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Hematopoiesis-supportive function of growth-arrested human adipose-tissue stromal cells under physiological hypoxia

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***Ex vivo* expansion of hematopoietic progenitors is considered as an attractive tool to increase the number of stem and progenitor cells (HSPCs) for cell therapy. The efficacy of *ex vivo* expansion is strongly depends on the feeder cell activity to mimic hematopoietic microenvironment. Here we demonstrated, that combination of mitomycin C-induced growth arrest and tissue-related O_2 (physiological hypoxia) modulated stromal capacity of adipose tissue derived stromal cells (ASCs). Growth arrest did not affect viability, stromal phenotype and multilineage potential of ASCs permanently expanded at tissue-related O_2. Meanwhile, the PCR analysis revealed an up-regulation of genes, encoded molecules of cell–cell (*ICAM1*, *HCAM/CD44*) and cell–matrix adhesion (*ITGs*), extracellular matrix production (*COLs*) and remodeling (*MMPs*, *HAS1*) in growth-arrested ASCs at physiological hypoxia in comparison with ambient O_2 (20%). The number of ICAM-1 positive ASCs was increased under low O_2 as well. These alterations contributed into the *ex vivo* expansion of cord blood HSPCs providing the preferential production of primitive HSPCs. The number of cobblestone area forming cell (CAFC) colonies was 1.5-fold higher at physiological hypoxia ($p < 0.05$). CAFCs considered as long-term culture-initiating cells (LTC-IC) known to support long-term hematopoiesis restoration *in vivo*. The presented data may be applicable in the development of upscale protocols of HSPC expansion.**

[**Key words:** Multipotent mesenchymal stromal cells; Adipose tissue; Growth arrest; Mitomycin C; Hematopoietic stem and progenitor cells; *Ex vivo* expansion; Physiological hypoxia; Adhesion molecules; Extracellular matrix]

Multipotent mesenchymal stromal cells (MSCs) are capable to provide the support of other cells, i.e., implementing stromal function. This phenomenon is most well studied in the bone marrow niche where the stromal cells (MSCs, reticular cells, and osteoblasts) are involved in the maintenance of hematopoietic stem and progenitor cells (HSPCs) (1,2). Dexter et al. (3) have proposed an approach which makes possible the HSPC *ex vivo* culture on a layer of bone marrow stromal cells. Preliminary expansion allows to achieve a significant HSPC amplification, which is especially important with the limited number of HSPCs per sample. The success of expansion depends directly on the type of stromal cells used and the way the supporting layer is prepared.

Currently, MSCs are most frequently used *in vitro* as the feeder for HSPC expansion (4–11). In addition to the feeder properties for hematopoietic cells, MSCs have high proliferative activity, and are also more accessible than other types of human feeder cells (e.g., ductal epitheliocytes or splenocytes) (12). MSCs were also used after directed differentiation into osteoblasts, thus creating a similarity of an endostal niche (13).

Bone marrow MSCs are most often applied for the HSPC *ex vivo* expansion, therefore their stromal activity has been described in the most detail (12). In addition to bone marrow, MSCs can be obtained from blood vessel walls, synovial membrane, placenta, cord blood, and the umbilical vein sub-endothelial layer (14). There are some differences between the MSCs from different sources concerning to the marker expression and potential for proliferation and differentiation, but in general their properties are quite similar, including their capability of supporting HSPCs (14,15). This makes it possible to use MSCs from different tissues to simulate the bone marrow niche conditions (4,6,16). Human adipose tissue stromal-vascular fraction MSCs (ASCs) have been shown to support hematopoiesis *in vitro* (5,17), which makes them a good alternative to the bone marrow MSCs and an easily accessible source of feeder cells for HSPC expansion for clinical use (18).

Preliminary preparation of cells is an important factor that determines the efficacy of stroma-dependent expansion. To create the conditions that simulate the HSPC tissue niche, it is necessary to consider the influence of various microenvironmental factors, among which the low O_2 level, physiological hypoxia, is the most important one (19). Earlier, we have demonstrated that the cultured ASCs ensured their uncommitted state at physiological O_2 level (5% O_2). This was manifested in a high proliferative activity, decelerated response to differentiation stimuli, glycolytic shift in ATP production, and others (20). Further analysis of umbilical cord blood HSPC expansion using ASCs precultured at 5% and 20% O_2 demonstrated that an increase in the number of both undifferentiated ($CD34^+$) and committed (CFU) HSPCs was more effective under

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physiological hypoxia (21). The total duration of ASC/HSPC co-culture was 7 days, therefore the ASC were not growth-arrested before the experiment. However, the protocols for MSC-associated HSPCs expansion, e.g., the cobblestone area forming cells (CAFC) multiplication, require a much longer culture duration (up to 5 weeks). This makes it necessary to arrest the MSC growth. Gamma-irradiation or treatment with mitomycin C (mmC) are the growth arrest gold standard. These methods are considered to be equivalent as both treatments inhibit DNA replication, though they do it in a different manner. MmC is an antibiotic which acts as a double-stranded DNA alkylating agent. It covalently crosslinks DNA, inhibiting DNA synthesis and cell proliferation and used to prepare the stromal layer for the embryonic stem cells and keratinocytes (22). The application of mmC has an important advantage: this product is authorized for use in clinical practice, is available, inexpensive, whereas irradiation is expensive and time-consuming (23).

In this paper, we characterize the functional activity of ASCs and their stromal function after mmC-induced growth-arrest at the different O_2 levels (ambient − 20% vs. physiological hypoxia − 5%).

