.*

***Cel pracy:*** *Celem pracy była analiza ilościowa owodniowych subpopulacji komórek macierzystych wykazujących ekspresję i koekspresję markerów powierzchniowych pluripotencji SSEA-3, SSEA-4, TRA-1-60 i TRA-1-81, w warunkach hodowli w różnych podłożach.*

***Materiał i metody:*** *Zastosowano techniki immunofluorescencyjne i mikroskopię fluorescencyjną do immunodetekcji i lokalizacji komórek macierzystych w prawidłowym łożysku ludzkim. Liczebność komórek SSEA-4⁺, SSEA-3⁺, TRA-1-60⁺ i TRA-1-81⁺ oraz komórek wykazujących koekspresję tych markerów, hodowanych w podłożach różniących się zawartością białek pochodzenia zwierzęcego, oceniano metodą cytometrii przepływowej.*

***Wyniki i wnioski:*** *Wykazano, że komórki o cechach embrionalnych komórek macierzystych występują w łożysku ludzkim w nabłonku owodni i w kosmówce, natomiast nie są obecne w doczesnej. Nabłonek owodni zawierał różne populacje komórek macierzystych, przy czym najliczniejsze były komórki SSEA-4⁺. Stwierdzono dysproporcje liczbowe w subpopulacjach komórek SSEA-4⁺, SSEA-3⁺, TRA-1-60⁺ i TRA-1-81⁺ oraz komórek wykazujących koekspresję tych markerów, a także brak różnic ilościowych pomiędzy subpopulacjami komórek hodowanych w badanych podłożach. Podsumowując, nabłonek owodni zawiera komórki macierzyste reprezentujące różne stadia rozwoju, ale może być atrakcyjnym źródłem komórek macierzystych SSEA-4+. Skład badanych podłoży hodowlanych, charakteryzujący się różną zawartością białek pochodzenia zwierzęcego nie miał wpływu na liczebność subpopulacji komórek macierzystych identyfikowanych w hodowli.*

Słowa kluczowe: **łożysko ludzkie** / **owodnia** / **pluripotencjalne komórki macierzyste** / / **markery pluripotencjalności** / **koekspresja** / **SSEA-3** / **SSEA-4** / / **TRA-1-60** / **TRA-1-81** / **podłoża hodowlane** /

**Abbreviations:**

BSA – bovine serum albumin; ESC – embryonic stem cells; FBS – fetal bovine serum; hESC – human embryonic stem cells; MSC – mesenchymal stem cells; PBS – phosphate buffered saline; SC –stem cells; SSEA-3 and SSEA-4 – Stage-Specific Embryonic Antigen 3 and 4, respectively; TRA-1-60 and TRA-1-81 – Tumor Rejection Antigen 1-60 and 1-81 respectively.

**Skróty:**

BSA – albumina surowicy wołowej; ESC – embrionalne komórki macierzyste; FBS – płodowa surowica bydlęca; hESC – ludzkie embrionalne komórki macierzyste; MSC – mezenchymalne komórki macierzyste; PBS – sól fizjologiczna buforowana fosforanami; SC – komórki macierzyste; SSEA-3 i SSEA-4, *ang.* – Stage-Specific Embryonic Antigen 3 i 4; TRA-1-60 i TRA-1-81, *ang.* – Tumor Rejection Antigen 1-60 i 1-81.

## Introduction

Recently published studies have reported the presence of a population of stem cells (SC), which exhibit characteristics of pluripotent embryonic cells, in the organs of adult individuals [1]. It is believed that pluripotent cells, which inhabit the organs during the fetal life of mammals, may become dormant in adult tissues and might become a source of tissue-specific precursor cells during the process of organ regeneration and for cell therapy [2, 3]. Demonstrating the presence of pluripotent cells with structural and functional capabilities comparable to embryonic stem cells (ESC), both in adult and perinatal tissues, presents a current scientific and medical challenge [4]. Confirmation of such presence would begin a new, scientifically justified, morally and


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ethically acceptable possibility to obtain these cells for therapeutic purposes and treatment of metabolic and genetic diseases [5, 6].

Perinatal tissues such as the cord blood, the placenta and the amniotic fluid, play a special role as a source of pluripotent cells due to the fact that, by nature, they are a reservoir of cells of fetal origin. The placenta is an organ formed in large part from fetal tissues - epiblast, postembryonic mesoderm and trophoblast - and in a relatively short period of time, which provides the possibility of preserving a significant number of cells with the characteristics of pluripotent ESC and similar potential to differentiation [7]. Human placenta is relatively easy to obtain, without additional risk to health and life of the donor or the fetus, and is subjected to mandatory recycling without ethical concerns. It may be taken during labor in a minimally invasive and fast way. The placenta is composed of a large number of cells per unit weight/volume, i.e. from 1 to 10 million cells/1g tissue depending on the placental layer. Approximately 80-160 million cells are obtained from human amniotic epithelial cells of a single placenta. The mean placental birth weight is about 500 g. Undoubtedly, the placenta has the best parameters in terms of mass, volume and cell number among all other currently used tissue sources of SC [8, 9]. Placental cells show a limited expression of the major histocompatibility complex antigens class I: HLA-A, HLA-B and HLA-C, as well as lack of MHC class II, which in the case of cells of fetal origin is a natural immunosuppression mechanism against the maternal defense system. That may be an important factor for the safe use of these cells and their progeny during transplantation [10, 11]. Finally, studies have shown that human amniotic epithelial cells have anti-inflammatory properties [12, 13] and placental SC have low tumorigenic properties [8, 11, 14].

The development of effective methods for cell isolation and culture, as well as controlling the process of cell differentiation, presents another problem [4]. Stem cells are currently defined by a combination of physical, genetic and functional properties. Universal markers for SC, that might be used for direct and efficient isolation of specific SC populations, have not been determined yet. Among the specific SC surface markers, an important role has been assigned to glycan epitopes that function as receptors and are involved in cell adhesion and signal transduction. Commonly used markers of human ESC (hESC) include TRA-1-60 and TRA-1-81 (Tumor Rejection Antigen 1-60 and 1-81) that belong to the class of keratan sulfate proteoglycans, and SSEA-3 and SSEA-4 (Stage-Specific Embryonic Antigen 3 and 4) which are carbohydrate epitopes of glycosphingolipids [15, 16, 17].

TRA-1-60 and TRA-1-81 are expressed on the surface of human tetracarcinoma SC, human embryonic germ cells and hESC [18], yet their molecular identity has remained elusive for decades.

Recently, the applicability of SSEA-3 and SSEA-4 in the characterization of SC has been questioned because of the probable incorporation of glycans into the cytoplasmic membrane, as a consequence of cell metabolism. It is likely that some glycans may appear on the cell surface as a result of their modified expression pattern, depending on the stage of cell differentiation. Finally, it was suggested that the changes of SSEA epitopes may depend on cell-culture medium relations, what is strictly connected with the use of animal-derived components [19, 20, 21]. In the light of these observations, verification of marker suitability and continuous search for such determinants of pluripotency would allow for the isolation of the sufficient number of possible homogeneous cells with characteristics of ESC.

## The aim

The aim of the study was a quantitative analysis of amniotic SC subpopulations cultured in two different media: Alpha MEM containing fetal bovine serum (FBS) and mTeSR1 containing animal-sourced bovine albumin, but no undefined serum animal-origin components. The identification of SC was based on the immunodetection of surface markers of pluripotency: SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 expression and co-expression.

