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Original article

Comparison of capacities to maintain hematopoietic stem cells among different types of stem cells derived from the placenta and umbilical cord

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ABSTRACT

Introduction: Cord blood is utilized as a useful source of cells for hematopoietic stem cell transplantation, but this can be problematic because there is a high rate of graft failure compared to when other graft sources are used. A previous study successfully avoided graft failure by simultaneously grafting cord blood and bone marrow mesenchymal stem cells (MSCs) that are considered to function in the hematopoietic stem cell niche of the bone marrow.

Organs of the fetal life support system such as the placenta and umbilical cord, which are discarded after delivery, contain an abundance of MSCs as well as cells that function in the hematopoietic stem cell niche. By identifying and collecting such cells and subsequently co-transplanting them with cord blood, an improvement in graft survival can be anticipated.

Methods: Three types of stem cells, amnion epithelial stem cells (AM-Epi), amnion mesenchymal stem cells (AM-Mes), and Wharton's jelly (WJ)-MSCs, all of which can be isolated and cultured from the placenta amnion or umbilical cord WJ, were investigated for the expression of hematopoietic stem cell niche markers and for their capabilities to maintain hematopoietic stem cells when co-cultured with cord blood hematopoietic stem cells.

Results: All types of isolated cells showed profiles that met the MSC minimal criteria according to surface marker analysis. In addition, all cell types expressed the hematopoietic stem cell niche marker stromal cell-derived factor-1 (SDF-1) (in order: AM-Epi > WJ-MSCs ≫ AM-Mes), although the expression declined with further passaging.

After 5 days of co-culturing with cord blood CD34+ cells, the percentages of CD34+, CD45− cells were: AM-Epi 37.8%, AM-Mes 38.8%, WJ-MSCs 27.3%, and fibroblasts 27.4%; and the number of CFU-GM colonies were: AM-Epi 255.5 ± 21.6, AM-Mes 246.3 ± 28.5, WJ-MSCs 118.3 ± 11.8, fibroblasts 147.8 ± 19.0, and NC 121.3 ± 6.5. Statistical analyses demonstrated that AM-Epi and AM-Mes produced significantly greater numbers of CFU-GM compared to WJ-MSC, fibroblasts, or NC ($p < 0.05$).

Conclusions: These findings indicated that cells derived from the fetal life support system such as AM-Epi and AM-Mes can be anticipated as potential cell sources for clinical application in cell therapies for the purpose of enhancing graft survival during hematopoietic stem cell transplantation.

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1. Introduction

Cord blood transplantation involves very little risk to the donor, and the process can be coordinated readily. Due to these advantages, there has been a rapid increase in the number of such transplantations performed in recent years. On the other hand, there is a major issue with this particular transplantation in that there is a high incidence of graft failure (10–20%) compared to other graft sources [1]. Some of the causes of the high rate of graft failure include fewer transplanted cells and the virtual absence of stromal cells that possess the ability to support hematopoietic cells compared to bone marrow transplantation. To prevent graft failure, a previous study simultaneously transplanted cord blood and bone marrow mesenchymal stem cells (MSCs) that support hematopoietic stem cells in the bone marrow microenvironment, and demonstrated that graft survival is improved using this co-transplantation procedure [2]. However, because bone marrow MSCs are normally difficult to collect and prepare from the recipient or from cord blood donors, transplantation is typically performed today using grafts from HLA-mismatched related donors. Moreover, a relatively invasive procedure is required to collect the bone marrow from healthy donors.

It has been gradually discovered that multipotent stem cells such as MSCs are present in the fetal life support system including the placenta and umbilical cord, and there is a high probability that these tissues contain cells that function as a part of the hematopoietic stem cell niche. Several different types of stem cells are present in the placenta including those that can differentiate into mesenchymal tissues such as bone, cartilage, and adipose tissue as well as those that possess a high immune tolerance [3]. In particular, the placenta amnion is physically located closest to the fetus, allowing for procedures such as tissue collection to be performed readily. The amnion, together with the chorion and decidua, form the fetal membrane and produce amniotic fluid to encase the fetus.

The amnion is composed of two types of multipotent stem cells – epithelial cells (amnion epithelial stem cells or "AM-epi") and mesenchymal cells (amnion mesenchymal stem cells, or "AM-Mes") – and both types of cells are known to differentiate into the three germ layers, exhibit high immune tolerance, and show anti-inflammatory effects [4–6]. Methods to isolate and culture these cells via enzymatic treatment have been established previously [7], and these cells are anticipated for their potential in future clinical application [8]. It has also been demonstrated that the placenta that is alongside the aorta-gonad-mesonephros region is an essential site for hematopoiesis during embryonic and fetal stages [9–11]. Furthermore, it has been reported that the human full-term placenta also contains hematopoietic stem cells, and that a full-term placenta cell line exhibiting MSC morphology supports the maintenance of hematopoietic stem cells [12]. The umbilical cord, which is covered in a sheath composed of amnion, contains two umbilical arteries and one umbilical vein, and its stroma is filled with a matrix called Wharton's jelly (WJ) that is composed of white mucopolysaccharides (Fig. 1). This WJ is rich in MSCs and is known to demonstrate multipotency and differentiate into bone, cartilage, adipose tissue, as well as the nerves [13]. Because it is possible to easily isolate and grow these cells using procedures such as the explant method or collagenase enzymatic treatment, they are highly anticipated for their potential application in the clinical setting [14].

These methods of cell collection from the fetal life support system do not entail invasive procedures to the donors. Moreover, because these tissues are typically discarded after delivery, few ethical issues are related to the clinical usage of these cells. In addition, it is possible to collect and prepare cells from the same individual as the cord blood donor. Therefore, stem cells derived from the fetal life support system can be anticipated as new sources of cells for cell therapy for the purpose of preventing cord blood graft failure. However, the location of cells that exhibit hematopoietic stem cell niche function and the capacity of such cells to support hematopoiesis after they are isolated and cultured have yet to be elucidated in detail in such tissues.

Therefore, in the present study, we focused on three different types of stem cells that can be isolated and cultured from the placenta amnion and umbilical cord WJ and assessed the changes in the expression of hematopoietic stem cell niche markers such as stromal cell-derived factor-1 (SDF-1) and nestin. Furthermore, I examined the capacity of these cells to support hematopoietic stem cells when they were co-cultured with cord blood hematopoietic stem cells. Based on these results, we endeavored to identify placenta- and umbilical cord-derived cells that maintain the hematopoietic stem cell niche.

2. Materials and methods

2.1. Human placenta and umbilical cord

Human placenta and umbilical cord were collected from pregnant women who underwent a planned C-section at 36–39 weeks of gestation at Nihon University Itabashi Hospital. Collected tissues were stored in phosphate-buffered saline (PBS) at room temperature and were used for experiments within 24 h of collection. Informed consent was obtained from the pregnant women prior to collecting a portion of the tissues that were planned to be discarded after delivery. All experiments using human fetal life support system (placenta and umbilical cord) were approved by the Clinical Research Review Board at Nihon University Itabashi Hospital and conducted in accordance with the principles of the Declaration of Helsinki (Approval number RK-111209-6).

2.2. Cell culture and preparation

2.2.1. HESS-5 cells

The stromal cell line HESS-5 was established from the bone marrow of irradiated C3H/HeN mice and was a generous gift from Dr. Takashi Tsuji of the Department of Biological Science and Technology at Tokyo University of Science [15]. Cells were cultured in Minimum Essential Medium (MEM) Alpha (Invitrogen) containing 10% horse serum (Lot 8092930, Invitrogen, Carlsbad, CA) in 5% CO_2 at 37 °C. Cells were split once a week at a ratio of 1:5.

2.2.2. Human fibroblasts

Fibroblasts derived from human skin were purchased from Lonza (Walkersville, MD) and were cultured in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS, SAFC Biosciences, St. Louis, MO) in 5% CO_2 at 37 °C. Fibroblasts were split at a 1:5 ratio approximately every 5 days, and fifth generation (P5) cells were used.