MATERIALS AND METHODS

Primary human mesenchymal stem cells Adipose tissue samples were obtained in the frame of Scientific Agreement from multidisciplinary clinic Soyuz (Moscow, Russia) after elective liposuction procedures under local anesthesia from healthy patients with written informed consent according to the Declaration of Helsinki. Adipose stromal cells (ASCs) were isolated from adipose tissue as previously described (24) with some modifications (25). The experimental protocols were approved by Biomedicine Ethics Committee of Institute of Biomedical Problems, Russian Academy of Sciences (Physiology Section of the Russian Bioethics Committee, Russian Federation National Commission for UNESCO, Permit 314/MCK/09/03/13). Briefly, tissue samples were treated with 0.075% collagenase IA (Sigma−Aldrich, St. Louis, MO, USA). After washing, cells were resuspended in growth medium, i.e. αMEM (Gibco, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Hyclone, GE Healthcare Life Sciences, South Logan, UT, USA), 250 μg/ml amphotericin, 5 μg/ml streptomycin, 5 U/ml penicillin, and 2 mM glutamine (MP Biomedicals Santa Ana, CA, USA). Directly after isolation, ASCs were placed in multigas CO_2 incubator (Sanyo) at 5% O_2, 5% CO_2, 90% N_2 (physiological hypoxia). After reaching 70−80% confluence cells were sub-cultured and ASCs of 2−4 passages were used in experiments.

Mononuclear cells from umbilical cord blood Cord blood mononuclear cells (cbMNCs) were isolated after written informed consent in the Cord blood bank Cryocenter (Moscow, Russia) using guidelines of the License of Federal Service on Surveillance in Healthcare and Social Development (Roszdravnadzor) (Permit FS 2010/342). Cryopreserved samples of CB-MNCs were provided as a part of Scientific Agreement between Cryocenter and Institute of Biomedical Problems.

Mitomycin C treatment Premonolayred ASCs were exposed overnight to mitomycin C (1.5 ug/ml) (Sigma−Aldrich, St. Louis, MO, USA) in growth medium. The cultures were washed with PBS thoroughly, trypsinized and subcultured in 60 and 35 mm Petri dishes (Corning Incorporated − Life Sciences, Oneonta, NY, USA) with a density of 6×10^3 cells/cm^2. Culture plates were placed into CO_2-incubator (20% O_2) or multigas incubator (5% O_2) (Sanyo, Osaka, Japan). After 72 h, mmC-ASCs reached 70−80% of confluence and were used for further analysis.

***In vitro* differentiation assay** Premonolayered mmC-ASCs were cultured in growth medium supplemented with specific adipogenic (1 μM dexamethasone, 0.5 mM IBMX, 10 μg/ml insulin, and 100 μM indomethacin) or osteogenic (0.2 mM ascorbic acid 2-phosphate, 10 mM glycerol 2-phosphate, and 0.1 μM dexamethasone) inductors (Mesenchymal Stem Cell Adipogenesis and Osteogenesis Kits, Merck KGaA, Darmstadt, Germany). After 7 days of induction, the cells were fixed with 4% formaldehyde in PBS. Adipogenic differentiation was determined by staining intracellular lipid droplets with 0.5% Oil Red O solution. Osteogenic differentiation was confirmed after histochemical evaluation of alkaline phosphatase activity (Leukocyte Alkaline Phosphatase Kit, Sigma−Aldrich).

Chemokine assessment Chemokine concentration in conditioned medium (CM) of mmC-ASCs was measured using Human Chemokine 6Plex (G-CSF, IL-8, MCP-1, MIG, MIP-1α, MIP-1β) assay (Bender MedSystems GmbH, Vienna, Austria) on FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA). CELLquest software (BD) was used for data acquisition. The concentration of each cytokine was linearly dependent on fluorescence intensity and was calculated using standard curves that were generated for each cytokine using Flow Cytomix Pro software (eBioscience, Thermo Fisher Scientific).

Quantitative RT-PCR analysis Total RNA was extracted with QIAzol Reagent (Qiagen, Venlo, Netherlands), purified by the phenol/chloroform method and reverse transcribed to cDNA with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. The expression of 84 genes encoding adhesion molecules and extracellular matrix was determined using the RT2 Profiler Extracellular Matrix & Adhesion Molecules Array (Qiagen) according to the manufacturer's protocol. Gene expression was normalized using five housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*) included in the kit. The gene expression level in the mmC-ASCs at physiological hypoxia was assessed in relation to mmC-ASCs at 20% O_2 by the $2^{-\Delta\Delta Ct}$ method. The significance of differences between groups was assessed by Student's *t* test. Differences were considered significant at $p \leq 0.05$.

CbMNC/mmC-ASCs and cbHSPC/mmC-ASCs coculture To enrich the population of cbHSPCs we used the experimental approach described by us previously (17). Briefly, confluent (70−80%) mmC-ASC layers were preformed at 20% and 5% O_2. MNCs from cord blood (1×10^6/ml) were seeded on these mmC-ASCs in RPMI 1640 (Gibco, Life Technologies, Thermo Fisher Scientific), supplemented with 10% inactivated FBS (Hyclone, GE Healthcare Life Sciences), 250 μg/ml amphotericin, 5 μg/ml streptomycin, 5 U/ml penicillin, and 2 mM glutamine (MP Biomedicals, Santa Ana, CA, USA) and cocultured at the same O_2 concentrations as mmC-ASCs monocultures. After 72 h, all floating cbMNCs were carefully washed away. One Petri dish in each set was fixed with cold methanol and the attached cbMNCs were analyzed after Giemsa differential staining. This ASC-associated population had been shown to be mostly composed of cbHSPCs (26,27). The dishes with attached cbHSPCs were further expanded 96 h. During mmC-ASC/cbHSPCs coculture, the new generation of floating cbHSPCs (flo-HSPCs) was observed. Flo-HSPCs were harvested, enumerated, and assayed by flow cytometry, seeded in colony-forming cell (CFC) and reseeding assays (Fig. 1).