## Materials and Methods

### Human placentas

Term placentas from 23 healthy donor mothers, aged 18-35 years, were obtained from the Perinatology and Gynecology Ward of the Central Clinical Hospital, Medical University of Silesia, Katowice.

The women gave their informed consent. Uncomplicated elective caesarean deliveries were performed between 37 and 38 weeks gestation, for medical reasons. If no evidence of placental insufficiency was found, the placentas were rapidly transferred to the laboratory in phosphate buffered saline (PBS) containing antibiotic-antimycotic solution (Penicillin 0.1 Units/ml, Streptomycin Sulphate 0.1 mg/ml, Amphotericin B 0.25 µg/ml; PAA) and EDTA (5 mM EDTA; Invitrogen) and rinsed several times with this mixture to remove blood.

Table I. Efficiency of cell isolation from human placental amnion.

| Placenta specification | | | | | |
|---|---|---|---|---|---|
| Number of placentas (n) | Mean mass of the placenta (g) | Average size (cm) | Mean mass of the amnion taken for cell isolation (g) | Mean number of cells isolated from sectioned amniotic tissue (mln) | Number of epithelial cells per 1 g of amnion (mln) |
| 23 | 593 | 20.1x18.3 | 12.69 | 117 | 9.22 |


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**Morphological studies**

Cross-sections excised from the pericentral area of the placenta were fixed in 10% formaldehyde, dehydrated in increasing concentrations of ethanol-xylene series and embedded in paraffin. The paraffin blocks were cut into 4 µm slices, which were then deparaffinized and rehydrated by passing through a series of xylene and alcohol, and stained with hematoxylin and eosin (H-E) by standard procedure.

***In vivo* immunodetection of stem cell markers**

Firstly, 10x20 mm tissue samples, containing entire cross-sections of the discoid placenta, were fixed in 4% buffered formalin (pH 7.4; 2 hr at room temperature), followed by infiltration with 30% sucrose (in PBS, overnight at 4°C) for cryoprotection. Then, the samples were embedded in tissue freezing medium (OCT Embedding Matrix, CellPath) and 7 µm thick cryosections were prepared. The sections were washed with PBS, blocked with 1% solution of bovine serum albumin (BSA) and incubated with primary antibodies: anti-SSEA-3 (rat IgM, MAB4303), anti-SSEA-4 (mouse $IgG_3$, MAB4304), anti-TRA-1-60 (mouse IgM, MAB4360) or anti-TRA-1-81 (mouse IgM, MAB4381) (all from Chemikon/Millipore), at a concentration of 10 µg/ml for 20 hr, at 4°C.

After the washing steps with PBS, the sections were exposed to appropriate secondary FITC-conjugated antibodies: mouse anti-rat IgM (11-0990, eBiosciences), goat anti-mouse IgG3 (110002, AbDSerotech) or goat anti-mouse IgM polyclonal antibody (NB7496, Novus Biologicals). Afterwards, in order to visualize the cell structure, F-actin was counterstained with 6.6 mM Rhodamine phalloidin (Invitrogen). For nuclei staining, slices were mounted with Hard Set Mounting Media containing DAPI (Vector Laboratories). In control specimens, the primary antibody was substituted with an equivalent concentration of isotype-specific immunoglobulins: rat IgM, k Isotype Ctrl (400801); mouse IgG3, k Isotype Ctrl (401301) or mouse IgM, k Isotype Ctrl (401601) (all from BioLegend).

**Isolation and culture of cells from the human amniotic membrane**

The amniotic membrane was manually separated from the chorion, washed in PBS, supplemented with antibiotics and antimycotics as described above, and cut into pieces (~20x20 mm). The fragments were then subjected to enzymatic digestion, at 37°C; 7 minutes with 2.4 U/ml dispase solution (Roche), twice 40 minutes with 0.05% trypsin-0.54 mM EDTA solution (PAA) and 60 minutes with 0.75 mg/ml collagenase solution (Roche). Cells which were recovered after digestion were collected by centrifugation (300 x g, 4°C) and suspended in a complete medium. Fractions collected after each digestion step were combined to constitute a mixture of the cells. From this process, the resulting cell suspensions were depleted of red blood cells by incubation with Lysing Buffer (BD Biosciences). After centrifugation and filtration through a 100 µm cell strainer, the cells were placed into culture flasks (1 x $10^5$ cells/$cm^2$). Each cell culture was maintained in: Alpha MEM (Alpha Modification of Minimum Essential Medium) (Sigma-Aldrich) supplemented by 10% FBS, 1% antibiotic-antimycotic solution and 1% L-glutamine (all from PAA); and in mTeSR1 medium (StemCell Technologies), in both cases at 37°C with water saturated atmosphere and 5% $CO_2$. Alpha MEM contains non-essential amino acids, sodium pyruvate, and additional vitamins [22] and is one of the most widely used synthetic cell culture medium for cultivation of a wide variety of cells grown in monolayer, while mTeSR1 is a standardized medium for feeder-independent maintenance of hESC in culture [23, 24]. mTeSR1 contains an animal-sourced protein - bovine albumin, but it is characterized by the elimination of undefined medium components and removal of the inherent variability associated with feeder cells and conditioned media. hESC maintained in mTeSR1 medium should be phenotypically homogenous and express high levels of antigens associated with pluripotency. The media were changed after 48 hr and every 3–4 days thereafter. The cells were grown for further 14 days under these conditions.

***In vitro* immunodetection of stem cell markers**

After 16 hours the cells were collected, centrifuged, suspended in PBS supplemented with 10% FBS and 5 mM EDTA and incubated (30 min., 4°C, in the dark) with anti-human primary antibodies: anti-SSEA-3 Rat IgM, anti-SSEA-4 Mouse IgG3, anti-TRA-1-60 Mouse (BALB/c) IgM, (560380) and anti-TRA-1-81 Mouse (BALB/c) IgM (560194) or with Rat IgM (553942), Mouse IgG3 (559806) and Mouse IgM (553474) (all from BD Pharmingen) isotype controls. All antibodies were conjugated with FITC and used in accordance with the manufacturer's protocol (20 µl solution per 100 µl cell suspension containing 1 x $10^6$ cells). Finally, the cells were fixed in 2% paraformaldehyde, and mounted in Vectashield Hard-Set Mounting Medium – with DAPI (Vector) in order to prevent photobleaching.

Both immunohistochemical and immunocytochemical reactions were analyzed in fluorescence microscope (Nikon Eclipse Ti-U), equipped with a camera (Nikon Digital Sight DS-SMC) running on the NIS-Elements AR 3.00 software (Nikon Instruments Inc.).

**Flow cytometry**

In order to complete flow cytometric analysis, the cells obtained from the amniotic membrane were harvested sequentially after 16 hours, and on days 7 and 14 of culture management in two different media. Immunostaining was performed for 30 minutes at 4°C in the dark, using PE anti-SSEA-3 rat IgM, Alexa Fluor 647 anti-Human TRA-1-81 mouse IgM, (560461, Human Pluripotent Stem Cell Sorting and Analysis Kit BD Pharmingen), PE anti-human TRA-1-60 mouse IgM, (560193, BD Pharmingen), Alexa Fluor 647 anti-human TRA-1-60-R mouse IgM, (330606, BioLegend) and PerCP anti-human/mouse SSEA-4 mouse $IgG_3$ (FAB1435C, R&D Systems), in combinations. In addition, matched isotype controls were used, namely: FITC Mouse IgM, PE Rat IgM, Alexa Fluor 647 Mouse IgM (560461, Human Pluripotent Stem Cell Sorting and Analysis Kit BD Pharmingen), PE Mouse IgM (401611, BioLegend), Alexa Fluor 647 Mouse IgM (401618, BioLegend) and PerCP Mouse IgG3 (IC007C, R&D Systems). Each time, at least 10 000 events were analyzed by flow cytometry (FACS Aria, Becton Dickinson) with the BD FACSDiVa software.

