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COMPARATIVE PHENOTYPIC ANALYSIS OF POPULATIONS OF HEMOTOPOIETIC PROGENITOR CELLS WITH DIFFERENT EXPRESSION LEVEL OF CD34 DERIVED FROM PLACENTAL TISSUE AND UMBILICAL CORD BLOOD

The investigation of placental HPCs and compare their properties with other fetal and adult HPCs are necessary for the evaluation the possibility of their clinical applying. It has been shown that placental tissue contains three populations with different level of CD34 expression such as $CD34^{+++}CD45^{low}$, $CD34^{++}CD45^{low}$ and $CD34^{+/low}CD45^{low}$. Similar to fetal liver placenta contain both population of $CD34^{++}CD45^{low/-}$ and $CD34^{+}CD45^{low/-}$cells that suggested about hematopoiesis in placental tissue. $CD34^{++}CD45^{low/-}$ population also express CD133, almost negative by lineage markers and had lymphocyte-like morphology that give evidence that this population contain primitive HPCs potentially stem cells. Also among placental cells are later progenitors with phenotype $CD34^{+/low}CD45^{+}$ as majority of these cells express hematopoietic lineage markers. Population with phenotype $CD34^{+++}CD45^{low}$ was observed only in placenta that suggests generated perhaps only in the placental tissue or migrate from the other sites of hematopoiesis and change the level of CD34 expression. Enzymatic treatment has insignificant effect on level of some cell surface protein in FACS analyze that worth to take into account in such experiments.

Keywords: hematopoietic progenitor cells, placental hematopoiesis, the expression of CD34 marker, umbilical cord blood.

Introduction. Nowadays the serious problem of hematology remains a deficiency of donors of hematopoietic progenitor cells (HPCs) for the transplantation in cases of hematologic malignancies and congenital disorders of hematopoiesis. Thereby the search of the new additional sources of HPCs is important for medicine. Recently it was shown that the human placenta plays an important role in fetal hematopoiesis [2, 8]. On the other hand the immunophenotype of placental-derived HPCs and their multipotency are still insufficient studied. The investigation of placental HPCs and compare their properties with other fetal and adult HPCs are necessary for the evaluation the possibility of their clinical applying. Thus the aim of this study was compare the phenotype of placental HPCs with umbilical cord blood HPCs.