Colony-forming cell assay Flo-HSPCs were collected on day 7 of coculture and 50×10^3 cells/ml were plated in methylcellulose-based medium MethoCult H4534 (Stemcell Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's protocol and cultured at 20% and 5% O_2. Hematopoietic colonies were scored after 14 days according manufacturer's manual.

Cobblestone area-forming cells CAFCs were defined as clusters of tightly packed cells that were non-refractory when viewed under a phase contrast microscope. Also, for more detailed analysis with Giemsa differential staining was applied.

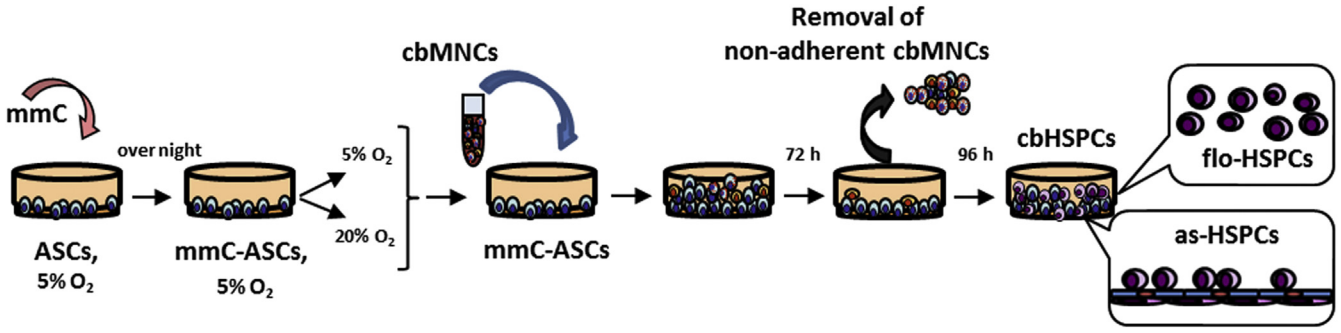

FIG. 1. Expansion of cbMNCs and their progeny HSPCs in co-culture with mitomycin C-treated ASCs. ASCs permanently expanded at physiological hypoxia (5% O_2) were overnight growth-arrested with mitomycin C (mmC) and further subcultured under 20% and 5% O_2. MNCs from human umbilical cord blood (cbMNCs) ($\times 10^6$ cells/ml) were inoculated on mmC-ASC pre-monolayer and cocultured 72 h under 20% and 5% O_2. Then non-adherent cbMNCs were removed. ASC-associated hematopoietic precursors (HSPCs) were further cultured for 96 h. Newly generated populations of floating (flo-HSPCs) and stroma-associated (as-HSPCs) were analyzed, accordingly.

Reseeding assay To evaluate the potential of newly-formed flo-HSPCs, 40×10^3 cells/ml were transferred onto new preformed ASC layers, again at 20% and 5% O_2, accordingly. After 72 h, the morphological observation of attached and floating cbHSPCs was performed, non-adherent cells were removed and co-cultures were further expanded during 14–21 days.

Light and fluorescent microscopy Bright-field, phase contrast, NAMC and fluorescent analysis of cultured cells was performed using Nikon Eclipse Ti–U microscope equipped with a Colour Digital Camera DS-Ri1. Images were saved and later processed with NIS-Elements Auto Research software (Nikon Instruments, Dusseldorf, Germany).

Fluorescent staining After 7 days, mmC-ASC/HSPC cocultures on coverslips were washed thoroughly with PBS and stained with anti-CD90 (FITC), CD45 (PE) and CD34 (FITC) antibodies for 30 min in CO_2-incubator. Then cells were washed with PBS trice, fixed with 4% formaldehyde in PBS for 15 min and mounted with Fluoroshield with DAPI (Sigma–Aldrich, St. Louis, MO, USA).

Flow cytometry Mouse anti-human monoclonal antibodies against CD45, CD34, CD73, CD90, CD105, CD54, CD44, CD29 (BD Biosciences) conjugated with FITC or PE were used for mmC-ASCs and cbHSPCs immunophenotyping. The staining procedures were executed according to manufacturer's instructions. Cells were analyzed with Accuri C6 flow cytometer (BD Biosciences). To evaluate fluorochrome unspecific staining, isotype controls for anti-IgG1 and anti-IgG2a were applied (BD Biosciences). Specimen analysis was performed with BD Accuri C6 software for collection, autocompensation, data processing and images acquisition.

Statistical analysis All data were derived from at least three independent experiments. The results are presented as mean ± standard error of the mean (M + S.E.M.). Comparisons between experimental results were determined by Mann–Whitney test for independent samples; $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of mitomycin C treated ASCs ASCs were isolated from human adipose tissue as described in the Materials and Methods section and permanently expanded under "physiological" hypoxia. Before the experiments, ASCs were treated with mmC and seeded into culture plates with $6 \times 10^3/cm^2$ density. During 72 h, mmC-ASCs were spreading out and formed a typical premonolayer (70–80% of confluence) (Fig. 2A). The efficacy of the growth arrest was confirmed by the absence of difference between initial ASC density after attachment ($5.5–5.8 \times 10^3/cm^2$) and ASC density when the experiments were finished ($5.3–6.0 \times 10^3/cm^2$). In flow cytometry, almost 100% of cells were CD73, CD90 and CD105 positive but CD45 negative (Fig. 2B). To confirm the multipotent state, mmC-ASCs were subjected to osteo- and adipodifferentiation. After 7 days in adipogenic medium, the mmC-ASCs with intracellular lipid droplets was detected (Fig. 2C). After osteoinduction, mmC-ASCs expressing alkaline phosphatase, a marker of early osteodifferentiation, was noted as well (Fig. 2D). These results confirmed that mitomycin C exposure did not affect ASC phenotype and multilineage commitment.