**Statistical analysis**

Statistical analysis was performed using Mann-Whitney U test. The level of significance was set at $p<0.05$.


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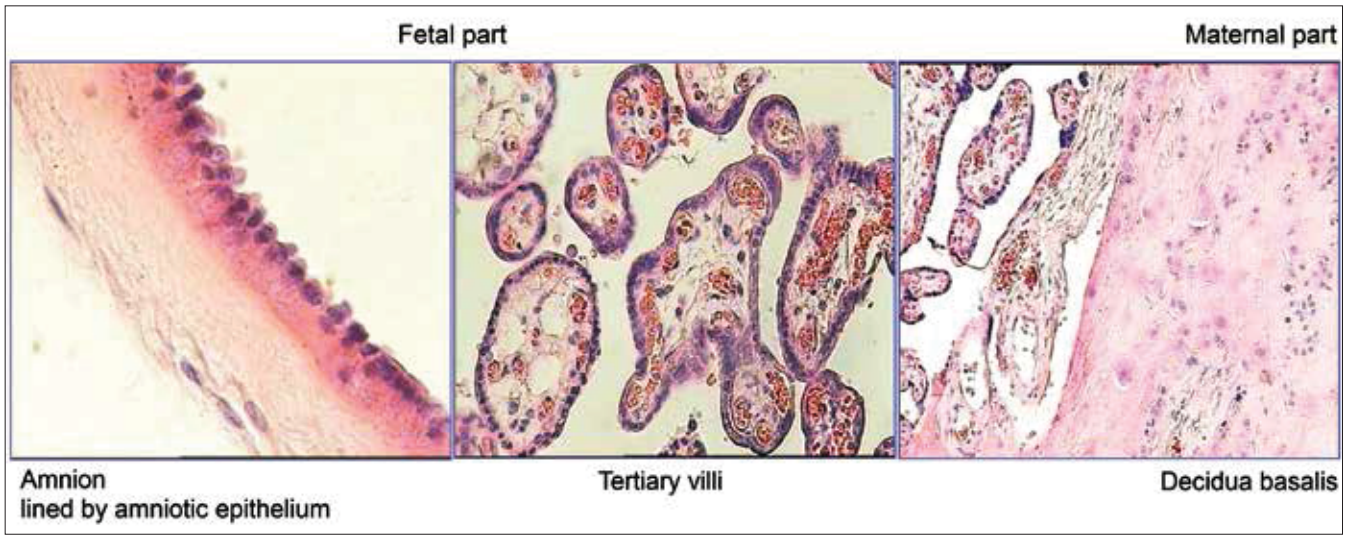


**Figure 1.** Topography of the human placenta at term. The tissues in which stem cell markers were identified are shown (H-E staining). Human amnion contains mesenchymal sparse stromal cells and it is lined by closely packed ectodermal epithelial cells.

## Results

In the first stage of the experiment we performed *in vivo* immunolocalization, followed by *in vitro* immunodetection and flow cytometric quantification of amniotic cells expressing markers of pluripotency isolated from normal term human placenta (Figure 1).

### Immunolocalization of SSEA-3$^+$, SSEA-4$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ cells in human placenta

It was shown that the cells characterized by SSEA-4 (Figure 2), SSEA-3 (Figure 3), TRA-1-60 (Figure 4) and TRA-1-81 (Figure 5) marker expressions were present mostly in the amniotic epithelium, and in a smaller number (SSEA-4 and SSEA-3) in the stem and tertiary villi. No immunoreactivity in the decidual cells was observed. Data gathered from the immunohistochemical reaction under the fluorescence microscope showed that SSEA-4$^+$ cells were the most numerous in the amnion. Cells expressing SSEA-3, TRA-1-60 and TRA-1-81 markers were spread between the cells of the amniotic epithelium, sometimes grouped in small clusters.

### Isolation of the amniotic cells and *in vitro* qualification and quantification of SSEA-3$^+$, SSEA-4$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ cells

The obtained mean efficacy of cell isolation was 9.22 million cells from 1g of human amniotic tissue (Table I). The isolation of the cells from the amniotic membrane was followed by the immunodetection of SC markers in the primary culture (Figure 6) and flow cytometric qualitative and quantitative analysis of SSEA-3$^+$, SSEA-4$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ subpopulations of human amniotic cells (Figure 7).

Our analysis showed that isolated amniotic cells expressed all four markers. SSEA-4$^+$ cells were the most numerous (62–95%), followed by cells with SSEA-3 marker (4.9–23.8%), TRA-1-81 (5–7%), and finally cells marked with TRA-1-60 (3-4% of the entire population). (Figure 9).

### *In vitro* SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 co-expression

In the next step of the experiment, we investigated the presence of a population of cells expressing the four evaluated ESC markers together in the amnion (Figure 7 and Figure 8).

The multi-parameter flow cytometry analysis revealed a very small number of cells (<0.3%) expressing SSEA-3 together with SSEA-4, TRA-1-60 and TRA-1-81. Different combinations of SSEA-4 marker with SSEA-3, TRA-1-60 and TRA-1-81 antigens, comprising from 3.5 to 6.65% of the primary culture, were found.

### Cell culture in Alpha MEM/FBS and mTeSR1 medium

Finally, we executed a quantitative analysis of SSEA-3$^+$, SSEA-4$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ subpopulations during a 14-day incubation period of the amniotic cells in two different media: Alpha MEM/FBS and mTeSR1. The quantitative data of the studied subpopulations, obtained from the flow cytometry on days 1, 7, and 14 of the incubation (Figure 9), did not reveal the presence of significantly different numbers of SSEA-3$^+$, SSEA-4$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ cells. In the primary culture and during the 14-day culture, both in mTeSR1 medium containing significantly reduced amount of proteins of animal origin and in Alpha MEM medium containing 10% FBS, we did not observe significant, falsified positive changes in the number of cells expressing both, SSEA and TRA markers.