2.2.3. CD34+ cells from human cord blood

Of the human cord blood specimens provided to the Cord Blood Processing and Storage Facility at Nihon University Itabashi Hospital, those that could not be prepared for further use or preserved due to an insufficient number of nucleated cells were used in the experiments within 24 h of collection. Cord blood monocytes were isolated using a gradient centrifugation method. For every 35 ml of cord blood, approximately 15 ml of blood cell isolation solution (Lymphoprep: density: 1.077 g/cm^3, Axis-Shield Poc, Oslo, Norway) was used. After placing the blood cell separation solution in a tube, cord blood was slowly layered on top and centrifuged for 30 min at 350–400 × *g*. Subsequently, the monocytes were fractionated by

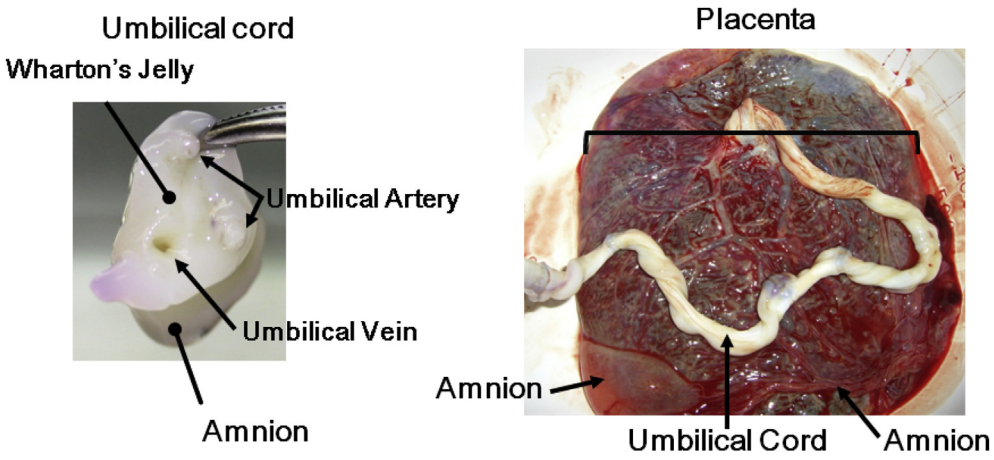

Fig. 1. Umbilical cord and placenta.

isolating the layer directly on top of the blood cell isolation solution. CD34+ cells were isolated from the collected human cord blood monocytes using magnetic beads. In the magnetic beads method, CD34+ cells were isolated using the CD34 MicroBeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and separation columns for positive selection (LS Columns, Miltenyi Biotec) according to the manufacturer's protocol for each kit. To collect cell fractions with greater purity, the above isolation protocol was conducted twice. Isolated CD34+ cells were frozen in CELLBANKER (Juji Field, Tokyo, Japan), stored in a liquid nitrogen tank, and thawed immediately prior to use for experiments. Experiments using human cord blood were approved by the Clinical Research Review Board at Nihon University Itabashi Hospital and the Ethics Committee at Tokyo Cord Blood Bank (Approval number 12-07-01).

2.2.4. *Placenta amnion-derived stem cells*

The placenta amnion was manually detached from the placenta after delivery and processed using the method previously described by Barbati et al. [7]. The collected placenta amnion specimen was washed with PBS. Because of the mesenchyme of amnion is spongy layer, washing the amniotic membrane in PBS before separation epithelial from mesenchyme, the mesenchyme layer is inflated and increases thickness. In this way, epithelial layer and mesenchyme layer can be easily separated (Fig. 2). AM-Epi and AM-Mes samples were prepared as described below:

First, the amnion epithelial and mesenchymal tissues were cut finely into 1-cm squares, submerged in DMEM containing 2.4 U/ml Dispase (Roche Diagnostics, Mannheim, Germany), and incubated with agitation for 20 min at 37 °C. After each sample was centrifuged at 150 × *g* for 15 min, the supernatant was discarded, 0.1% Collagenase type II (Sigma–Aldrich) was added, and the samples were agitated for 60 min at 37 °C. Subsequently, the samples were strained with a 40-μm filter into a centrifuge tube and centrifuged at 150 × *g* for 15 min. These obtained samples were used in the experiments as either AM-Epi or AM-Mes according to the originating tissue. Cells were cultured in MEM Alpha (Life Technologies) containing 10% FBS in 5% CO_2 at 37 °C. Once the cells reached 80–90% confluence, they were collected with 0.05% Trypsin-EDTA

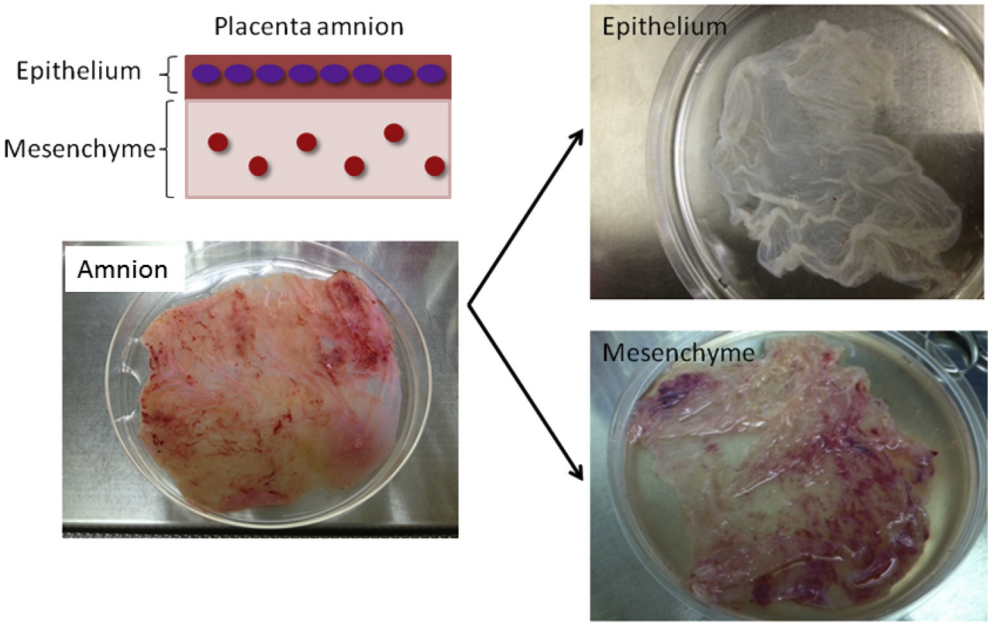

Fig. 2. Structure of the placenta amnion.

(Life Technologies) and subcultured on a plastic dish. Cells were split at a 1:5 ratio approximately every 5 days. Cells cultured up to P3 were used in the experiments.

2.2.5. Umbilical cord WJ-derived MSCs

Umbilical cord WJ-derived MSCs (WJ-MSCs) were prepared using the explant method previously described by McElreavey et al. [16]. The umbilical cord samples were washed with PBS after collection, and the umbilical cord artery and vein were mechanically extracted and removed. WJ was finely minced using a scalpel or ophthalmic scissors into ~5-mm diameter pieces that were placed in a plastic dish and subsequently cultured in 10% FBS MEM Alpha in 5% CO_2 at 37 °C. Once the adhered cells reached 80–90% confluence, they were collected with 0.05% Trypsin-EDTA and subcultured into a plastic dish. Cells were subsequently split at a 1:5 ratio approximately every 5 days. Cells cultured up to P3 were used in the experiments.

2.3. Immunohistochemistry

Frozen sections of human umbilical cord and placenta: Tissues were washed with PBS after collection, finely minced to about 1 cm in diameter, and fixed in 4% paraformaldehyde (Muto Pure Chemicals, Tokyo, Japan) at 4 °C for 24 h. Umbilical cord tissue was dehydrated by submerging in saline containing sucrose, embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan), and frozen in liquid nitrogen. The placenta was embedded in O.C.T. compound without the dehydration process after fixing, and frozen in liquid nitrogen. Tissue blocks were sectioned using a cryostat (BRIGHT5040, Hacker Instruments & Industries, Winnsboro, SC) at 10-μm thickness and mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan).

Placenta amnion and umbilical cord samples for immunostaining: First, tissues were minced finely and digested enzymatically. Subsequently, cells (1×10^4/100 μl) were centrifuged with CytoSpin 3 (Thermo Fisher Scientific, Waltham, MA) for 5 min at 600 rpm, spread onto glass slides, and fixed with 4% paraformaldehyde (room temperature, 15 min).

Cultured cell sample preparation for immunostaining: Cells were cultured for approximately 48 h in 24-well multiplates (Becton Dickinson, Bedford, MA) containing a round cover glass (Matsunami Glass) in each well. After the cells reached ≥80% confluence, they were fixed in 4% paraformaldehyde (room temperature, 15 min) and stained. Subsequently, the cover glasses were removed and mounted on glass slides.

Immunofluorescent staining of both tissue samples and cells was performed in the following order: blocking, incubation with primary antibody, incubation with secondary antibody, nuclear staining, and application of mounting medium. For blocking, samples were incubated in Tris-buffered saline (TBS) containing 10% goat serum (Vector Laboratories, Burlingame, CA) and 1% BSA without agitation for 1 h at room temperature in a humid box. The following primary antibodies were used: mouse anti-human cytokeratin19 (CK19) antibody (Progen Biotechnik GmbH, Heidelberg, Germany) as an epithelial marker; rabbit anti-human SDF-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human nestin antibody (Sigma–Aldrich) as hematopoietic stem cell niche markers; and rat anti-human stage-specific-antigen 3 (SSEA3) antibody (R&D Systems, Minneapolis, MN) and mouse anti-human stage-specific-antigen 4 (SSEA4) antibody (R&D Systems) as embryonic stem cell markers. Samples were incubated overnight with primary antibodies (diluted 1:100 to 1:500 with 1% BSA-TBS) in a humid box at 4 °C. On the following day, samples were washed with PBS and incubated with fluorescent-labeled secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-rat IgM, Alexa Fluor 680 goat anti-rabbit, Invitrogen) diluted with 1% BSA-TBS at 1:500 for 1 h at room temperature in a humid box. The samples were washed with PBS again, stained with 5 μg/ml Hoechst 33342 (Sigma–Aldrich) for nuclear staining for 15 min at room temperature, and subsequently washed with distilled water. FluoromountG (Southern Biotech, Birmingham, AL) was using as the mounting medium, and all samples were visualized and imaged with a confocal laser scanning microscope (FV10i-SW, Olympus, Tokyo, Japan).