Mitomycin C treated ASCs under different O_2 levels To evaluate the effect of O_2 level on mmC-ASC functions, cells after mitomycin C treatment were seeded on culture plates and placed

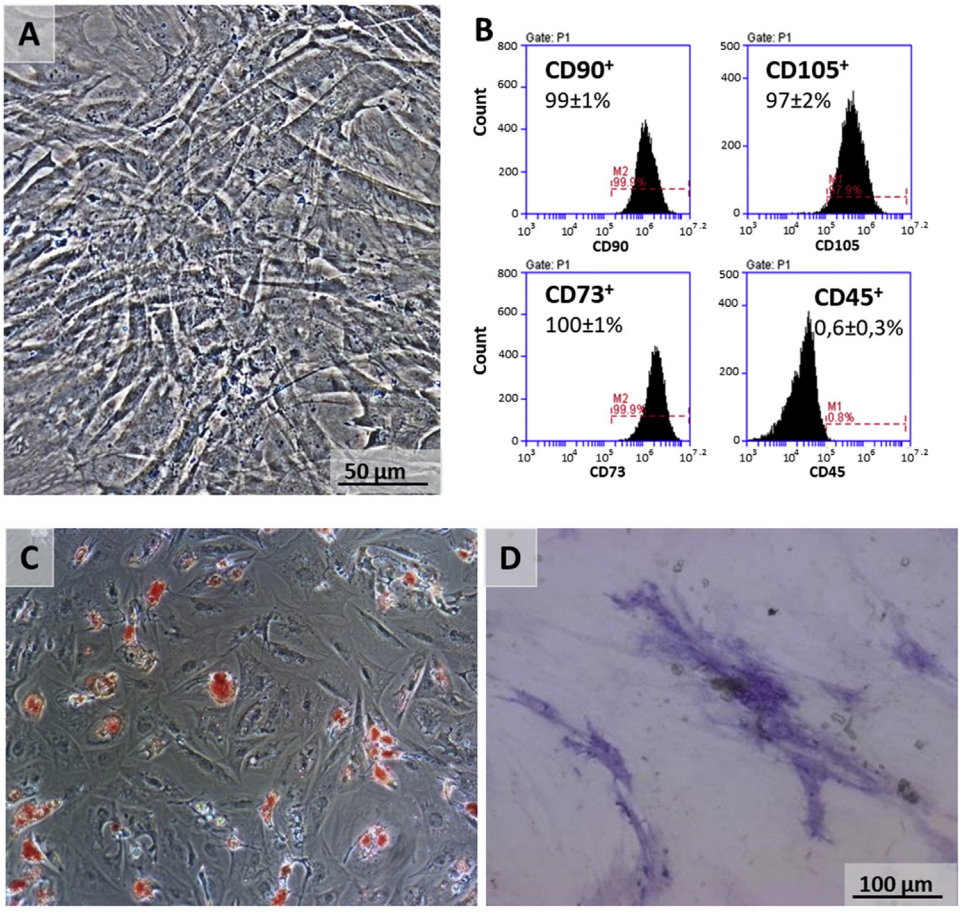

FIG. 2. Mitomycin C-treated ASCs displayed stromal phenotype at physiological hypoxia. (A) mmC-ASCs retained typical fibroblast-like morphology in premonolayer. Phase contrast. (B) Cell surface markers on mmC-ASCs. Flow cytometry, representative plots. The percentage of positively-stained ASCs is presented in upper left corner of plots (mean ± S.E.M. from three independent experiments). (C) Intracellular lipid droplets in mmC-ASCs after adipoinduction (oil Red O, phase contrast). (D) Histochemical evaluation of alkaline-phosphatase mmC-ASCs after osteoinduction (bright field).

into CO_2-incubator (20% O_2) and multigas incubator (5% O_2, physiological hypoxia). Independent of the O_2 levels, more than 90% of mmC-ASCs were attached to the culture plastic, spread out and, after 72 h, formed a preconfluent monolayer (70–80%). These mmC-ASCs were used to evaluate the functional activity.

Viability and phenotype MmC-ASCs displayed high viability at 20% and 5% O_2 (under both O_2, less than 5% of mmC-ASCs were stained with Trypan blue in exclusion test). They stably expressed the CD90, CD73, and CD105 stromal markers and did not bear hematopoietic 45 antigen (not shown). Almost 100% of mmC-ASCs bore HCAM (CD44) adhesion molecule and integrin 1 beta (CD29) on the surface, the mean fluorescence intensity (MFI) of staining was independent of the O_2 levels. At 5% O_2, the proportion of mmC-ASCs expressing ICAM-1 (CD54) adhesion molecule was increased, while the MFIs were similar at different O_2 levels (Fig. 3A).

Cytokine production MSCs are known to produce hematopoiesis-associated chemokines. Among those, a special attention is paid to C–C (MCP-1,-3, MIP1-a,-b, RANTES) and C–X–C (IL-8, IP-10) families. Multiplex analysis of chemokines in the mmC-ASC-conditioned medium demonstrated the presence of a significant amount of MCP-1 and IL-8 at both O_2 levels (Fig. 3B).

Thus, independently of O_2 levels, mmC-ASCs displayed high viability, retained stromal phenotype, secreted hematopoietic chemokines. An increased proportion of ICAM-1-positive mmC-ASCs at 5% O_2 may contribute to the enhancement of their stromal function.

Hematopoiesis-supportive potential of mitomycin C-treated ASCs The ability of MSCs to maintain hematopoiesis *ex vivo* put forward of their use as a feeder layer. Previously, we have described the stroma-dependent expansion of cbHSPCs on ASCs at the different O_2 levels. For those experiments, we have applied non-growth-arrested ASC premonolayer (21). Accordingly, when interacting with HSPCs, ASCs continued to proliferate, which was not applicable for prolonged coculture.