## Discussion

The applicability of SSEA-3 and SSEA-4 in the characteristics of SC has recently been questioned due to the fact that some glycans are likely to appear on the cell surface as the result of metabolic incorporation. It was also found that, a clear-cut switch in the core structures of glycosphingolipids occurs during hESC differentiation into embryoid bodies, causing cross-reactivity of antibodies against SSEA-3 and SSEA-4 with


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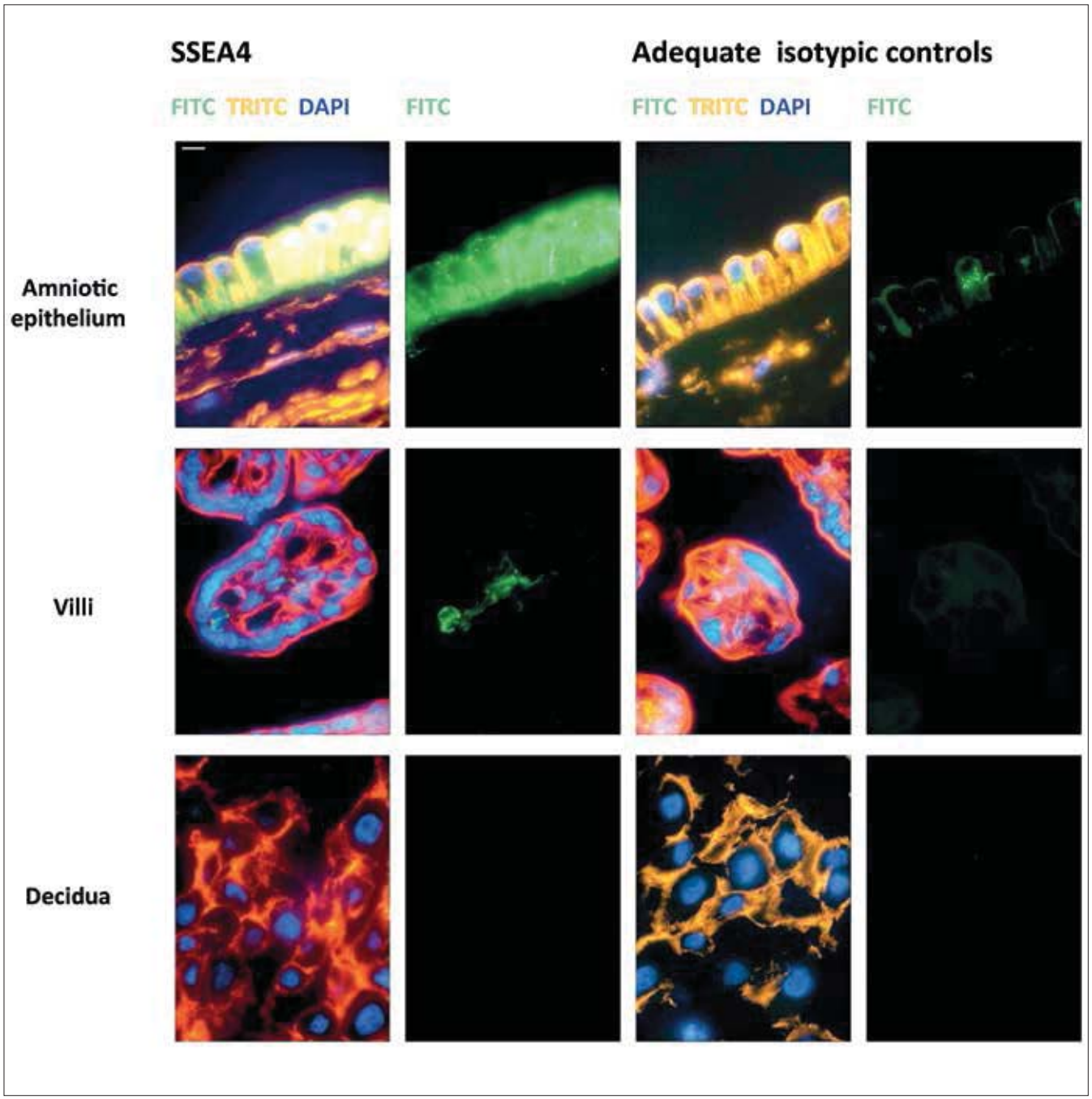


**Figure 2.** Immunolocalization of SSEA-4⁺ cells in human placenta at term. FITC (green) - anti-SSEA-4 antibody (left columns) and adequate isotypic controls (right columns) conjugated with FITC; TRITC (red-orange) - F-actin stained with Rhodamine phalloidin; DAPI (blue)- cell nuclei stained with DAPI. Specific staining for SSEA-4 is shown separately in the left column FITC. Scale = 10 μm.

these molecules [25]. In addition, the presence of SSEA antigens on the surface of cells in culture conditions has been suggested to be the consequence of changes in their nature, depending on the cell-medium interactions involving: the incorporation of glycosphingolipids of FBS, induction of gene expression of glycosyltransferases or FBS-induced activity of enzymes involved in the biosynthesis of SSEA-3 [21].

FBS itself contains a detectable amount of globoseries glycosphingolipids and a molecule corresponding to the size of SSEA-4 could be detected [19]. Recently, a number of cell culture media formulations for maintaining hESC and retaining the advantages of being fully-defined and serum-free, have been described. We were able to verify the hypothesis that a culture environment can change the expression pattern of markers of pluripotency during a 14 day amnion-derived SC culture in two different media.

At the first stage of the experiment we chose and quantified the cells expressing SSEA-3 and SSEA-4, as well as uncontested markers of pluripotency, namely: TRA-1-60 and TRA-1-81, which are specific indicators for ESC [16, 18, 19, 26].


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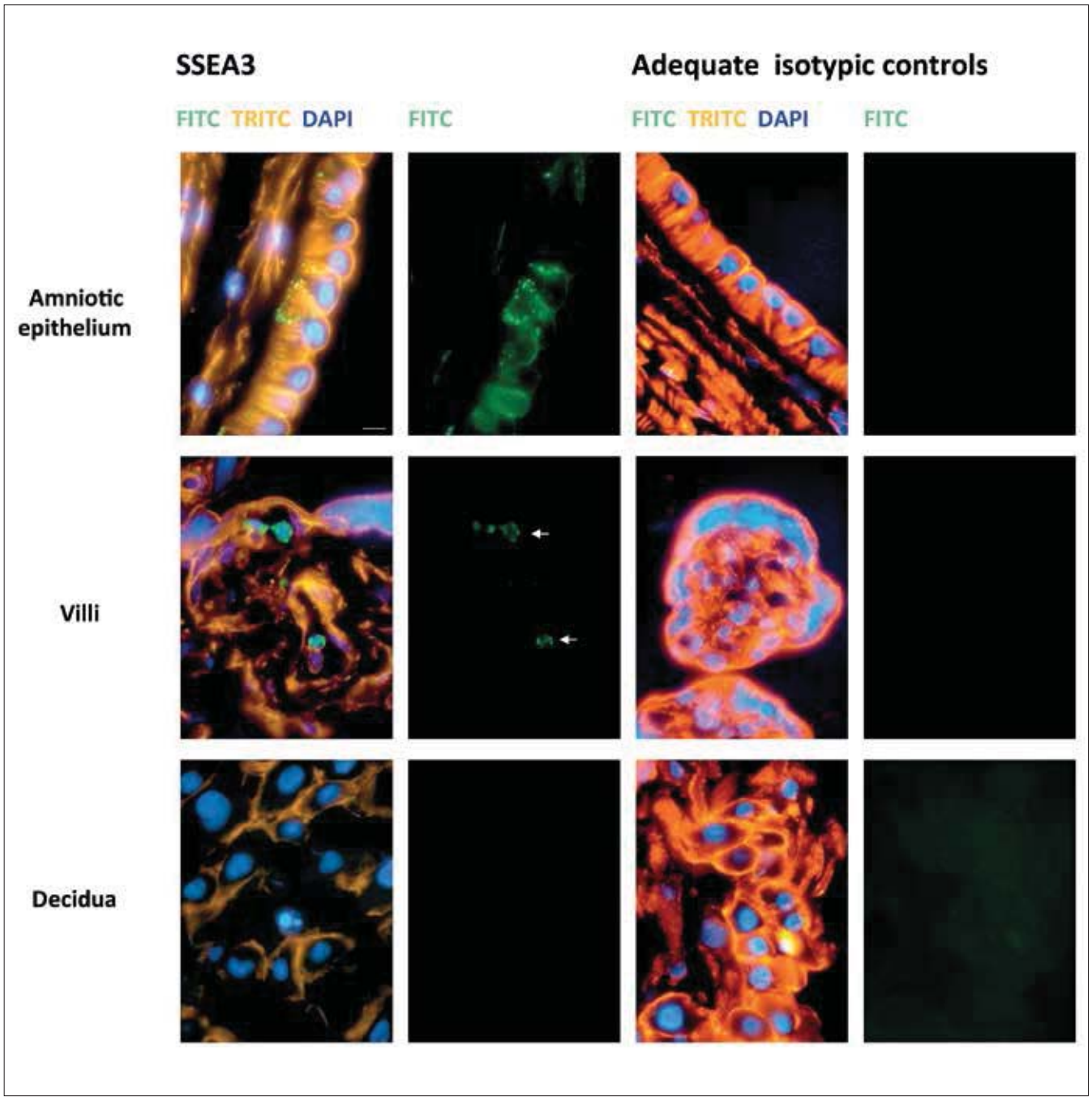