2.4. Flow cytometry

AM-Epi, AM-Mes, and WJ-MSCs (P2) were suspended in PBS with 0.2% BSA, and samples of 1×10^6 cells/100 μl/tube were prepared. To block non-specific binding, 0.1 ml PBS containing 10 μl normal rabbit serum (Funakoshi, Tokyo, Japan) was added to each tube and incubated without agitation at room temperature for 10 min. After washing the cells with PBS (0.2% BSA), 5 μl phycoerythrin (PE)-labeled anti-human CD90, CD73, CD11b, CD19, or HLA-DR antibody (BD Biosciences) or allophycocyanin (APC)-labeled anti-human CD105, CD34, or CD45 antibody (BD Biosciences) was added to each cell suspension tube and incubated in the dark for 30 min at 4 °C. For negative controls, PE- or APC-labeled mouse IgG1 antibody (BD Biosciences) was used following the above protocol. After cells were washed and suspended in 0.4 ml PBS (0.2% BSA), they were strained with a mesh filter into 5-ml FACS tubes (BD Falcon, Franklin Lakes, NJ). In each tube, 5 μl of 7-aminoactinomycin D (7AAD, BD Biosciences) was added to detect dead cells. Cell surface antigens were screened with a FACS Calibur Flow Cytometer (Becton Dickinson), and the results were analyzed with CellQuest software (Becton Dickinson).

2.5. Co-culture experiment of CD34+ cells from human cord blood

Cells derived from the placenta and umbilical cord and CD34+ cells from human cord blood were co-cultured using a modified protocol of a method described by Tsuji et al. [17,18]. Human fibroblasts (Lonza) were used as negative control cells, and the mouse stromal cell line HESS-5 was used as positive control cells. AM-Epi, AM-Mes, WJ-MSCs (P3 for each cell type), HESS-5, or human fibroblasts were seeded onto 6-well plates and cultured until reaching confluence (1×10^5/well). Subsequently, these cultured cells were used as feeder cells and cultured together with CD34+ cells from human cord blood (1×10^5 cells/well). For the growth and maintenance of hematopoietic stem cells, Myelocult H5100 medium (MEM containing 12.5% horse serum, 12.5% FBS, 10^{-4} M 2-mercaptoethanol; Stem Cell Technologies, Vancouver, Canada) with 50 ng/ml Stem Cell Factor (Wako) was used. After 5 days of co-culture, non-adherent human cord blood cells were collected. After the cells were washed with MEM Alpha and enumerated with a hemocytometer, the growth rates of cells in reference to the pre-co-culture cell count were determined. Furthermore, cell surface markers on human cord blood cells after co-culture were analyzed using flow cytometry. Specifically, this flow cytometry analysis was conducted using the aforementioned method with double staining with fluorescein isothiocyanate (FITC)-labeled anti-human CD45 antibody (BD Biosciences) and APC-labeled anti-human CD34 antibody (BD Biosciences).

Negative controls were prepared for each sample, and quadrant areas were determined.

2.6. Colony forming unit-granulocyte/macrophage (CFU-GM) assay

CFU-GM assay was conducted to assess the hematopoietic activity of cord blood CD34+ cells after co-culturing with the three

different types of cells derived from the fetal life support system. After AM-Epi, AM-Mes, WJ-MSCs, HESS-5, or human fibroblasts were co-cultured for 5 days with cord blood CD34+ cells, non-adherent human cord blood cells were collected, seeded on a 35-mm dish at 2×10^4 cells/ml in the methylcellulose-based medium MethoCult H4034 Optimum (Stem Cell Technologies), and cultured in 5% CO_2 at 37 °C. Colonies that formed after 14 days of culture were enumerated (CFU-GM). Samples were compared in triplicate, and statistical significance was assessed.

2.7. Statistical analysis

Comparisons of quantitative data with the CFU-GM assay were conducted using one-way analysis of variance (ANOVA) and a post-hoc test (Tukey method), and $p < 0.05$ was considered statistically significant. GraphPad Prism software (Ver5.0a) was used for analysis.

3. Results

3.1. Immunohistochemical study of the umbilical cord and placenta tissues

To identify the location of cells that possess hematopoietic niche function in the human umbilical cord and placenta tissues, frozen sections of each tissue were created and immunostained with the hematopoietic stem cell niche marker SDF-1. Representative immunostained images of the umbilical cord tissue are shown in Fig. 3. Cells that compose WJ in the umbilical cord tissue were weakly positive for SDF-1 (Fig. 3A). SDF-1 was expressed at high levels in amnion epithelial cells that were positive for CK19, an epithelial marker (Fig. 3B). In contrast, endothelial cells and smooth muscle cells that compose the umbilical arteries and vein were both SFD-1 negative.

A representative immunostaining image of the placenta tissue is shown in Fig. 4. In the placenta amnion, which makes up the outer-most layer of the placenta, CK19+ amnion epithelial cells and CK19− amnion mesenchymal cells directly underneath the epithelial cells were identified (Fig. 4A). SDF-1 expression was high in the CK19+ amnion epithelial cells and extremely low in amnion mesenchymal cells (Fig. 4A). Trophoblasts that compose the placenta villi were also positively stained for SDF-1 (Fig. 4B). In contrast, vascular cells and mesenchymal cells that are present in the villi were SDF-1 negative.

These results demonstrated that SDF-1 is expressed in a differential manner depending on the site within the umbilical cord and placenta tissues and is expressed at high levels especially in the amnion epithelium that is present at the outer-most layer of the umbilical cord and placenta.

3.2. Immunocytological analysis of cells derived from the fetal life support system

Because stem cells that are present in the fetal life support system exhibit high proliferative activities, they can be cultured and grown after they have been isolated from the tissues with collagenase enzymatic treatment or the explant method. In the present study, with consideration for the ease of isolating and culturing cells from tissues and for the expression of SDF-1, we isolated cells from the placenta amnion epithelium, placenta amnion mesenchyme, and umbilical cord WJ. The three different types of stem cells (AM-Epi, AM-Mes, and WJ-MSCs) obtained after the isolation procedures were cultured and morphologically compared by conducting immunocytochemical analyses using hematopoietic stem cell niche markers as well as embryonic stem cell markers. From 1 g of placenta amnion epithelium or mesenchyme, approximately 3×10^5 stem cells were isolated through enzymatic digestion. From 1 g of umbilical cord, approximately 3×10^5 stem cells were isolated using the explant method.

The immunostaining images of AM-Epi derived from placenta amnion epithelium are shown in Fig. 5. In cytospin samples of cells isolated from the amnion epithelium with enzymatic digestion, the majority of the cells expressed CK19 (Fig. 5, Cytospin, CK19), verifying that these cells are indeed derived from the amnion epithelium. CK19+ cells had high levels of SDF-1 expression (Fig. 5, Cytospin, SDF-1). The hematopoietic stem cell niche marker nestin and the human embryonic stem cell marker SSEA4 were also expressed in many cells (Fig. 5, Cytospin, nestin, SSEA4). Staining

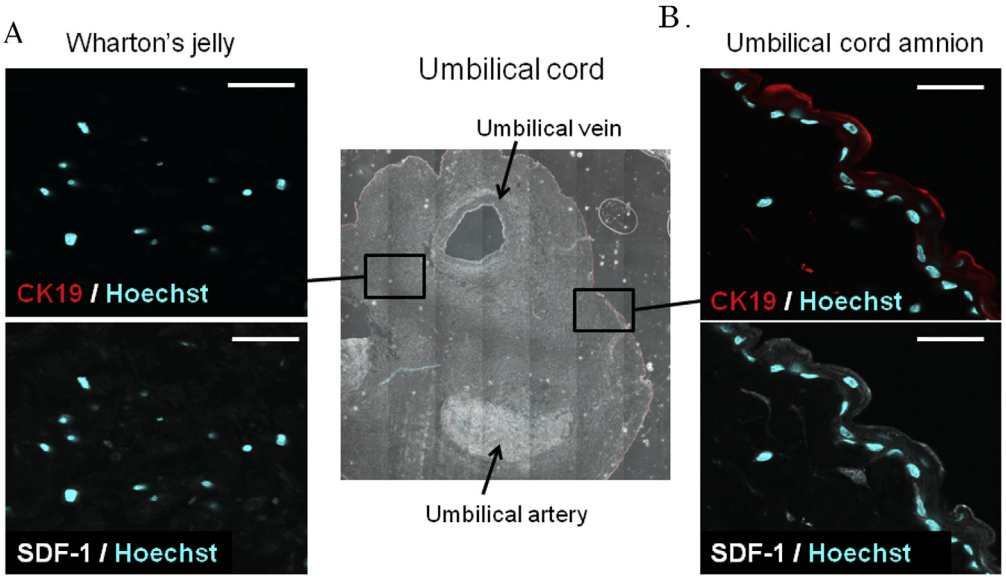

Fig. 3. Immunostaining of human umbilical cord tissue. Frozen sections of human umbilical cord tissue were prepared and immunostained with anti-cytokeratin19 (CK19) antibody or with anti-SDF-1 antibody. Nuclei were stained with Hoechst 33342 (Hoechst). Figure shows the Wharton's jelly (A) and the umbilical cord amnion (B). The arrows indicate the umbilical artery and umbilical vein. Scale bars: 50 μm.