MSCs are involved in the regulation of hematopoiesis, creating a specific tissue environment. In addition to soluble mediators, MSCs produce extracellular matrix components and also contact with hematopoietic cells and matrix through the surface molecules of several superfamilies (integrins, selectins, sialomucins, immunoglobulins) (28). The increased/decreased production of various MSC metabolites can regulate both HSPC self-renewal and differentiation (29).

To characterize the mmC-ASC stromal activity, we profiled the activity of adhesion molecule and extracellular matrix genes, and the efficacy of cbHSPC expansion at the different O_2 levels.

Differential gene expression of ASCs after mitomycin treatment Using Extracellular Matrix & Adhesion Molecules PCR Array, the expression of 84 genes encoded molecules which are involved in cell adhesion and extracellular matrix metabolism was determined.

In the mmC-ASCs under 5% O_2, the several genes were upregulated versus cells at 20% O_2 (Table 1). Those were primarily the genes encoding cell–cell (*ICAM1*, *HCAM/CD44*) and cell–matrix (*ITGs*) molecules. Furthermore, the transcription of several collagen molecules (*COLs*) and enzymes governing matrix remodeling was upregulated: interstitial (*MMP1*) and membrane-bound (*MMP14*, *ADAMTS1*) metalloproteases, as well as hyaluronansynthase (*HAS1*) involved in proteoglycan synthesis. These data may evidence the enhancement of mmC-ASC stromal activity at 5% O_2.

To test this assumption, mmC-ASCs were used for cbHSPC expansion at 20% and 5% O_2.

Expansion of cbHSPCs on mitomycin C-treated ASCs As it was mentioned earlier, proliferating hematopoietic cells could adhere to the feeder layer (26,27). Above feature can be used to select the cells potentially capable of expansion. Of all cbMNCs inoculated onto mmC-ASCs, about 10% of the cells were attached during 72 h of coculture, which averaged 49.7 ± 6 and 49.1 ± 18.0 × 10^3/cm^2 of total nucleated cells (TNCs) at 20% and 5% O_2, respectively. During the next 96 h of coculture,

A

Adhesion molecules	Positive mmC-ASCs, %		MFI arb. un. * 10^3	
	20% O_2	5% O_2	20% O_2	5% O_2
ICAM-1 (CD54)	11±2	27±1 *	210±70	220±30
HCAM (CD44)	100±1	100±1	2510±900	2670±1300
Integrin beta 1 (CD29)	99±1	100±2	830±200	1040±30

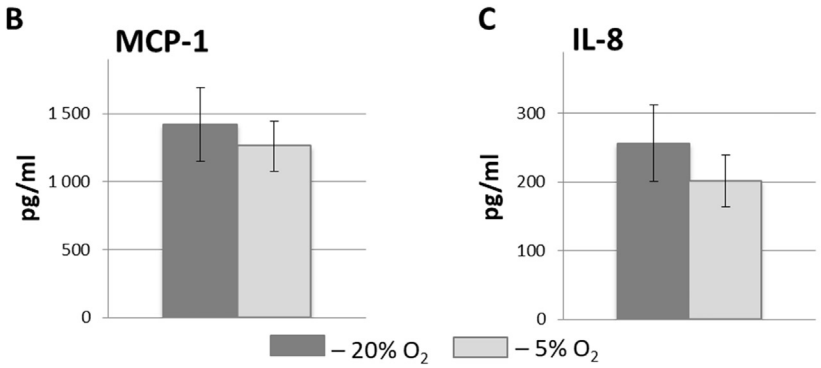

FIG. 3. Characterization of mitomycin C-treated ASCs at different O_2 levels. (A) Expression of adhesion molecules. The percentage of positively stained mmC-ASCs and arbitrary units of a mean fluorescence intensity (MFI) was determined by flow cytometry. The data are representative of three independent experiments (*$p < 0.05$ vs. 20% O_2). (B, C) Paracrine activity. Concentrations of MCP-1 (B) and IL-8 (C) were measured in conditioned medium of mmC-ASCs with ELISA. The data are representative of three independent experiments.

TABLE 1. Differential expression of mmC-ASC genes encoding the molecules mediating the interaction of HSPCs with microenvironment.

Gene	Description	Fold regulation, 5 vs. 20% O_2
	Cell-to-cell adhesion	
ICAM1	Intercellular adhesion molecule 1	1.5
CD44	Homing-associated cell adhesion molecule (HCAM/CD44)	1.5
	Extracellular matrix	
COL6A1	Collagen, type VI, alpha 1	1.7
COL6A2	Collagen, type VI, alpha 2	1.8
COL8A1	Collagen, type VIII, alpha 1	1.8
	Cell–matrix adhesion	
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	1.8
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	1.9
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	1.5
ITGA6	Integrin, alpha 6	1.5
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	1.9
ITGB5	Integrin, beta 5	1.6
	Extracellular matrix remodeling	
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.8
HAS1	Hyaluronan synthase 1	1.6
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	1.6
MMP14	Matrix metallopeptidase 14 (membrane-inserted)	1.7

The genes whose expression significantly (more than 1.5-fold, $p < 0.05$) changed in mmC-ASCs at 5% vs. 20% O_2 are shown.

from those attached cells two cbHSPC populations were raised, the floating (flo-HSPCs) and mmC-ASC-associated (as-HSPCs). In both populations cbHSPCs had a high viability of greater than 90%.

Flo-HSPCs Flo-HSPCs were small rounded cells, bright with phase-contrast microscopy (Fig. 4A and B). Flow cytometry revealed uncommitted $CD34^+$ precursors among flo-HSPCs, with the proportion exceeding significantly their initial share among CB-MNCs (Fig. 4C). In addition to early precursor immunophenotyping, the functional activity of flo-HSPCs was evaluated using the CFC and cobblestone area forming cell (CAFC) tests. These assays made it possible to identify the progenitor cells of different commitment, which are an important criteria for the evaluation of the effects of microenvironmental factors on hematopoietic cells.