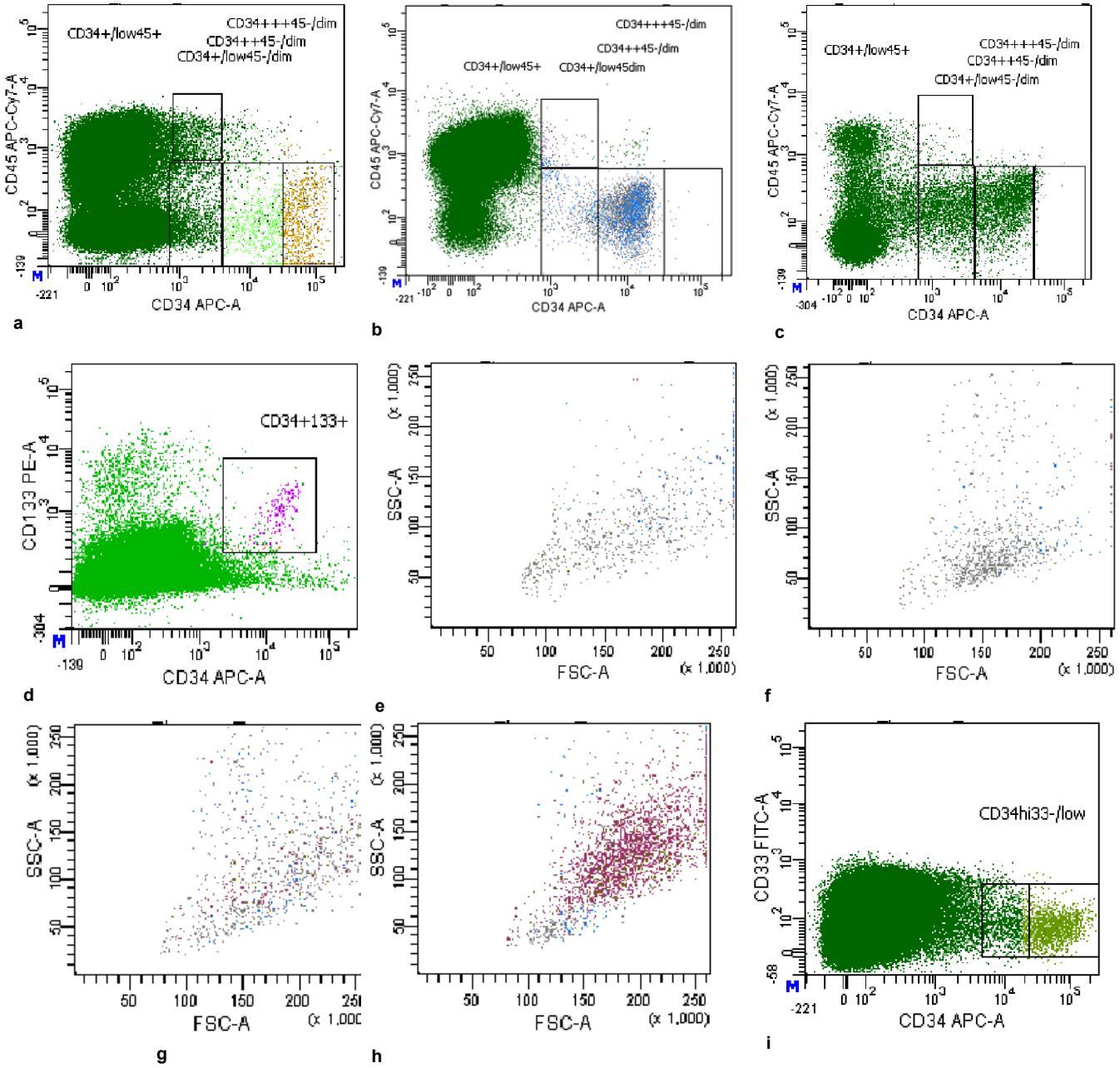
Materials and methods. *Placental and cord blood mononuclear cells isolation.* Placentas were obtained after natural childbirth or by Cesarean section on the 39 – 41 weeks in 23 – 36 years woman after informed consent. Umbilical cord blood was collected using standard cord blood collection techniques. All samples were tested on aerobic, anaerobic bacterial and fungal contamination. Maternal blood was tested for evidence of infection agent such as HIV-1/2, HCV, HBV, CMV and Treponema pallidum. Placental tissue was additional assay for Chlamidium trachomatis, Mycoplasma genitalis, Ureaplasma urealyticum and Ureaplasma parvum, HSV-1/2, CMV. The umbilical cord was cut and removed along with amniotic sac and deciduas from the placenta. Placenta was minced on small fragments by scissors. Remains placental blood was extensively washed from fragments of placental tissue on shaker until tissue became colorless. Placental tissue was treated with 0,2% collagenase I (Serva, Germany), 0,35 mg/ml hyaluronidase (Sigma, USA), 100 U/ml DNase I (Sigma, USA), 1 mg/ml BSA for 30-40 min at +37°C. After that placental cells were isolated by filtration through a 70μm cell strainer (Becton Dickinson, USA). Remaining tissue was incubated with fresh portion of enzymes for 20-40 min at +37°C. Mononuclear cells from placenta and umbilical cord were recovered by Ficoll density gradient fractionation (Density 1,077g/ml, Biochrome, Germany), washed twice, and filtered through a 40 μm cell strainer . Mononuclear cells from umbilical cord blood were treated with the same mix of enzymes for 50 min at +37°C and ones washed in PBS .

Flow cytometry analysis. For immunophenotyping placental and cord blood cells were stained with the following fluorochrome conjugated monoclonal antibodies (Becton Dickinson, USA): anti-CD34 APC, anti-CD90 FITC, anti-CD45 APC-Cy7, anti-CD105 PerCP-Cy 5.5, anti-CD73 PE, anti-CD14 Pacific Blue, anti-CD31 PE, anti-CD133 PE, anti-45RA FITC, anti-CD7 PE, anti-CD19 PE-Cy7, anti-CD33 FITC, anti-CD235a PE. Measurements were performed on cell sorter BD FACSAria (Becton Dickinson, США).

Statistical analysis. Results are expressed as mean with 95%-confidence interval. Statistical significance was determined using the U-Mann– Whitney test.