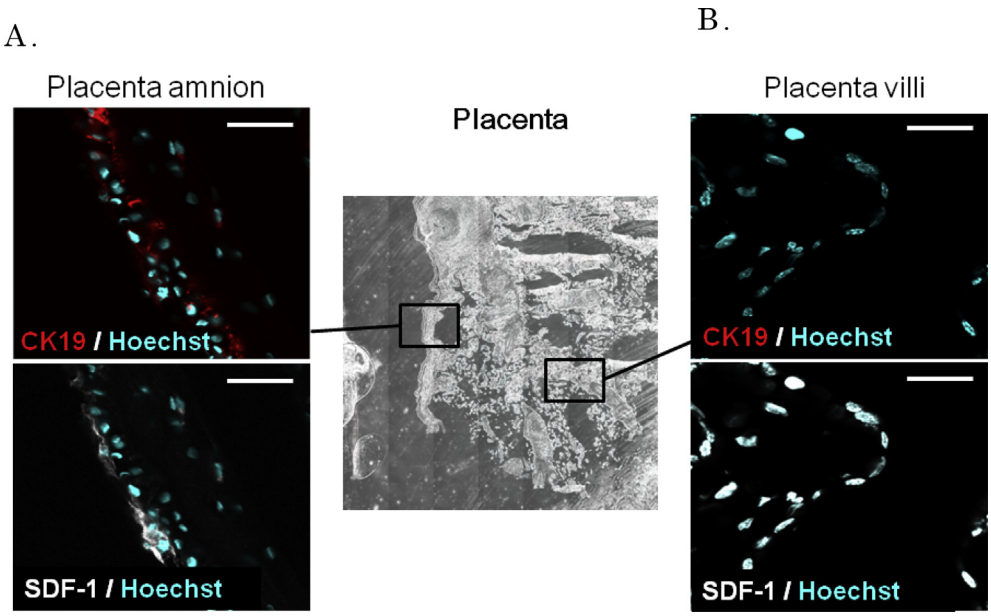

Fig. 4. Immunostaining of human placenta tissue. Frozen sections of human placenta tissue were prepared and immunostained with anti-CK19 antibody or with anti-SDF-1 antibody. Nuclei were stained with Hoechst 33342 (Hoechst). Figure shows the placenta amnion (A) and placenta villi (B). Scale bars: 50 μm.

for another embryonic stem cell marker, SSEA3, was negative (Fig. 5, Cytospin, SSEA3). Splitting the isolated cells resulted in satisfactory cell growth from P0 through P3. With passaging, the cytoplasm expanded and hypertrophied, and the nuclei tended to enlarge gradually. CK19 expression was evident up to P1 but decreased from P2 and was virtually undetectable at P3 (Fig. 5, P0–P3, CK19). SDF-1 and nestin expression also showed a tendency to diminish after culturing, and although expression of both was detectable up to P1, they markedly declined from P2 (Fig. 5, P0–3, SDF-1, and nestin). Similarly, SSEA4 expression tended to decrease after culturing; however, continual positive expression was evident up to P3 in some cells (Fig. 5, P0–3, SSEA4). Virtually no SSEA3-positive cells were detected after culturing (Fig. 5, P0–3, SSEA3).

Immunostaining of placenta amnion stroma-derived AM-Mes is shown in Fig. 6. Most cells in the cytospin samples were CK19– (Fig. 6, Cytospin, CK19), verifying that these cells are derived from the amnion mesenchyme. SDF-1 expression in AM-Mes was markedly lower compared to that in AM-Epi (Fig. 6, Cytospin, SDF-1). Nestin and SSEA4 were expressed in many cells, similar to AM-Epi (Fig. 6, Cytospin, nestin, and SSEA4). Similar to AM-Epi, SSEA3 was negative in the majority of AM-Mes (Fig. 6, Cytospin, SSEA3). Splitting the isolated cells resulted in satisfactory growth from P0 to P3, and both the cytoplasm and nuclei tended to become larger with passaging. SDF-1 was virtually undetectable from P1 with subculturing (Fig. 6, P0–P3, SDF-1). Nestin and SSEA4 expression also tended to diminish after culturing, and expression of both markedly declined and could not be detected from P1 (Fig. 6, P0–3, nestin, and SSEA4). Virtually no cells positive for SSEA3 were present after culturing (Fig. 6, P0–3, SSEA3).

Immunostaining of WJ-MSCs derived from umbilical cord WJ is shown in Fig. 7.

Because WJ-MSCs were collected using the explant method, immunocytochemical analysis was conducted with P1 cells. Almost all cells were CK19–, confirming that samples were not contaminated with epithelial cells (Fig. 7, P1, CK19). The cellular proliferative capacity was satisfactory up to P3, and similar to AM-Epi and AM-Mes, the cytoplasm and nucleus of WJ-MSCs tended to become enlarged with passaging (Fig. 7, P1–3, CK19). Although SDF-1 was evident at P1, albeit at a low level, its expression markedly declined with passaging, similar to AM-Epi and AM-Mes (Fig. 7, P1–3, SDF-1). Nestin showed a relatively greater expression at P1 compared to that of AM-Epi or AM-Mes. However, its expression decreased from P2 and became virtually undetectable (Fig. 7, P1–3, nestin). SSEA4 was also expressed in most P1 cells; however, its expression showed a tendency to decrease with passaging (Fig. 7, P1–3, SSEA4). SSEA3 expression was negative, similar to AM-Epi and AM-Mes (Fig. 7, P1–3, SSEA3).

These results showed that in these stem cells derived from the fetal life support system, expression of SDF-1, nestin, and SSEA4 was observed immediately after isolation, verifying the differences in cell origins. However, cells that were grown through subculturing demonstrated a decline in the expression of these markers and resulted in smaller morphological differences among the cell types.

3.3. Cell surface marker profile of stem cells derived from the fetal life support system

In cell surface marker analysis with flow cytometry, all cell types, AM-Epi, AM-Mes, and WJ-MSCs (P2), were positive for the MSC markers CD73, CD90, and CD105 but were negative for the blood cell markers CD34, CD45, CD19, CD11b, and HLA-DR (Fig. 8). This particular profile met the minimal criteria for MSCs [19]. In addition, there were no obvious differences among these three types of isolated stem cells in the percentages of CD73, CD90, or CD105 + cells. These results indicated that all three cell types, AM-Epi, AM-Mes, and WJ-MSCs, exhibit MSC-like morphology at P2.

3.4. Co-culture experiment with cord blood CD34+ cells

To assess the abilities of the three types of stem cells derived from the fetal life support system to maintain hematopoietic stem cells, P3 cells were used as feeder cells and co-cultured with human cord blood CD34+ cells, and the extent of cord blood cell proliferation and maintenance capacity of hematopoietic stem cells was analyzed. CD34+ cells isolated from cord blood monocytes using magnetic beads were analyzed with flow cytometry to confirm that