Using semi-solid methylcellulose-based medium (Methocult) formulated to promote optimal growth and differentiation of hematopoietic progenitor cells, hematopoietic CFCs were detected. Among the flo-HSPCs collected at day 7, the CFC number at 5% O_2 significantly exceeded the CFCs under 20% O_2 (Fig. 4D).

When reseeded on a new mmC-ASCs layer, a part of flo-HSPCs were attached and again gave rise to the floating and stroma-associated populations (Fig. 4E and F). Upon HSPC reseeding, the CAFC areas were formed faster versus the initial coculture. They could be found as early as in 72 h after reseeding.

Stroma-associated HSPCs Stroma-associated HSPCs (as-HSPCs) were present both on the surface of mmC-ASCs and under the feeder layer (Fig. 5A–D). Among sub-stromal HSPC subpopulation, two types of cells could be distinguished: (i) small rounded 12–16 μm cells and (ii) large flattened 28–43 μm cells (Fig. 5B, C, E, F). The small cells had a large rounded basophilic nuclei containing one or two distinct nucleoli, and a thin rim of the cytoplasm. In the larger cells, nuclei were predominantly elongated or bean-shaped, contained one to two large nucleoli, and were shifted to the periphery of the cells. The cytoplasm was strongly flattened and was lighter in the center. Both cell types were located alone or in the groups of 4–16 cells. After 7 days of culture, clusters of CAFCs were formed in the sub-stromal space (Fig. 5C, F). At 5% O_2, the number of such CAFC associations was significantly higher versus standard culture conditions (20% O_2) (Fig. 5G).

CAFCs are considered to be primitive HSPCs capable of long-term hematopoiesis initiation *in vivo*. Detection of these cells in hematopoietic cultures is a common technique that allows to evaluate the proportion of actively proliferating early hematopoietic progenitors *in vitro*. Since we obtained CAFCs after 7 days of coculture, we can assume that the above cells in the CAFC hierarchy relate to CAFC-7. Those cells are capable of commitment in multi- and biopotent CFUs: CFU-GEMM, CFU-GM, BFU-E *in vitro*, and CFU-S *in vivo* (30). For their proliferative activity, CAFCs from the 7–11 day's coculture correspond to CFU-S12 that form colonies in the spleen of irradiated mice on day 12–14 after transplantation (31).

HSPCs associated with mmC-ASCs were immunophenotyped on coverslips in Petri dishes and in suspension after trypsinization of coculture (Fig. 5H and I). In both cases, blood-borne cells were identified due to expression of pan-leukocyte antigen CD45. ASCs were assumed to be CD90 positive, CD45 negative. The further analysis revealed that among $CD45^+$ cells the HSCPs with $CD90^-/CD45^+$ and $CD90^+/CD45^+$ were presented (Fig. 5H). Among $CD90^-/CD45^+$[Fig. 5] cells, there were both large flattened cells and small rounded cells; the HSPCs expressing both markers were represented by small rounded cells only (Fig. 5H, inset). CD90 is known to be expressed not only by stromal cells, but also by very early HSPCs (32,33), thus $CD45^+/CD90^+$ cells can be assumed to be earlier precursors than the $CD45^+/CD90^-$. These observations were confirmed with flow cytometry (Fig. 5I). More than 90% of cells were viable after trypsinisation of mmC-ASC/HSPC coculture. Since the suspension contained two cell types, the gating of populations for analysis was performed based on the expression of CD90 and CD45 antigens. $CD90^+/CD45^-$ ASCs were found in the lower right quadrant (LR), and the $CD90^+/CD45^+$ cells were observed in the upper right quadrant (UR). The majority of the latter carried the CD34 antigen as well, i.e. these cells were the undifferentiated hematopoietic progenitors. The $CD90^-/CD45^+$ HSPC population was found in the lower left quadrant (LL). The share of $CD34^+$ cells among as-HSPCs comprised about 60%, significantly exceeding the initial proportion of $CD34^+$ undifferentiated precursors in CB-MNC population.

DISCUSSION

In this study, we demonstrated that the growth-arrested ASCs retain their stromal phenotype, ability to lineage-specific differentiation, and can maintain umbilical cord blood HSPCs independent of the O_2 levels in the microenvironment. Compared to 20% O_2, a significantly more hematopoietic CFUs were found among the