RESULTS. In previous investigation we have shown that ISHAGE protocol is more appropriate for FACS analysis of HPCs derived from native and cryopreserved placental tissue [9]. Analysis by this protocol shown that content of population HPCs that have phenotype $CD34^{+}CD45^{low}$ with lymphocyte-like morphology (SSC^{low}) among $CD45^{+}$ cells from native placental tissue was 0,60 % (0,39 – 0,86 %, n = 13). The percentage of $CD34^{+}CD45^{low}SSC^{low}$ among $CD34^{+}CD45^{low}$ was 78,5% (70,5 – 85,6%, n=13).We have shown that placental tissue consist of three populations that differ in expression level of CD34. We designate they as $CD34^{+/low}CD45^{low/-}$, $CD34^{++}CD45^{low/-}$ and $CD34^{+++}CD45^{low/-}$. Two populations $CD34^{+/low}CD45^{low/-}$ and $CD34^{++}CD45^{low/-}$ were determined both in placental tissue and in cord blood (Fig.1a,b). $CD34^{++}CD45^{low/-}$ population from placental tissue were also positive by CD133 (Fig.1d). The level of expression of CD14 on placental cells with phenotype $CD34^{+/low}CD45^{low/-}$ and $CD34^{++}CD45^{low/-}$ was 7,24 % (3,27 – 12,62%, n=4) and 3% (0,48 – 7,57%, n=4) in contrast to the same populations of cells from cord blood where it almost missing. Population of cells with phenotype $CD34^{+++}CD45^{low/-}$ was present in placental tissue and almost absent in both cord blood and fetal liver (Fig.1a-c). The percentage of $CD34^{+++}CD45^{low/-}$ cells among all viable mononuclear cells was 0,28% (0,05-0,70) in placental tissue and 0,006% (0,003-0,01) in cord blood. FASC analysis was shown that $CD34^{++}CD45^{low/-}$ cells isolated from placental tissue in general have lymphocyte-like morphology ($FSC^{low}SSC^{low}$) whereas both $CD34^{+}CD45^{low/-}$ and

$CD34^{+++}CD45^{low}$ cells are very heterogeneous by morphology (Fig.1e-g). Also increasing of expression of CD45 on $CD34^{+}$-cells lead to increasing their granularity and size (Fig.1h). The multiparameter flow cytometry analysis demonstrated that $CD34^{+++}CD45^{low}$ cells had immunophenotype $CD33^{-/low}$, $CD14^{-/low}$, $CD235^{-}$, $CD19^{-}$, $CD7^{-/low}$, $CD45RA^{-}$ (Fig.1i-n). The level of expression of CD14 on $CD34^{+++}CD45^{low}$ placental cells were 4,25 % (1,49 – 8,35%, n=4). Expression of CD14 was increased with decreasing of expression level of CD34 on $CD45^{low/-}$cells from placental tissue. Similarly the expression of CD7 and CD19 increased with decreasing expression level of CD34 on $CD45^{low/-}$cells from placental tissue and cord blood. Unlike cord blood and fetal liver placenta contain the population $CD34^{+/low}CD45^{+}$ cells. This population characterized by higher levels of lineage markers expression compare to population with lower level of CD45 and with higher level of CD34. The level of expression of CD14 together with CD33 on $CD34^{+/low}CD45^{+}$ cells was 73,5% (54,15 – 88,39%, n=4), the expression level of CD7 and CD19 on this population were 14,17% (8,38 – 21,18%, n=4) and 3% (1,38 – 5,24%, n=4) respectively. Furthermore level of expression hematopoietic lineage markers increased on placental CD34 positive cells with increasing expression level of CD45 and decreasing CD34 (Fig.1o-r).