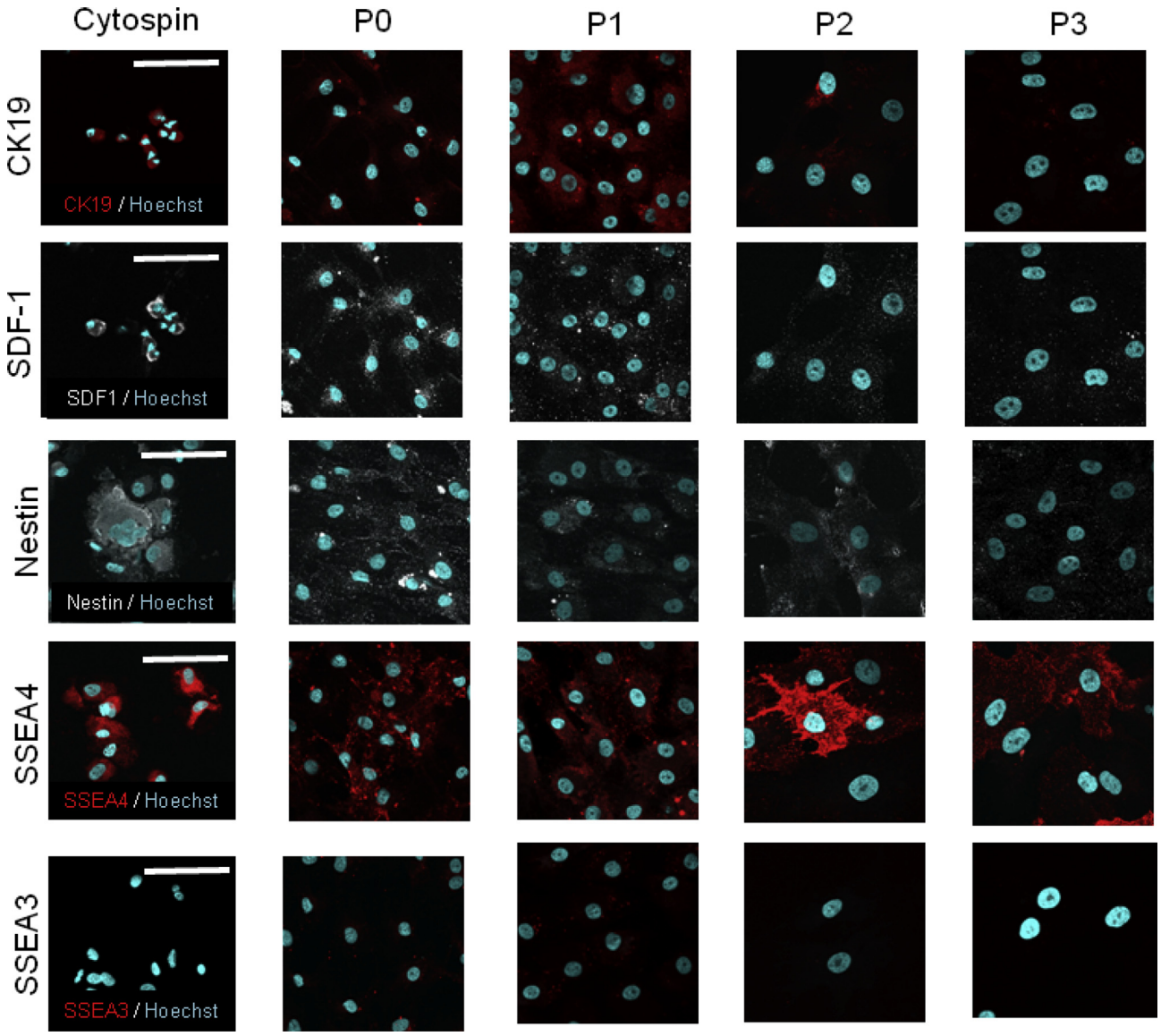

Fig. 5. Immunostaining of placenta amnion epithelial stem cells. After manually detaching the human placenta amnion epithelium, cells were isolated from the tissue via enzymatic treatment and were analyzed using immunocytochemistry. Immunostaining with cytokeratin19 (CK19), SDF-1, nestin, SSEA4, and SSEA3 are shown in order from the top to bottom. Passage 0 (P0), passage 1 (P1), passage 2 (P2), and passage 3 (P3) cells immediately after cytospin isolation are shown from left to right. Nuclei were stained with Hoechst 33342 (Hoechst). Scale bars: 50 μm.

the purity was ≥90%. After 5 days of co-culture, cord blood cells were collected and quantified. The cell count increased 5.8-fold in AM-Epi, 7.6-fold in AM-Mes, and 6.5-fold in WJ-MSCs. A 4.6-fold increase in the cell count of the positive control HESS-5 was observed. In contrast, cell proliferation was unstable when negative control fibroblasts were used as feeder cells; in some experiments, the cell count increased, and in other experiments, no changes were observed compared to the number of seeded cells. Moreover, when cord blood CD34+ cells were cultured alone without feeder cells, very little cell proliferation was evident. The same experiment was conducted three times using stem cells derived from the fetal life support system from the same donor, and similar trends of increased cord blood cells with co-culture were demonstrated in all three experiments (Fig. 9).

After co-culturing the cells, the percentages of the CD34+ CD45− cell fraction, which is a marker for hematopoietic progenitor cells, were analyzed with flow cytometry. The results demonstrated that this fraction accounted for 37.8% in AM-Epi, 38.8% in AM-Mes, 27.3% in WJ-MSCs, 60.0% in HESS-5, and 27.4% in fibroblasts. Both AM-Epi and AM-Mes, albeit not to the level of positive control HESS-5, had significantly greater percentages than negative control fibroblasts (Fig. 10). The same experiment was conducted an additional two times using stem cells derived from the fetal life support system from the same donor, and similar results were obtained. These results indicated that AM-Epi, AM-Mes, and WJ-MSCs support the proliferative and maintenance capacities of CD34 + cells from human cord blood.

3.5. Hematopoietic activity of cord blood CD34+ cells after co-culture

To determine the differentiation potential of cord blood CD34+ cells into blood cells that were co-cultured with three different types of stem cells derived from the fetal life support system, cord

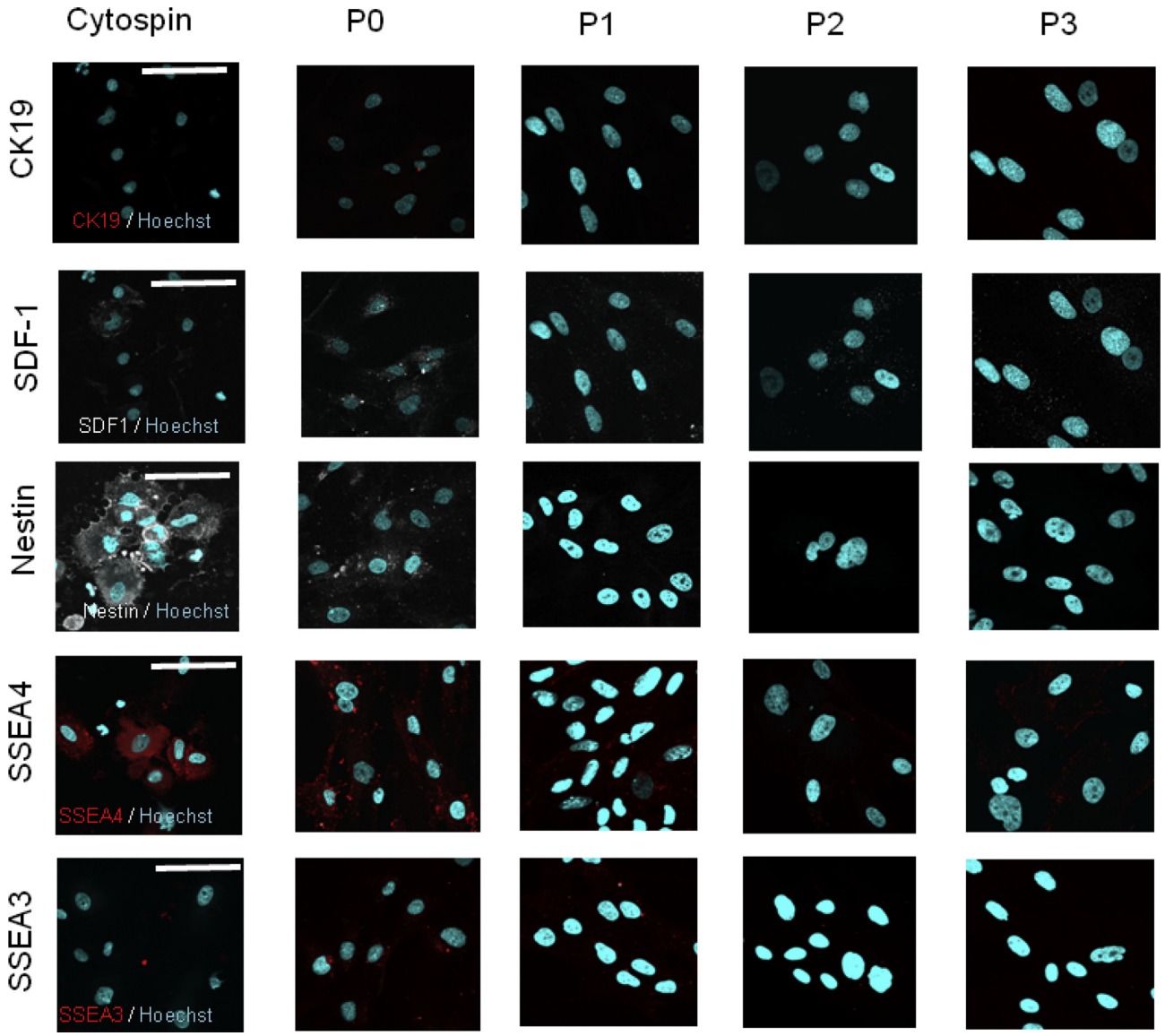

Fig. 6. Immunostaining of placenta amnion mesenchymal stem cells. After manually detaching the human placenta amnion mesenchyme, cells were isolated and cultured after enzymatic digestion, and subsequently analyzed by immunocytochemistry. Immunostaining with cytokeratin19 (CK19), SDF-1, nestin, SSEA4, and SSEA3 are shown in order from top to bottom. Passage 0 (P0), passage 1 (P1), passage 2 (P2), and passage 3 (P3) cells immediately after cytospin isolation are shown from left to right. Nuclei were stained with Hoechst 33342 (Hoechst). Scale bars: 50 μm.

blood cells were collected after co-culturing and subsequently examined with the CFU-GM assay to compare the number of colonies formed among the different cell types (Fig. 11). CFU-GM colonies for each cell type were (mean ± SD): 255.5 ± 21.6 for AM-Epi, 246.3 ± 28.5 for AM-Mes, 118.3 ± 11.8 for WJ-MSCs, 271.3 ± 39.2 for HESS-5, 147.8 ± 19.0 for fibroblasts, and 121.3 ± 6.5 for negative control (NC) without feeder cells. The CFU-GM assay with cord blood CD34+ cells before co-culture resulted in a colony population of 345.0 ± 33.9, demonstrating that the colony formation capacity tended to be high even before the co-culture experiments, although there were some variations among individual samples. Statistical analysis showed that both AM-Epi and AM-Mes had significantly greater CFU-GM colonies compared to WJ-MSCs, NC, or fibroblasts ($p < 0.05$). These results indicated that AM-Epi and AM-Mes exhibit high capacities to maintain hematopoietic stem cells and that the capacity levels were greater than that of fibroblasts or WJ-MSCs.