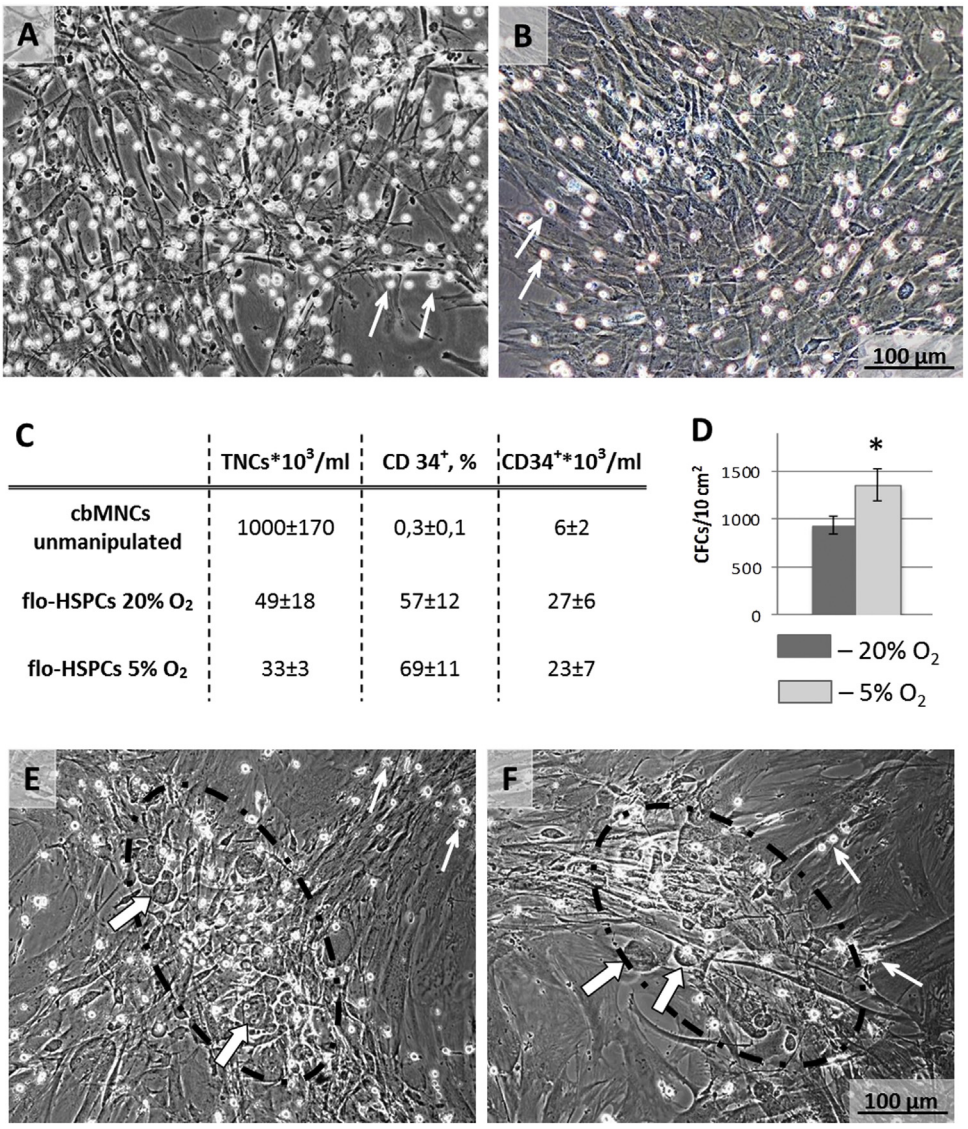

FIG. 4. Floating HSPCs in coculture with mmC-ASCs after 7 days of *ex vivo* expansion at different O_2 levels. (A,B) flo-HSPCs appeared as phase bright with phase contrast microscopy (white arrows). (C) Evaluation of $CD34^+$ precursors among cbMNCs and flo-HSPCs, flow cytometry data are representative of three independent experiments. TNCs, total nucleated cells. (D) Estimation of CFUs among flo-HSPCs using semi-solid methylcellulose medium (Methocult, Stemcell Technologies) assay. The data are representative of four independent experiments ($^*p < 0.05$ vs. 20% O_2). (E,F) Reseeding of flo-HSPCs gave rise to new generation of floating and stroma-associated HSPCs. Phase contrast. Flo-HSPCs are indicated with thin white arrows; as-HSPCs are indicated with thick white arrows. The dotted line surrounds the CAFC cluster. (A,E) 20% O_2; (B,F) 5% O_2.

floating HSPCs, as well as a significantly more CAFC colonies were observed among the ASC-associated HSPCs at physiological hypoxia (5% O_2). At least some of these effects could be due to a change of mmC-ASCs functional activity connected with the up-regulation of genes responsible for cell adhesion and extracellular matrix production/remodeling under physiological hypoxia.

The stimulating effects of *ex vivo* hypoxia on the expansion of hematopoietic cells both in monoculture and in coculture with stromal cells have been convincingly shown previously (19,34,35). It was demonstrated that hypoxic expansion enhanced the number of CFUs and improved its resistance to irradiation and the potential to restore hematopoiesis in lethally irradiated animals (36,37). Furthermore, low oxygen level preferentially maintained the viability and proliferation of low differentiated hematopoietic cells versus the committed precursors (37,38). These effects may be due to the up-regulation of *HIF-1α* and HIF-dependent genes (*VEGF* and *ABCG2*), as well as to the activation of CXC chemokine receptor 4 (CXCR4) expression (31).

In addition, *in vitro* physiological hypoxia affects not only the hematopoietic cells, but also the MSCs. Being propagated under hypoxia, MSCs have demonstrated decreased osteogenic and adipogenic potentials (25,39), stimulation of chondrodifferentiation, and an increase in the proliferative activity and in the number of fibroblast colony forming units (25,40,41). These data emphasize the role of oxygen as an important factor that determines the fates of the cells that belong to the stromal and hematopoietic lineages.

HSPC stratification in coculture with MSCs may be associated with the local O_2 level. As it was demonstrated under standard conditions (20% O_2) bone marrow MSC-adherent HSPCs proliferated actively giving rise to the floating population (27). Those cells that migrated under the stromal monolayer divided rarely and retained the immature $CD34^+CD38^-$ phenotype versus the other fractions of hematopoietic precursors in coculture (27). The microareas of hypoxia were formed under feeder layer, which was demonstrated using pimonidazole (42). Under the hypoxic protocol, decreased adhesion of hematopoietic cells to the MSCs was