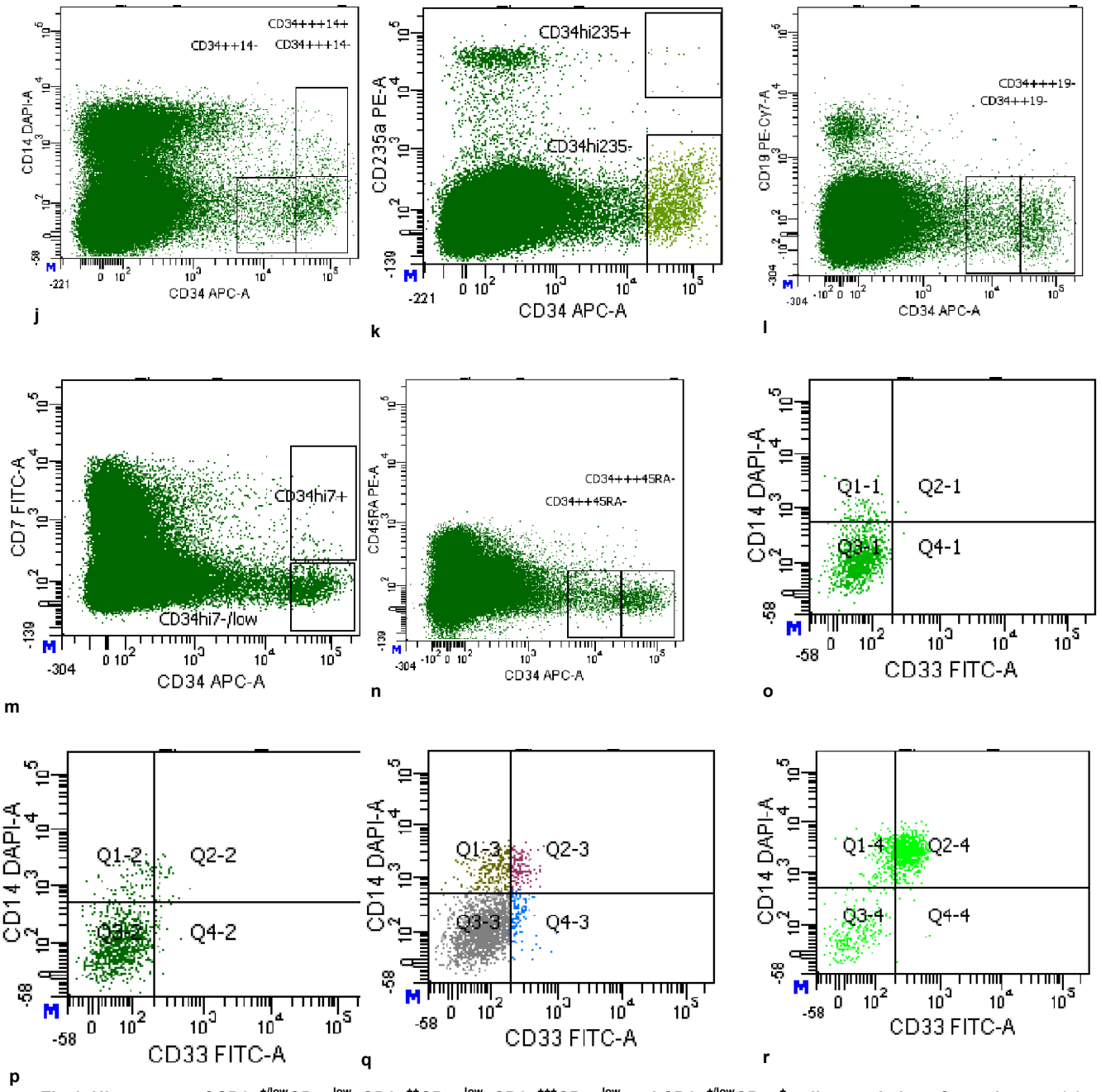


Fig 1. Histograms of CD34$^{+/low}$CD45^{low}, CD34^{++}CD45^{low}, CD34^{+++}CD45^{low} and CD34$^{+/low}$CD45^{+} cells populations from placenta (a), cord blood (b) and fetal liver (c). Histogram of expression of CD34 and CD133 in placental tissue (d). Histograms of FSC/SSC (morphology of cells) for populations with phenotype CD34^{+++}CD45^{low} (e), CD34^{++}CD45^{low} (f), CD34$^{+/low}$CD45^{low} (q) and CD34$^{+/low}$CD45^{+} (h) from placenta tissue. Histograms of expression of CD34 and lineage markers in placental tissue such as CD33 (i), CD14 (j), CD235 (k), CD19 (l), CD7 (m) and CD45RA (n). Histograms of expression of both CD14 and CD33 on cells populations with phenotype CD34^{+++}CD45^{low} (o), CD34^{++}CD45^{low} (p), CD34$^{+/low}$CD45^{low} (q) and CD34$^{+/low}$CD45^{+} (r) from placenta tissue. The results are representative of four experiments

We investigated the effect of enzymatic treatment that we used for isolation of placental cells on the expression level of some hematopoietic markers in umbilical cord blood. In most analysis we didn't observed a significant differences in the level of the expression of markers. However we have shown that the amount of cells in populations with phenotype CD7^{+}CD45RA^{+} CD45^{+}CD45RA^{+} were significant increased in enzymatically treated samples (Tab.1).