**Figure 3.** Immunolocalization of SSEA-3$^+$ cells in human placenta at term. FITC (green) - anti-SSEA-3 antibody (left columns) and adequate isotypic controls (right columns) conjugated with FITC; TRITC (red-orange) - F-actin stained with Rhodamine phalloidin; DAPI (blue) - cell nuclei stained with DAPI Specific staining for SSEA-3 is shown separately in the left column FITC. Scale = 10 μm.

It is known that the compositions of SC of the amniotic membrane comparing with other placental tissues are quite different and it seems that the amniotic membrane retains pluripotent SC characteristics. It may reflect the different origin of the amnion (from pluripotent epiblast) and the chorion (from trophectoderm), as well as the distance between the amniotic cells and the centers of organogenesis where they develop, the extracellular matrix is produced, and growth factors are secreted. It was also confirmed *in vivo* by Izumi et al., [27] that most epiblast-derived fetal amniotic epithelial cells are positive for SC markers in the early second trimester of the pregnancy (17-19 weeks) and some of them remain in the term amnion. The authors observed that the percentage of TRA-1-60$^+$ and TRA-1-81$^+$ cells decreased over the course of pregnancy, from about 30-40% to about few percent.

The above mentioned results, as well as our results which identified the expression of SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 in fetal tissues of human term placenta, especially in the amniotic epithelium, indicate that even at term, the ratios of the cells expressing all four markers in the human amnion (from few to more than 50%) are considerably higher than in other placental tissues, as well as in adult SC (0.01-0.1%), for


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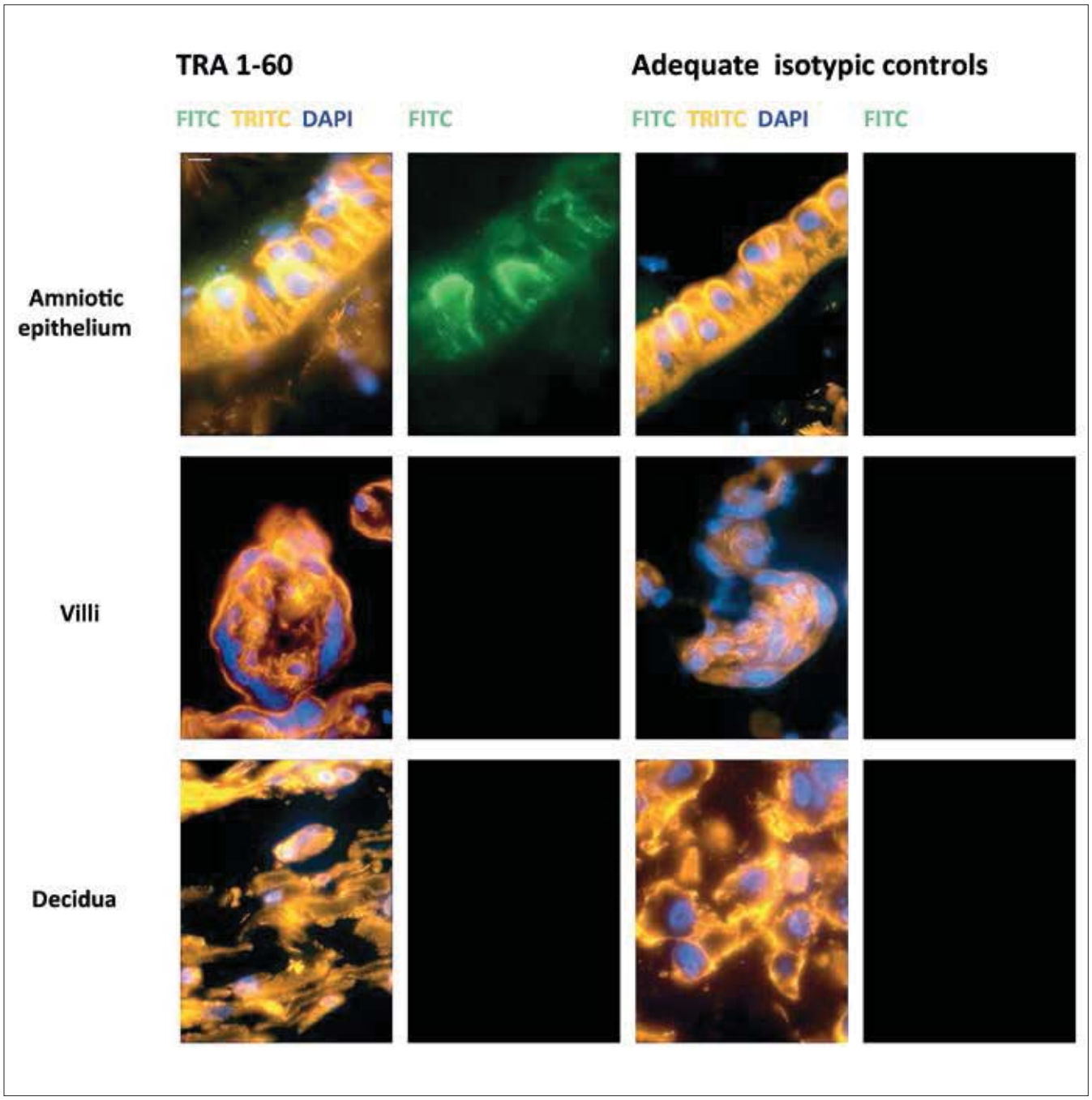


**Figure 4.** Immunolocalization of TRA-1-60$^+$ cells in human placenta at term. FITC (green)- anti-TRA-1-60 antibody (left columns) and adequate isotypic controls (right columns) conjugated with FITC; TRITC (red-orange)- F-actin stained with Rhodamine phalloidin; DAPI (blue)- cell nuclei stained with DAPI. Specific staining for TRA-1-60 is shown separately in the left column FITC. Scale = 10 µm.