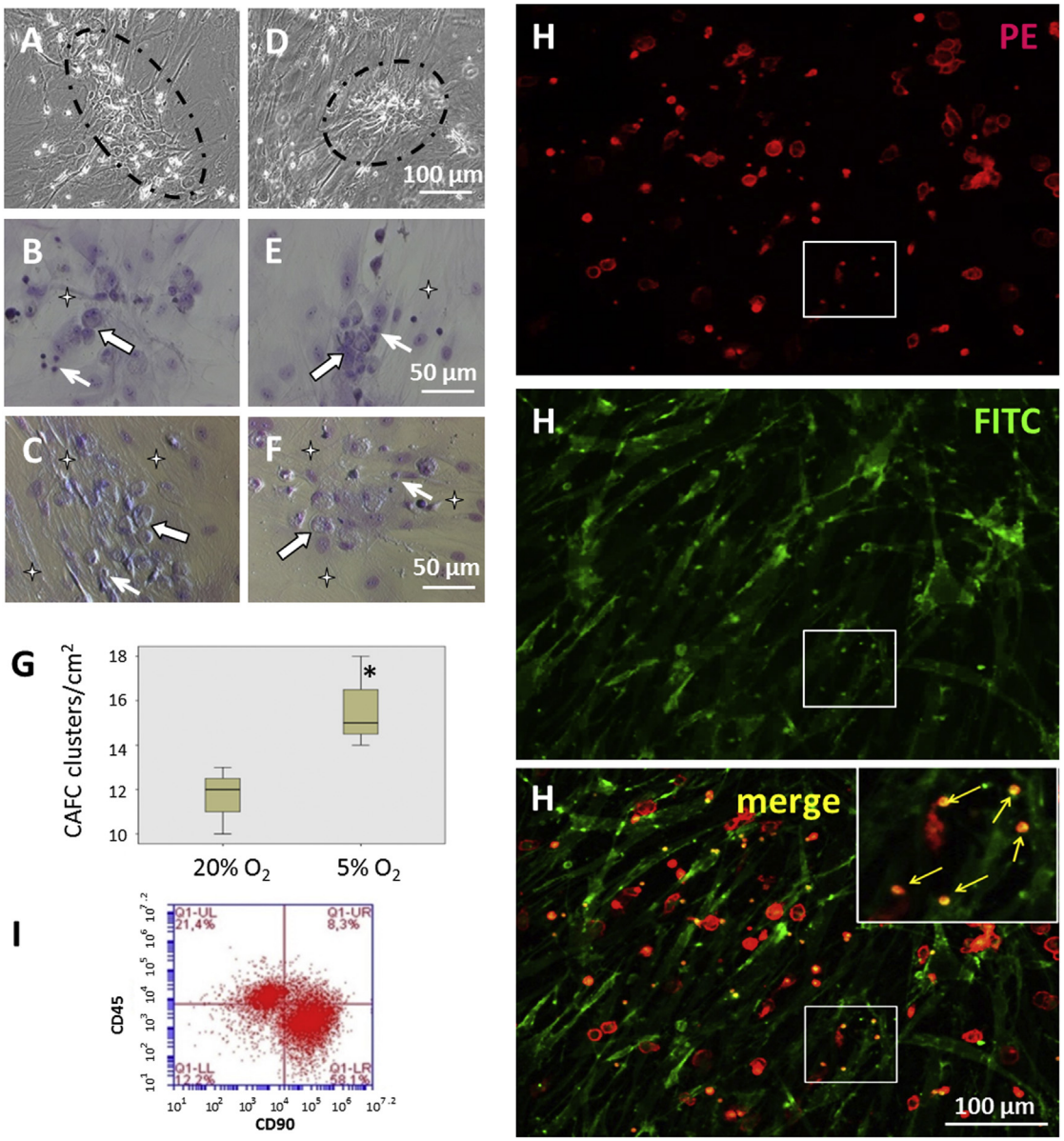

FIG. 5. Mitomycin C-ASCs-associated HSPCs after 7 days of *ex vivo* coculture at different O_2 levels. (A,D) Colonies of HSPCs on and beneath of mmC-ASCs. Phase contrast. The dotted line surrounds the CAFC cluster. (B,E) Heterogeneity of ASC-associated HSPCs. Differential Giemsa staining of mmC-ASC-associated HSPCs: small-sized HSPCs (thin white arrows); large-sized HSPCs (thick white arrows). HSPCs formed groups resembling cobblestone areas under both O_2. Bright field. (C,F) Heterogeneity of ASC-associated HSPCs on and beneath of mmC_ASCs: small-sized HSPCs (thin white arrows); large-sized HSPCs (thick white arrows). DIC microscopy well demonstrates, that small and large HSPCs are combined in CAFC-like areas beneath of mmC-ASCs indicated with asterisks. (G) Enumeration of CAFC clusters. *Significant difference from the values at 20% O_2, $p < 0.05$. (H) Immunocytochemical indentification of mmC-ASC and HSPCs in coculture. Upper image: CD45 cells$^+$ (red staining, PE); middle image, CD90$^+$ cells (green staining, FITC); low image: merge, double-stained cells are yellow. Inset: small rounded double-stained HSPCs (yellow) expressing both markers are marked with arrows. (I) Flow cytometry of mmC-ASC/HSPC suspension, representative plot.

observed, but these conditions facilitated cell migration under the stromal monolayer. Under hypoxia, an increased production of vascular endothelial growth factor A (VEGF) was detected that apparently mediated the increased permeability of the MSC monolayer (42).

Stromal cells provide the formation of specific hematopoietic microenvironment due to the production of paracrine mediators, direct cell contacts, and extracellular matrix production (2,43). In mmC-ASCs under physiological hypoxia, profiling of extracellular matrix and associated molecule gene expression confirmed an up-regulation of hypoxia-dependent genes encoding adhesion molecules, matrix enzymes, collagens, integrins. The above changes may be assumed to contribute into stimulation of mmC-ASC stromal activity in relation to stroma-associated HSPCs under physiological hypoxia, we have described here.

Thus, the growth arrest of ASCs expanded under physiological hypoxia allows to obtain the cells with modified feeder capacities. Application of above ASCs in combination with physiological hypoxia as a feature of HSPC tissue niche provides pronounced stromal effects with predominant maintenance of very primitive HSPCs. These HSPCs are considered as long-term culture-initiating cells (LTC-IC) known to support long-term hematopoiesis restoration *in vivo*. The results presented here may be on demand to create the effective and controlled methodological approaches that ensure

production of a large number of hematopoietic stem and progenitor cells for needs of regenerative medicine.

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