SPANDIDOS PUBLICATIONS

Adipocyte differentiation of human marrow mesenchymal stem cells reduces the supporting capacity for hematopoietic progenitors but not for severe combined immunodeficiency repopulating cells

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Abstract. Bone marrow stromal cells provide a microenvironment for hematopoiesis. Adipocytes are the major stromal cell phenotype in bone marrow, but their function in hematopoiesis is poorly understood. In this study, we compared the hematopoietic-supporting capacity of adipocytes and their progenitor, mesenchymal stem cells (MSCs), by culturing human cord blood (CB) $CD34^+CD38^-$ hematopoietic progenitor cells (HPCs) on a layer of adipocytes or MSCs. CB $CD34^+CD38^-$ cells cultured on MSCs generated higher proportions of $CD34^+CD38^-$ HPCs and colony-forming cells than those cultures on a layer of adipocytes, indicating an inferior hematopoietic support by adipocytes. However, CB $CD34^+CD38^-$ HPCs cultured on MSCs and adipocytes were equally capable of reconstituting human hematopoiesis in non-obese diabetic/severe combined immunodeficient disease (NOD/SCID) mice. These findings show that differentiation of MSCs into adipocytes is accompanied by the loss of capacity to support mature HPCs, but not transplantable SCID-repopulating cells.

Introduction

Human bone marrow contains hematopoietic stem cells (HSCs) and their progeny (hematopoietic progenitor cells, HPCs) which continuously provide blood cells in the periphery, and stromal cells which provide a supportive scaffolding or microenvironment for hematopoiesis (1). Human $CD34^+$ cells include several classes of HPCs, such as relatively mature *in vitro* colony-forming cells (CFCs) and immature transplantable SCID-repopulating cells (SRC) that can engraft in non-obese diabetic/severe combined immunodeficient disease (NOD/SCID) mice (2,3). Bone marrow stromal cells comprise a variety of cell types, such as macrophages, fibroblasts, endothelial cells, adipocytes, reticular cells and osteogenic precursors (1,4). Adipocytes are most abundant in adult human bone marrow, but there have been conflicting reports on their role in hematopoiesis (4). Adipocyte differentiation from murine preadipocytes has been shown to maintain hematopoiesis *in vitro* (5). In human long-term bone marrow culture, stromal adipocytes have been reported to be essential for long-term hematopoiesis, indicating that bone marrow adipocytes exert a positive effect on hematopoiesis (6). On the other hand, it has been shown that the ability of a preadipose cell line to support growth of hemopoietic stem cells decreases when the preadipocytes differentiate into adipocytes (7), and continuous human marrow hematopoiesis *in vitro* is not stimulated by adipocytes (8). Thus, the precise role of adipocytes in hematopoiesis remains to be determined.

Bone marrow stromal cells contain undifferentiated cells of mesenchymal origin which have the potential to differentiate into adipocytes, osteoblasts, chondrocytes, and stromal fibroblasts, which are referred to as mesenchymal stem cells (MSCs) (9). MSCs in bone marrow are few in number, but can be expanded *in vitro* to form a monolayer in culture of homogeneous populations. These cells have been shown to be able to support hematopoietic differentiation of $CD34^+$ hematopoietic progenitor cells (10). It has also been reported that MSCs support the hematopoietic cells, including long-term culture initiating cells (LTC-IC) after differentiation toward stromal and osteogeneic lineages (11).

In the present study, we compared the hematopoietic-supporting capacity of adipocytes and their progenitor, MSCs. We co-cultured cord blood (CB) $CD34^+CD38^-$ cells (an immature population of $CD34^+$ cells) (12) on MSCs or adipocytes, and found that the hematopoiesis-supporting

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capacity of MSCs decreased with adipocyte differentiation. However, CB $CD34^+CD38^-$ cells co-cultured with adipocytes preserved their ability to engraft in NOD/SCID mice, suggesting that adipocytes maintain the ability to support transplantable SCID-repopulating cells.

Materials and methods

Cytokines. Recombinant human (rh)-interleukin (IL)-3, rh-stem cell factor (SCF), rh-granulocyte colony-stimulating factor (G-CSF), rh-granulocyte/macrophage (GM)-CSF, rh-thrombopoietin (TPO) and rh-erythropoietin (Epo) were a generous gift from the Kirin Brewery Co. Ltd. (Tokyo, Japan). Flt3 ligand (FL) was purchased from R&D Systems (Minneapolis, MN).