example in bone marrow [28]. The proportion of amniotic cells which react with the SSEA-4, TRA-1-60, TRA1-81, and SSEA-3 antibodies, together with the fact that anti-SSEA-3 and anti-SSEA-4 antibody recognizes different parts of the ganglioside and that anti-TRA-1-60 and anti-TRA-1-81 antibody recognizes different epitopes, suggest that amniotic SC are at various stages of differentiation. It indicates that the amniotic cell population consists of different subsets. Similarly, as in the study by Miki et al., [29] no specific pattern of the SC marker-positive cells distribution was observed in different parts of the amnion. The authors suggested that amniotic SC are not organized by external regulatory signals. They showed also that almost all amniotic epithelial cells react with antibodies specific for SSEA-4 and 10-20% with antibodies to TRA-1-60 and TRA-1-81. Amniotic mesenchymal cells, and cells observed in any other part of the human placenta, chorion and decidua basalis, were negative for SSEA-4, TRA-1-60 and TRA-1-81. In histological sections taken from the human placenta, SSEA-3$^+$ cells were not observed in any layer of the placenta *in vivo*. That finding is in contrast to our observations that both SSEA-4$^+$ and SSEA-3$^+$ cells are present in the amniotic epithelium and that small subpopulations of these cells are present in stem and tertiary villi. It should be also noted


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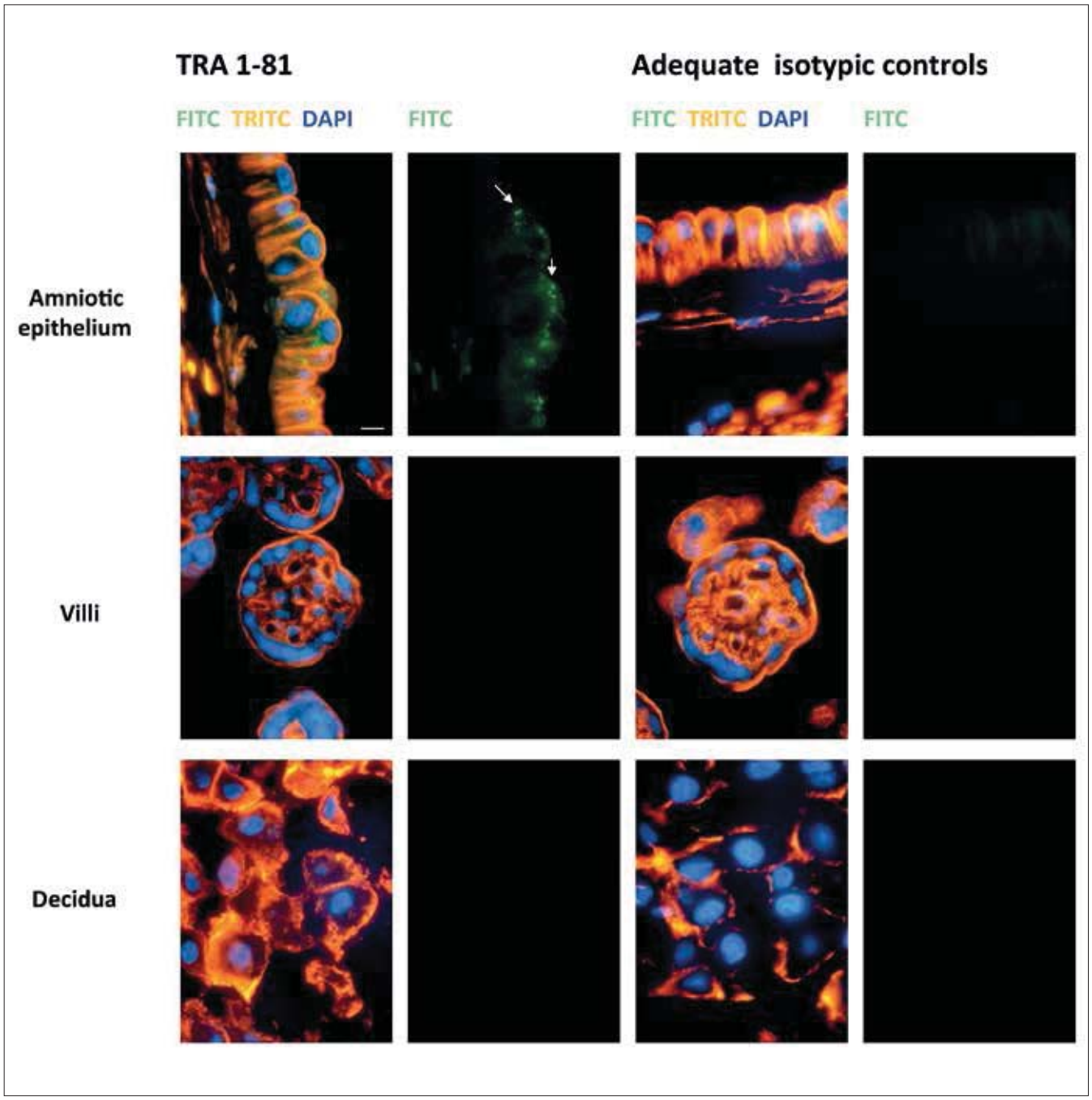


**Figure 5.** Immunolocalization of TRA-1-81$^+$ cells in human placenta at term. FITC (green)- anti-TRA-1-81 antibody (left columns) and adequate isotypic controls (right columns) conjugated with FITC; TRITC (red-orange) - F-actin stained with Rhodamine phalloidin; DAPI (blue) - cell nuclei stained with DAPI. Specific staining for TRA-1-81 is shown separately in the left column FITC. Scale = 10 μm.

that Huang et al., found that all above mentioned antigens, as well as Oct-4, can be detected in MSC derived from the decidua basalis, ranging from 33.5% to 44.6% [30].

In earlier studies, Miki et al., found and quantified *in vitro* amniotic SSEA-4$^+$ (43.9%), TRA-1-81$^+$ (9.9%), TRA-1-60$^+$ (9.8%) and SSEA-3$^+$ (8.8%) cells, despite the fact that the latter were not identified *in vivo* [31]. The difference in amniotic SSEA-3$^+$ *in vivo* and *in vitro* identification may result from a temporary appearance of this antigen on the cells. It was probably not influenced by components of the culture media (including 10% FBS) because in our study we identified SSEA-3$^+$ cells both *in vivo* and *in vitro*. Our results, based on one-marker identification *in vitro*, showed amnion-derived SC to be phenotypically heterogeneous, with the dominance of the SSEA-4$^+$ cells (62–95%). Subpopulations of cells with SSEA-3, TRA-1-81, and TRA-1-60 markers were significantly less numerous (4.9–23.8%, 5–7% and 3-4%, respectively). These observations confirm that amniotic epithelium offers the largest potential reservoir of cells expressing markers of pluripotency, especially SSEA-4, among other tissues of the human placenta.

Data analyzing the co-expression of antigens that are pluripotency markers on amniotic SC are scarce.