4. Discussion

4.1. Immunohistochemical analysis of cells derived from the fetal life support system

Immunohistochemical analysis of the human umbilical cord and placenta showed that cells positive for the hematopoietic stem cell niche marker SDF-1 were detected in the umbilical cord amnion, umbilical cord WJ, placenta amnion, and placenta villi. In the placenta tissue, SDF-1 was expressed at high levels especially in the CK19+ amnion epithelium, but virtually no expression was observed in the CK19− amnion mesenchyme. Amnion epithelial cells are derived from embryonic ectoderm, and amnion mesenchymal cells arise from embryonic mesoderm. Due to such differences in origins, these cells may inherently have differential expression of SDF-1. On the other hand, the *in vivo* hematopoietic stem cell niche marker nestin and the embryonic stem cell marker

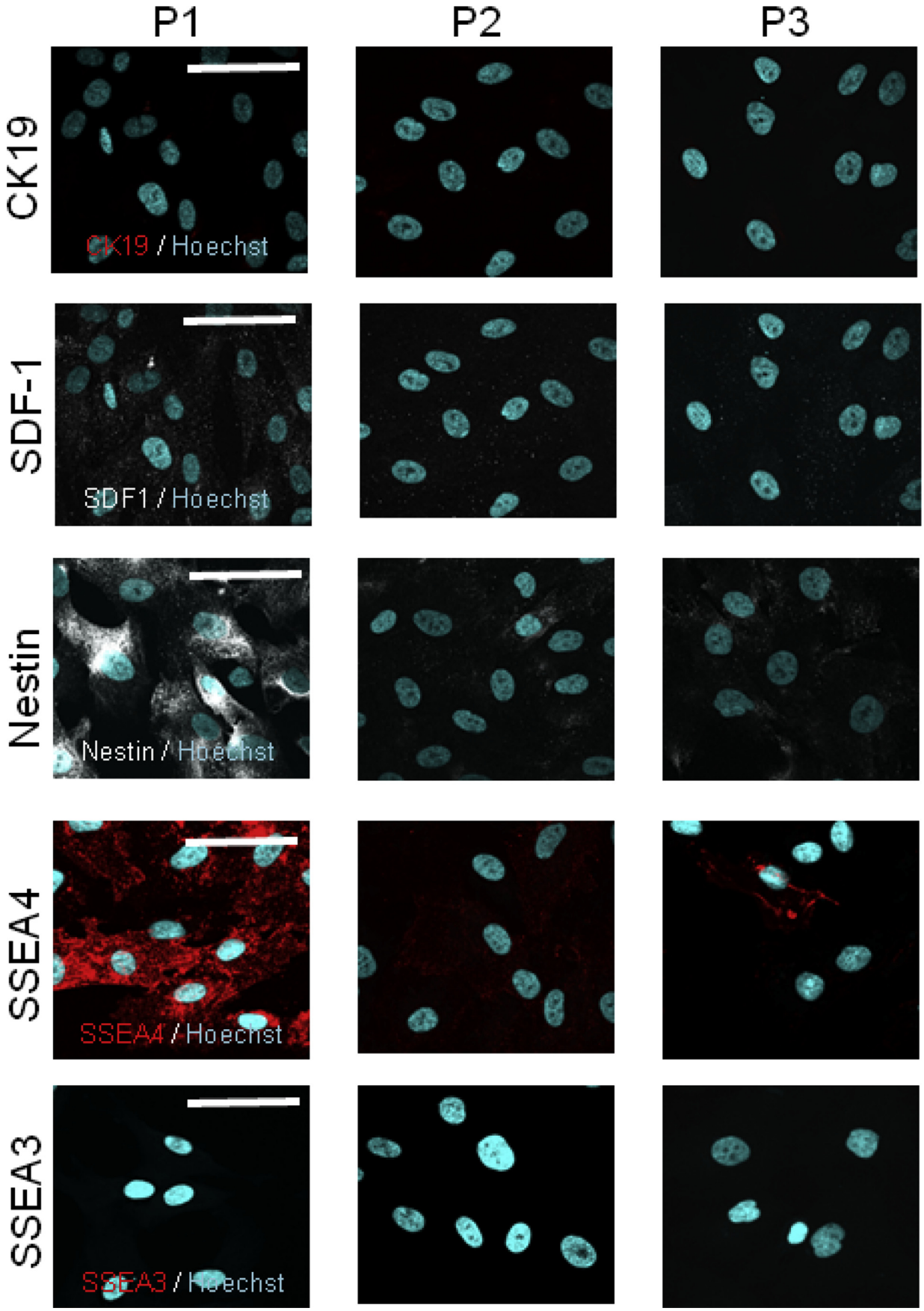

Fig. 7. Immunostaining of WJ-derived mesenchymal stem cells. Cells were isolated and cultured from the human umbilical cord WJ by the explant method and analyzed with immunocytochemistry. Immunostaining with cytokeratin19 (CK19), SDF-1, nestin, SSEA4, and SSEA3 are shown in order from top to bottom. Passage 1 (P1), passage 2 (P2), and passage 3 (P3) cells are shown from left to right. Nuclei were stained with Hoechst 33342 (Hoechst). Scale bars: 50 μm.

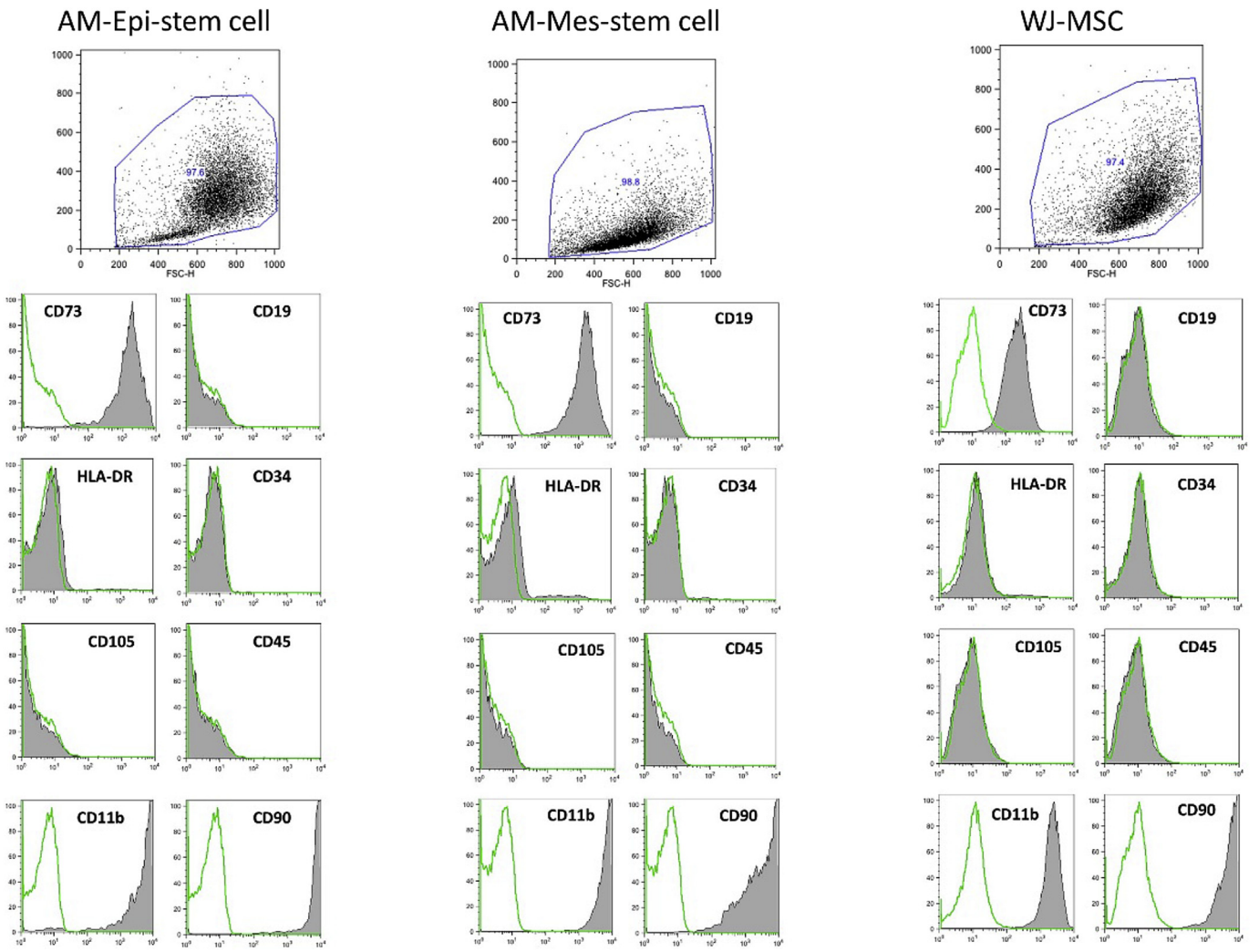

Fig. 8. Surface marker profile of stem cells derived from the fetal life support system. Surface markers of AM-Epi, AM-Mes, and WJ-MSCs isolated from the fetal life support system were analyzed using flow cytometry. The red line indicates the isotype control.