***Table* 1. The influence of enzymatic treatment on the selection of umbilical cord blood cells with some phenotype**

Markers	Level of markers expression in umbilical cord blood, %	Level of markers expression in umbilical cord blood after enzymatic treatment, %
CD7CD45RA	16,7 (10,4-24,3) *	25,2 (21,5-29,7) *
CD45CD45RA	40,4 (31,3-49,8) *	52 (44,7-59,1) *
CD33	12,6 (3,9-25,2)	19,6 (10,0-31,5)
CD34	0,9 (0,6-1,2)	1,1 (0,7-1,6)
CD14	10,2 (1,1-27,0)	17 (8,2-28,3)
CD19	19,5 (8,7-33,2)	16,2 (7,9-26,8)

* – significantly different, n=4, p≤0,05. All data not presented

Discussion. The analysis of the phenotype of placental mononuclear cell fraction once more confirmed that placental tissue contains hematopoietic progenitor cells. The percentage of $CD34^{+}CD45^{low}SSC^{low}$ among $CD34^{+}CD45^{low}$ that is lower than in cord blood evidence about necessity of gating the $CD34^{+}CD45^{low}$ cells by morphology as described in previous our work [9].

Placental tissue contains HPCs with different expression level of CD34 that we suggested evidence about different stage of immaturity. Similar to fetal liver placenta contain both population of $CD34^{++}CD45^{low/-}$ and $CD34^{+}CD45^{low/-}$cells that suggested about hematopoiesis in placental tissue. On the other hand among placental cells are more mature HPCs with phenotype $CD34^{+/low}CD45^{+}$ in contrast to fetal liver and cord blood. Majority of these cells express hematopoietic lineage markers that indicate they are later progenitors. We have shown that expression of lineage markers on HPCs such as CD14, CD7 and CD19 increased with decreasing of expression level of CD34. The fact that we find progenitors of different stage of differentiation into mature placental tissue suggests that such cells continue to generate in the placenta and/or migrate to the placental tissue and didn't disappear until the moment of birth.

Also we shown that CD133 mostly was expressed by placental and cord blood $CD34^{++}CD45^{low/-}$ that give evidence about primitively of this population of cells. Their stage of immaturity was confirmed by the level of lineage markers CD33, CD235, CD19, CD7 and CD45RA on the verge of absence however some cells among this population expressed CD14. Interestingly placenta contain $CD34^{+++}CD45^{low/-}$ cells that as well as $CD34^{++}CD45^{low}$ didn't express lineage markers but in contrast to $CD34^{++}CD45^{low/-}$ cells were negative for stem cells markers CD133 and have heterogeneous morphology. The absence of such cells in cord blood and fetal liver suggests that they perhaps generated only in the placental tissue or migrate from the other sites of hematopoiesis and change the level of CD34 expression. We propose that population of placental HPCs which have the very high level of cell-cell adhesion protein CD34 ($CD34^{+++}CD45^{low/-}$) allows them to interact with placental cell niche. It was studied at a single cell level the characteristics of a populations of $CD34^{+++}$ hematopoietic progenitor cells that autors defined in human umbilical cord blood and these cells have extensive proliferative and replating capacity *in vitro* [3].