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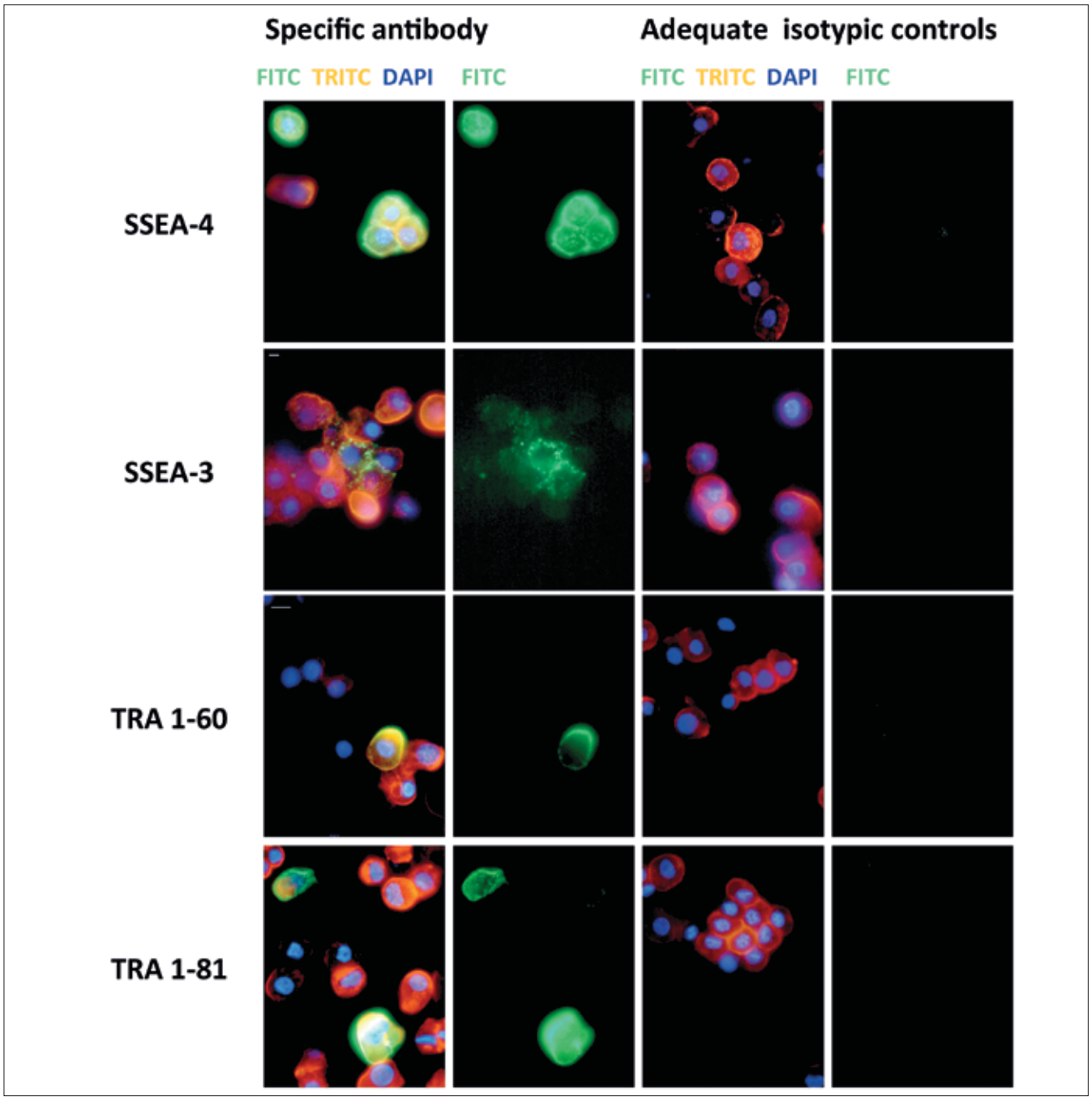


**Figure 6.** Immunodetection of SSEA-4$^+$, SSEA-3$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ cells in primary culture of amniotic cells. FITC (green) - specific antibodies (left columns) and adequate isotypic controls (right columns) conjugated with FITC; TRITC (red-orange) - F-actin stained with Rhodamine phalloidin; DAPI (blue) - cell nuclei stained with DAPI. Specific staining for SSEA-4, SSEA-3, TRA-1-60 and TRA-1-81 is shown separately in the left column FITC. Scale = 10 µm.

In our study, the multi-parameter flow cytometry analysis revealed a very small number of cells (<0.3%) expressing SSEA-3 together with SSEA-4, TRA-1-60 and TRA-1-81, in the human amnion. Different combinations of the SSEA-4 marker with SSEA-3, TRA-1-60 and TRA-1-81 antigens, comprising from 3.50 to 6.65% of the primary culture, were found. It seems that SSEA-4$^+$ subpopulation is heterogeneous and only about 5.7% of the amniotic cells contain both, SSEA-4 and SSEA-3 antigen. The possibility that SSEA-3, TRA-1-60 and TRA-1-81 are not the main SSEA-4 partners in the co-expression on the amniotic cells cannot be ruled out. Other authors found that SSEA-4 staining was not confined to the TRA-1-60$^+$ cells in colonies of various pluripotent hESC lines stained with various markers at different time points. TRA-1-60$^+$ cells exhibited overlapping specific Oct-4 and/or Nanog staining. Co-expression of all markers was detected only in small areas [32].

As a consequence, amniotic SC, like hESC, may be divided into many different cell subpopulations and diversified in their clonogenic self-renewal and pluripotent properties [33]. It seems also that human amnion cells display some characteristic properties of SC, regardless whether they are located in the amniotic stroma or the epithelium, and different embryological origin of


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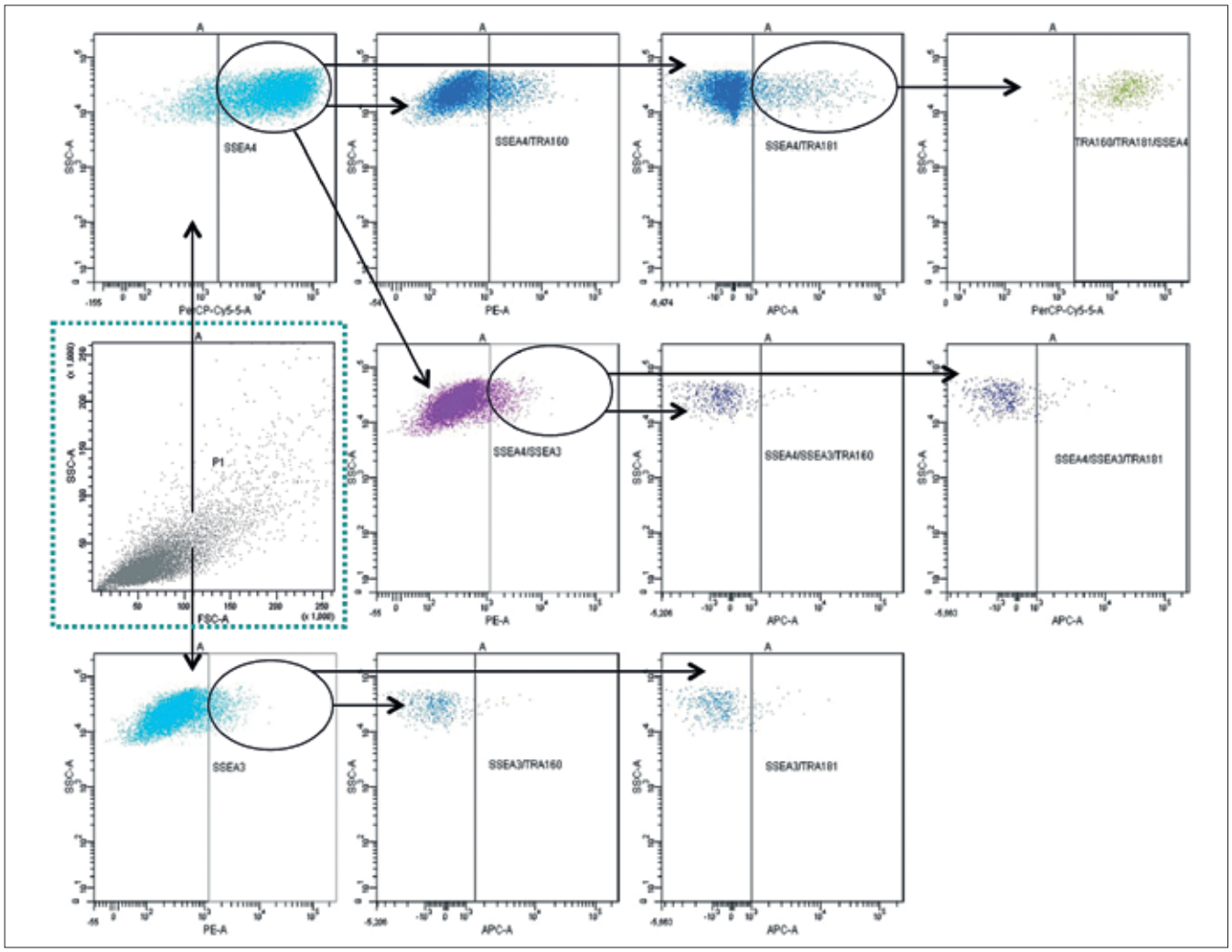