SSEA4 were expressed in epithelial cells and mesenchymal cells from the placenta amnion as well as in cells isolated from the umbilical cord WJ. These findings are consistent with previous reports that demonstrated expression of various stem cell markers in the amnion epithelium, amnion mesenchyme, and WJ-derived cells [4,5,20,21]. These findings suggested that these cells may contain stem cell components that display hematopoietic niche functions. Robin et al. found that a cell line generated from human full-term placenta that possess the ability to maintain hematopoietic stem cells expresses MSC markers as well as several pericyte markers, and postulated that these cells may originate from pericytes that are present in the villous capillary [12]. In the present study, we observed SDF-1 expression in the villous trophoblast cells but not in the capillary endothelium or in its vicinity in the placenta villi tissue. In terms of the isolation and *ex vivo* proliferation of cells that exhibit hematopoietic niche function, because the villous tissue is composed of numerous different types of cells such as maternal cells (e.g., maternal blood) and fetal cells, it would be extremely difficult to isolate and grow specific cells. For this reason, we excluded cells derived from villous tissues for examination in the present study. The identification and analyses of hematopoietic niche cells located in this region are future topics of investigation.

In this study, I cultured cells from the epithelium and mesenchyme of the placenta amnion and from the umbilical cord WJ, and conducted a morphological analysis by preparing three types of stem cells (AM-Epi, AM-Mes, WJ-MSCs) using previously established isolation and culture methods. Because there are no previous studies that simultaneously compared cells derived from the same donor, this study provides a novel perspective. Our results showed that all three types of cells showed decreased expression of SDF-1, nestin, and SSEA4 with further subculturing. Previous reports have also demonstrated decreases in stem cell markers as well as diminished multipotency with passaging [22,23]. These findings suggested that culturing and repeatedly subculturing the cells may reduce or eliminate the physiological functions of stem cells derived from the fetal life support system. Based on these results, we believe it is desirable to conduct further functional analysis using cells that have gone through fewer passaging (P0 or P1) if possible in all types of the stem cells derived from the fetal life support system. SDF-1 is a chemokine whose expression is triggered by hypoxic stimuli [24], and its expression may change depending on the cell culture conditions. In the future, it may be necessary to compare SDF-1 expression of these stem cells under hypoxic stimuli conditions or to examine other markers of the hematopoietic stem cell niche.

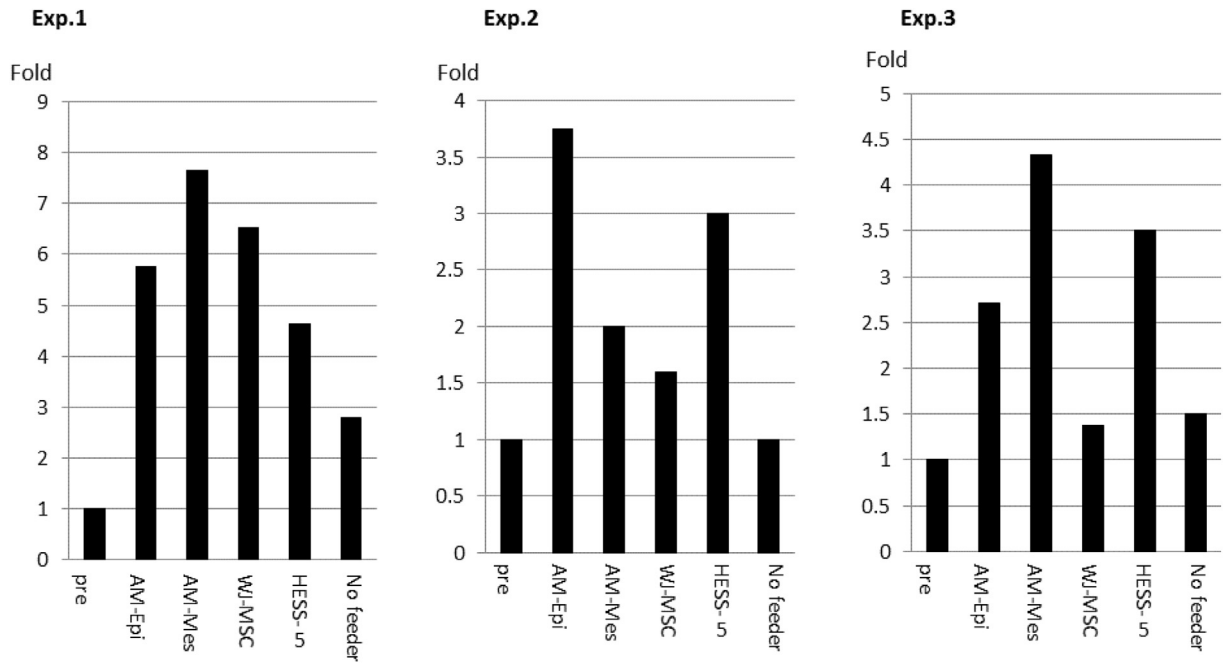

Fig. 9. Cord blood CD34+ cell counts after co-culture. Post-co-culture CD34+ cell counts normalized to pre-co-culture counts are shown. AM-Epi, AM-Mes, and WJ-MSCs all showed a similar level of proliferation as the positive control HESS-5. The same experiment was conducted three times using stem cells derived from the fetal life support system from the same donor, and similar trends in fold-increases of cord blood cells were demonstrated with co-culture.

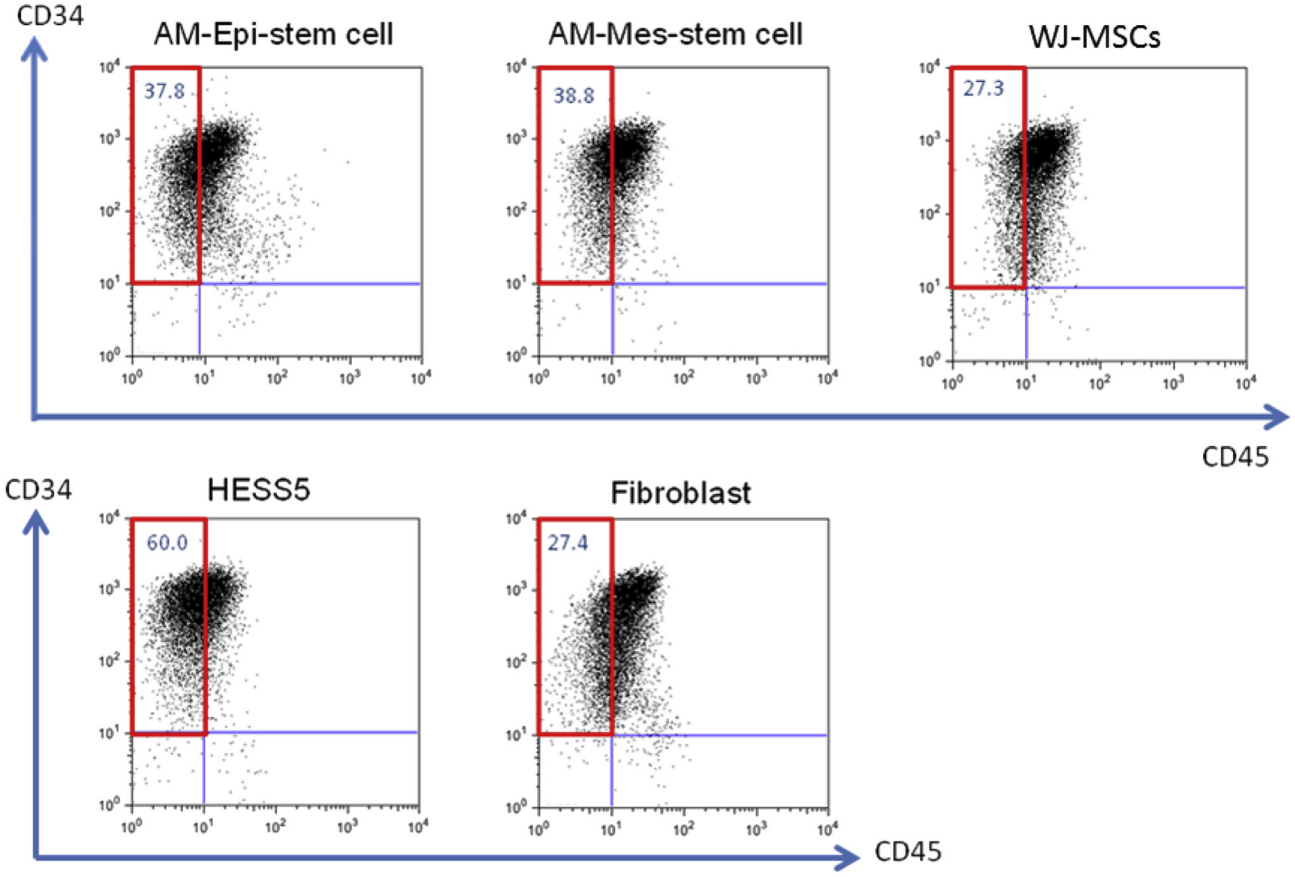

Fig. 10. Capacity to maintain CD34+ cells by stem cells derived from the fetal life support system. Human stem cells derived from the fetal life support system (AM-Epi, AM-Mes, and WJ-MSCs), positive control (HESS-5), and negative control (human fibroblasts) were co-cultured with cord blood CD34+ cells for 5 days. Subsequently, cord blood cells were collected and analyzed with flow cytometry to determine the percentage of hematopoietic stem cell fraction (CD34+ CD45− cells). The red frame represents the CD34+ CD45− fraction.