Thus placenta contain primitive HPCs potentially stem cells with phenotype $CD34^{++}CD45^{low/-}$. These observations are in agreement with reports showing that cells with high level of CD34 contain primitive hematopoietic progenitors including stem cells and primitiveness of progenitor cells was closely related to levels of CD34 [4, 5, 6, 7, 10]. Barcena et al. found two population $CD34^{++}CD45^{low}$ and $CD34^{+}CD45^{low}$ in chorionic villi and in the chorioamniotic membrane of different stage of placental development. $CD34^{++}CD45^{low}$ cells express markers of multipotent primitive hematopoietic progenitors and hematopoietic stem cells and demonstrate myeloid and erythroid potential *in vitro*, generated $CD56^{+}$ natural killer cells and $CD19^{+}CD20^{+}sIgM^{+}$ B cells in polyclonal liquid cultures while $CD34^{+}CD45^{low}$ content more committed progenitors [2]. Fetal bone marrow cells also contain population of cells with different expression level of CD34 ($CD34^{hi}$ and $CD34^{lo}$) wherein only $CD34^{hi}$ cells has phenotype of the most primitive hematopoietic cells such as $Thy\text{-}1^{+}$,$HLA\text{-}DR^{low}$, $CD38^{low}$, $CD45RA^{-}$, also expressed low levels of antigens CD13 and CD33 but no detectable cell surface antigens of more mature cells (CD2, CD10, CD14, CD15, CD16, CD19, glycophorin A) in addition $CD34^{hi}$ cells support long-term B lymphopoiesis and myelopoiesis *in vitro* and mediate T, B, and myeloid repopulation of human tissues implanted into SCID mice [5]. In adult bone marrow CD34 antigen density decreased concurrently with maturation and increasing CD38 antigen density [10]. It was reported that fetal liver contain cells with high level of CD34 and they were enriched for $Thy\text{-}1^{+}$, $CD117^{+}$, $CD123^{+}$, $HLA\text{-}DR^{+}$, $CD7^{-}$, $CD38^{-}$, $CD45^{-}$, $CD71^{-}$,$CD115^{-}$ and able to reconstitute lymphoid and myeloid lineages in SCID-hu mice [6].

We have shown that enzymatic treatment that we used for placenta in some cases change the expression of some hematopoietic markers in umbilical cord blood that detected by FACS. Also it was reported that the intensity of expression of CD3, CD4, CD8, αβ and γδ T cell receptors that examined by FACS was decreased by 25-40% in peripheral blood lymphocyte that were incubated with collagenase type 1 A and Dispase [1]. It is worth noting that in our case the expression level was increased in the case of enzymatic treatment. Thus when comparing the expression of markers by FACS in the two samples, one of which was treated of enzymes while the other didn't it worth to take into account that the resulting difference may be caused only by the effect of enzymatic treatment. We consider that in such cases worth to treat all samples for obtaining reliable data. In our investigation we were treated the cells from cord blood with enzymes that we used for placenta and then analyzed by FACS.

Conclusions. Placental tissue contains three populations that differ in expression level of CD34 such as $CD34^{+/low}CD45^{low/-}$, $CD34^{++}CD45^{low/-}$ and $CD34^{+++}CD45^{low/-}$. Similar to fetal liver placenta contain both population of $CD34^{++}CD45^{low/-}$ and $CD34^{+}CD45^{low/-}$cells that suggested about hematopoiesis in placental tissue. $CD34^{++}CD45^{low/-}$ population also express CD133, almost negative by lineage markers and had lymphocyte-like morphology that give evidence that this population contain primitive HPCs potentially stem cells. Also among placental cells are later progenitors with phenotype $CD34^{+/low}CD45^{+}$ as majority of these cells express hematopoietic lineage markers. Population with phenotype $CD34^{+++}CD45^{low}$ was observed only in placenta that suggests generated perhaps only in the placental tissue or migrate from the other sites of hematopoiesis and change the level of CD34 expression. Enzymatic treatment has insignificant effect on level of some cell surface protein in FACS analyze that worth to take into account in such experiments.

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ПОРІВНЯЛЬНИЙ ФЕНОТИПІЧНИЙ АНАЛІЗ ПОПУЛЯЦІЙ ГЕМОПОЕТИЧНИХ ПРОГЕНІТОРНИХ КЛІТИН З РІЗНИМ РІВНЕМ ЕКСПРЕСІЇ *CD34*, ОТРИМАНИХ З ТКАНИНИ ПЛАЦЕНТИ І ПУПОВИННОЇ КРОВІ