**Figure 7.** Flow cytometric analysis of amnion-derived cells exhibiting co-expression of SSEA-4, SSEA-3, TRA-1-60 and TRA-1-81. Arrows show the consecutive steps of the analysis starting from the primary culture (P1 population).

these layers. Both populations had a similar immunophenotype and potential for *in vitro* differentiation into major mesodermal lineages [34]. Lack of significant quantitative differences in the number of SC based on the expression and co-expression of SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 markers observed in our study may indicate that the expression of these markers *in vitro* was not influenced by the components of the Alpha MEM/FBS and mTeSR1 culture media.

Based on different number of amniotic SSEA-4$^+$ and SSEA-3$^+$ cells identified in presented studies, the question arises whether these cells exhibit more or less pronounced pluripotency. During hESC differentiation, SSEA-3 and SSEA-4 successively disappeared from the surface of the cells and SSEA-4, but not SSEA-3, was found on MSC [19]. That absence did not seem to exert a significant influence on cell pluripotency [20]. Human primordial germ cells were positive for both, SSEA-3 and SSEA-4, but human amniotic stem cells CD117$^+$, able to generate cell lineages of different germ layers, were positive for SSEA-4 and negative for SSEA-3 [17].

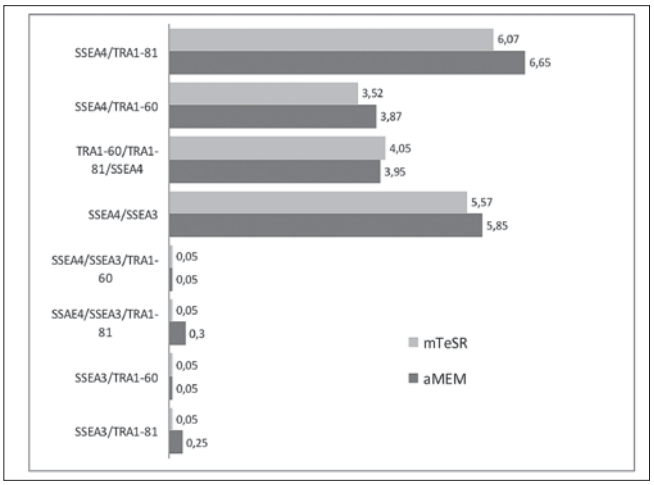


**Figure 8.** Quantitative data of the co-expression analysis of stem cell markers on amnion-derived cells. The results show a number of positive-staining cells as a percent of primary population. Amniotic cells taken from the placenta were cultured in both mTeSR1 and Alpha MEM/FBS. The values are means of measurements from two placentas. The differences between the cultures in two different media were not significant.


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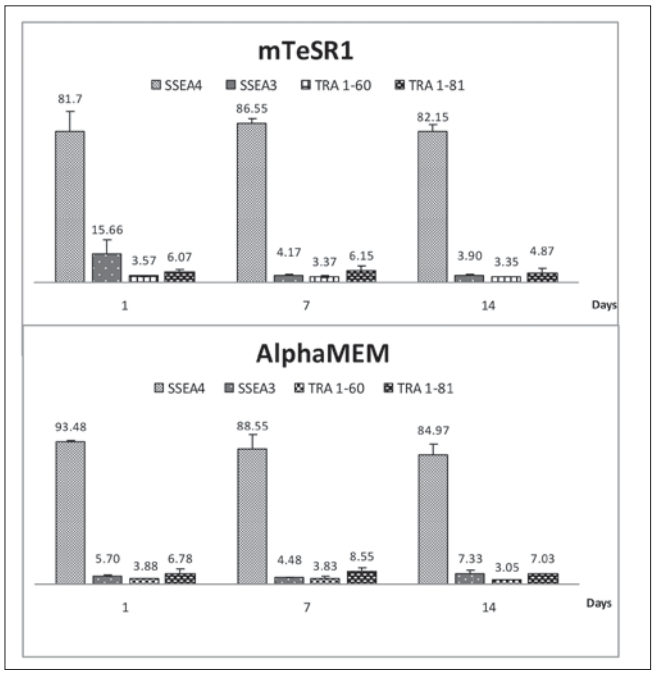


**Figure 9.** The percent of SSEA-4$^+$, SSEA-3$^+$, TRA-1-60$^+$ and TRA-1-81$^+$ cells during 14 days of amnion-derived cells culture in two different media. The flow cytometric analysis was performed on day 1, 7 and 14 of the culture; n=7 for SSEA-4$^+$ cells and n=5 for SSEA-3$^+$ cells (mTeSR1, day 1st), and n=2 for all other measurements in mTeSR1 and Alpha MEM/FBS.

Moreover, hESC generated from both, SSEA-3$^+$ and SSEA-3$^-$ cells retained their pluripotent potential and chromosomal stability, and were able to generate teratomas containing all three germ layers upon injection into immunodeficient mice [33]. On the other hand, both SSEA-3 and SSEA-4 antigens, as well as TRA-1-60 and TRA-1-8, were also found on human first-trimester fetal blood, the liver, and bone marrow mesenchymal stem cells (MSC). The data on their presence on adult MSC are inconclusive [21, 35, 36].

## Conclusion

To sum up, this preliminary study shows that the evaluation of SSEA-3 expression and its sialylated derivative - SSEA-4, as well as the recognized SC markers: TRA-1-60 and TRA-1-81 which can be authoritative indicators of pluripotent cells in the primary culture, has not been influenced by media components. Amniotic SC expressing SSEA epitopes can be identified independently of the FBS presence in the content of the culture media.

We have recently reported that human amnion might have attracted attention as an alternative source of SSEA-4$^+$ SC, but to a lesser degree of SSEA-4$^+$ cells exhibiting co-expression of the known markers of pluripotency: SSEA-3$^+$, TRA-1-60$^+$ and TRA-1-81$^+$. The cells with characteristics of ESC – SSEA-4$^+$, TRA-1-60$^+$, TRA-1-81$^+$, as well as the cells expressing the SSEA-3 marker, could be identified in the fetal part of the human placenta, not only in the amnion but also the chorion.

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