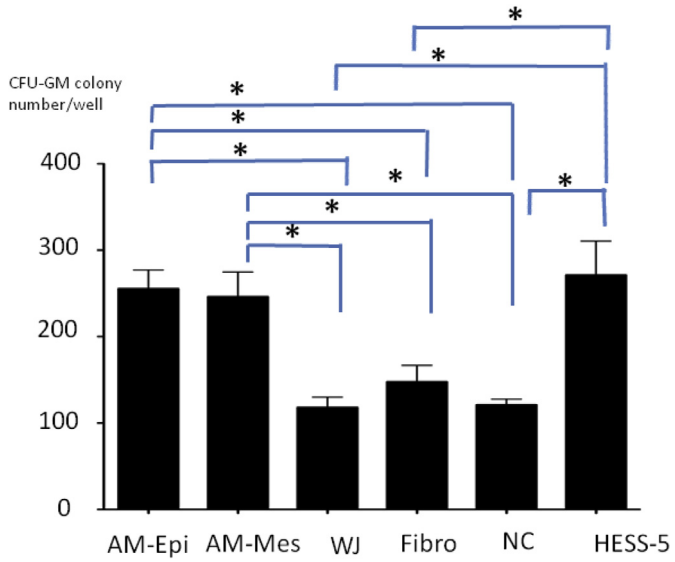

Fig. 11. Hematopoietic activity of CD34+ cells in the cord blood after co-culture. Human stem cells derived from the fetal life support system (AM-Epi, AM-Mes, and WJ-MSCs), positive control (HESS-5), cell control (human fibroblasts), and negative control (NC) without feeder cells were co-cultured with cord blood CD34+ cells for 5 days. Subsequently, cord blood cells were collected and analyzed with the CFU-GM assay. *$p < 0.05$ (One-way ANOVA, Tukey's post-hoc test).

4.2. Surface marker profile of stem cells derived from the fetal life support system

The three types of stem cells derived from the fetal life support system (AM-Epi, AM-Mes, WJ-MSCs) that were isolated and cultured from the same donor in the present study all exhibited a cell surface marker profile that met the minimal criteria for MSCs [19]. This is consistent with previously reported results on AM-Epi, AM-Mes, and WJ-MSCs [4,21,25]. Although AM-Epi originates from epithelial cells, they lost the expression of epithelial markers and began expressing mesenchymal markers with subculturing. A previous study showed that this phenomenon of a morphological transition into mesenchymal cells is observed when preparing AM-Epi samples, indicating that a similar modification is also occurring at the gene level [22]. This phenomenon of epithelial–mesenchymal transition is thought to be induced through some type of stimulus during cell culture. However, there is a possibility that a portion of mesenchymal cells contained in the amnion epithelium fraction are selected through culturing and consequently proliferate. Thus, the mechanistic elucidation of this phenomenon is a future subject of investigation.

One of the characteristics of cell lines derived from the fetal life support system that possess the ability to support hematopoiesis is that they express MSC markers and exhibit differentiation potential into bone and adipose tissue [12]. Moreover, it has also been shown in mouse bone marrow that cells that function in the hematopoietic stem cell niche exhibit the morphology and function of MSCs [22]. In the future, it will be necessary to investigate the multipotency of stem cells derived from the fetal life support system prepared in the present study, and to elucidate the association between MSC function and the capability of maintaining hematopoietic stem cells.

4.3. Capacity to maintain hematopoietic stem cells in stem cells derived from the fetal life support system

In the present study, we compared the ability to maintain hematopoietic stem cells among different stem cells derived from the fetal life support system. The results showed that the proliferative capacity of cord blood CD34+ cells was high when co-cultured with any of the three cell types (AM-Epi, AM-Mes, or WJ-MSCs), and the levels were similar as the proliferative capacity of the HESS-5 positive control. In addition, the ability to maintain hematopoietic stem cells was shown to be greater in AM-Epi and in AM-Mes compared to WJ-MSCs based on flow cytometry and CFU-GM assay results. Robin et al. [12] generated a cell line from human placenta without separating different cell types and co-cultured these cells with CD34+ cells from human cord blood, and subsequently found that CD34+ cells increased 2.2- to 7.7-fold. This proliferative capacity is near equivalent to our results with isolated AM-Epi and AM-Mes (5.8-fold for AM-Epi and 7.6-fold for AM-Mes). The numbers of CFU-GM colonies of cord blood cells co-cultured with AM-Epi and with AM-Mes were similar to when the cells were co-cultured with HESS-5, which possess a high ability to maintain hematopoietic stem cells, suggesting that the functional capabilities of AM-Epi and AM-Mes to maintain hematopoietic stem cells are also high. Although there are several previous studies that assessed the capacity to maintain hematopoietic stem cells in co-culture experiments of bone marrow MSCs or WJ-MSCs with hematopoietic stem cells [26–30], none of them have made comparisons among different types of stem cells derived from the fetal life support system from the same donor in a co-culture system. Thus, the observation that AM-Epi and AM-Mes have greater capacities to maintain hematopoietic stem cells than WJ-MSCs is a novel finding of this investigation.

Lu et al. [31] reported *in vitro* that umbilical cord-derived MSCs and bone marrow MSCs have similar abilities to maintain hematopoietic stem cells. In the present investigation, the capacity to maintain or support CFU-GM was low in WJ-MSCs at a similar level as fibroblasts or the negative control without feeder cells. Although the reason for this is not clear, it is postulated that the samples may have contained cells that do not support hematopoietic stem cells because WJ-MSCs were prepared using the explant method as opposed to the collagenase enzymatic treatment. Recently, it was reported in a co-transplantation experiment of human hematopoietic stem cells and human umbilical cord-derived MSCs into NOD-SCID mice that the heterogeneity of MSCs is an important factor that influences hematopoietic stem cell engraftment [32]. In the future, a comparison of WJ-MSCs prepared with the enzymatic method may be necessary. Additionally, although we co-cultured each cell type with 1×10^5 cord blood CD34+ cells in the present study, it may also be necessary to study the changes in proliferation and maintenance capacity using different numbers of cells in the co-culture protocol. In the future, to verify whether or not cells obtained through co-culture maintain their hematopoietic activities *in vivo*, it will be necessary to transplant co-cultured cord blood cells into immunodeficient mice with ablated bone marrow to examine the engraftment and to assess whether or not a rapid improvement in hematopoietic recovery occurs.

Recently, clinical studies have been conducted in which umbilical cord-derived MSCs were transplanted at the same time as hematopoietic stem cell transplantation, and these studies showed that simultaneous MSC grafting can be performed safely and that this simultaneous grafting promotes hematopoietic stem cell engraftment, leading to early hematopoietic recovery [33–35]. It is also desirable to investigate whether or not hematopoietic stem cell engraftment rates improve and whether or not hematopoietic recovery is promoted by AM-Epi, AM-Mes, and WJ-MSCs when they are co-transplanted with human hematopoietic stem cells.

Recently, there have been few studies that investigated the mechanism of action of hematopoietic stem cell engraftment enhancement by MSCs derived from the fetal life support system.

Lee et al. simultaneously transplanted human hematopoietic stem cells and human umbilical cord-derived MSCs in NOD-SCID mice, and found that the expression of MCP-1, RANTES, EGF, and VEGF from MSCs is important in hematopoietic stem cell engraftment [32]. Moreover, Chhabra et al. reported that the PDGF-B signaling pathway plays a key role in maintaining hematopoietic stem cells in placental trophoblasts [36]. In the future, by comprehensively investigating and elucidating the molecular mechanism regarding hematopoietic stem cell maintenance by stem cells derived from the fetal life support system, it is anticipated that it would be possible to identify cells with greater grafting effects and to establish optimal methods for cell preparation, collection, preservation, and transplantation.

5. Conclusion

We tried to identify stem cells from organs of the fetal life support system such as the placenta and umbilical cord, and analyzed capacities to maintain hematopoietic stem cells. As a result, AM-Epi and with AM-Mes possessed a high ability to maintain hematopoietic stem cells, suggesting that the functional capabilities of AM-Epi and AM-Mes to maintain hematopoietic stem cells are also high. These findings indicated that cells derived from the fetal life support system such as AM-Epi and AM-Mes can be anticipated as potential cell sources for clinical application in cell therapies for the purpose of enhancing graft survival during hematopoietic stem cell transplantation.

Conflict of interest

The authors declare that they have no conflict of interest.

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