Дослідження плацентарних гемопоетичних прогеніторних клітин (ГПК) і порівняння їх з властивостями ГПК плоду і дорослого організму необхідні для оцінки можливості їх клінічного застосування. Було показано, що тканина плаценти містить три популяції з різним рівнем експресії CD34, такі як $CD34^{+++}CD45^{low}$, $CD34^{++}CD45^{low}$ і $CD34^{+/low}CD45^{low}$. Як і в фетальній печінці, в плаценті містяться популяції з фенотипом $CD34^{++}CD45^{low/-}$ і $CD34^{+}CD45^{low/-}$, що дозволяє говорити про кровотворення в плацентарній тканині. $CD34^{++}CD45^{low/-}$ популяція також експресує CD133, практично негативна по лінійним маркерами і має лімфоцитоподібну морфологію. Це свідчить про те, що така популяція містить примітивні ГПК, потенційно – стовбурові клітини. Також серед плацентарних клітин присутні більш пізні прогенітори з фенотипом $CD34^{+/low}CD45^{+}$. Більшість таких клітин експресує гемопоетичні лінійні маркери. Популяція з фенотипом $CD34^{+++}CD45^{low}$ спостерігається лише в плаценті, клітини з таким фенотипом, імовірно, утворюються тільки в плацентарній тканині і/або мігрують з інших сайтів кровотворення, змінюючи при цьому рівень експресії CD34. Ферментативна обробка має незначний вплив на рівень експресії поверхневих білків при FACS аналізі, що варто враховувати в подібних експериментах.

Ключові слова: гемопоетичні прогеніторні клітини, плацентарний гемопоез, експресія CD34 маркера, пуповинна кров.

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СРАВНИТЕЛЬНЫЙ ФЕНОТИПИЧЕСКИЙ АНАЛИЗ ПОПУЛЯЦИЙ ГЕМОПОЭТИЧЕСКИХ ПРОГЕНИТОРНЫХ КЛЕТОК С РАЗЛИЧНЫМ УРОВНЕМ ЭКСПРЕССИИ *CD34*, ПОЛУЧЕННЫХ ИЗ ТКАНИ ПЛАЦЕНТЫ И ПУПОВИННОЙ КРОВИ

Исследование плацентарных гемопоэтических прогениторных клеток (ГПК) и сравнение их со свойствами ГПК плода и взрослого организма необходимы для оценки возможности их клинического применения. Было показано, что ткань плаценты содержит три популяции с различным уровнем экспрессии CD34, такие как $CD34^{+++}CD45^{low}$, $CD34^{++}CD45^{low}$ и $CD34^{+/low}CD45^{low}$. Как и в фетальной печени, в плаценте содержатся популяции с фенотипом $CD34^{++}CD45^{low/-}$ и $CD34^{+}CD45^{low/-}$, что позволяет говорить о кроветворении в плацентарной ткани. $CD34^{++}CD45^{low/-}$ популяция также экспрессирует CD133, практически отрицательна по линейным маркерам и имеет лимфоцито-подобную морфологию. Это свидетельствует о том, что такая популяция содержит примитивные ГПК, потенциально стволовые клетки. Также среди плацентарных клеток присутствуют более поздние прогениторы с фенотипом $CD34^{+/low}CD45^{+}$. Большинство таких клеток экспрессирует гемопоэтические линейные маркеры. Популяция с фенотипом $CD34^{+++}CD45^{low}$ наблюдается только в плаценте, клетки с таким фенотипом, предположительно, образуются только в плацентарной ткани и/или мигрируют из других сайтов кроветворения, изменяя при этом уровень экспрессии CD34. Ферментативная обработка оказывает незначительное влияние на уровень экспрессии поверхностных белков при FACS анализе, что стоит учитывать в подобных экспериментах.

Ключевые слова: гемопоэтические прогениторные клетки, плацентарный гемопоэз, экспрессия CD34 маркера, пуповинная кровь.

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DEVELOPMENT OF PIG CUMULUS-OOCYTE COMPLEXES AT CONSTANT AND OSCILLATING TEMPERATURE AND PH

It is shown that replacement of constant temperature and pH of culture conditions by oscillating ones does not significantly decrease diameter of COCs as a result of their maturation in in vitro culture for 24 hours. Increase in content of follicular fluid from 10 % to 20 % in the medium of their maturation NCSU does not influence the gain in COCs diameter.

Key words: cumulus-oocyte complex, follicular fluid.

Introduction. Improving the growth and development of the live object remains relevant because the problem associated with the increase of its productivity and improvement of its viability. You can select two opposite to each other, strategic approaches to its solution: genetic and epigenetic, which, like any opposites, deny and complement each other and are interconnected by passages. Gene expression takes place only in certain environments [23]. It is pointed out, in particular, by such concepts as expressivity and penetrance. The result of the development of a biological object is not only preformed, in particular, in the form of genetic program but also is determined by the epigenesis, in particular, by the influence of external conditions. And then, by the creation of conditions of the envi-