Mice. Ten-week-old female NOD/shi/SCID mice were purchased from Clea Japan (Tokyo, Japan). The mice were kept on racks under specific pathogen-free conditions with a laminar air flow and were supplied with sterile food and drinking water.

Culture of mesenchymal stem cells. Human bone marrow was aspirated from the iliac crest of normal subjects after obtaining informed consent. Mononuclear cells (MCs) were separated by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden). MCs were cultured in a Mesenchymal stem cell growth medium (MSCGM) (Cambrex Biosciences, Walkersville, MD) (13). The medium was changed every week, and adherent cells with a fibroblast-like appearance were maintained. For adipogeneic differentiation, MSCs were cultured in an adipogeneic medium containing insulin, dexamethasone and indomethacin (hMSC differentiation kit-Adipogenic) (Cambrex Biosciences). For osteogeneic differentiation, MSCs were incubated in an osteogeneic medium containing dexamentasone, ascorbate and ß-glycerophosphate (Osteogenic differentiation kit). For chondrogeneic differentiation, MSCs were cultured in a chondrogeneic medium containing tumor growth factor (TGF)ß-3, dexamethasone and ascorbate (Chondrogeneic differentiation kit). Adipogeneic, osteogeneic and chondrogeneic differentiation were confirmed by staining with Oil red O, von Kossa and Safranin O, respectively (14,15).

Isolation of lineage-negative cord blood cells. Umbilical cord blood (CB) was obtained from normal full-term deliveries after obtaining the mother's consent. Mononuclear cells (MCs) were separated by density gradient centrifugation using Ficoll-Paque (Amersham). The MCs were subjected to deplete lineage positive cells using the Human progenitor cell enrichment kit (StemCell Technologies, Inc., Canada) which included antibodies to lineage-specific antigens (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and Glycophorin A). The lineage negative cord blood cells were frozen in α-medium supplemented with 10% dimethylsulfoxide, 12% hydroxyethyl starch (CP-1 cryoprotectant) (Kyokuto Pharmaceutical Co., Tokyo, Japan) and 8% human serum albumin at -80˚C.

Flow cytometric analysis and cell sorting. Cells were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies at 4˚C for 30 min and subjected to an immunofluorescence analysis using an EPICS XL flow cytometer (Coulter, Miami, FL). The monoclonal antibodies used were PE-conjugated antibodies to CD34 (Coulter), CD45 (Coulter) and CD106 (Pharmingen, San Diego, CA); and FITC-conjugated antibodies to CD38 (Coulter), CD49, CD54 (Immunotech, Marceille, France), CD73 (Pharmingen) and CD105 (Serotec Oxford, UK). Dead cells were gated out with a forward vs side scatter window and propidium iodide staining. Isotype-matched antibodies were used as control in all experiments. $CD34^+CD38^-$ cells were isolated by sorting lineage negative cord blood cells using FACSAria (Becton-Dickinson).

Co-culture of CB $CD34^+CD38^-$ cells and MSCs or adipocytes in the presence of cytokines. A monolayer of MSCs was established in wells of a 12-well plate (Coster), and adipocytes were induced as described above. $CD34^+CD38^-$ cells ($1x10^3$ cells/well) were cultured without a stromal layer or on a layer of MSCs or adipocytes in 1 ml of serum-free medium, StemPro-34 (Gibco BRL, Grand Island, NY), supplemented with StemPro-34 nutrient supplement (Gibco BRL), 2 mM glutamine and penicillin/streptomycin in the presence of 100 ng/ml SCF, 100 ng/ml TPO and 100 ng/ml FL. After 7 and 14 days of culture, the cells were harvested by vigorous pipetting and subjected to CFC assay and flow cytometric analysis.

RT-PCR analysis of cytokines. Total RNA was extracted from the monolayer stromal cells using an Isogen RNA extraction kit (Wako Pure Chemical Co., Osaka, Japan) and was dissolved in diethylpyrocarbonate (DEPC; Sigma)-treated water. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using primers described previously (13) and the RNA PCR kit AMV ver. 2.1 (Takara Shuzo, Shiga, Japan). The amplified products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed.

Colony-forming cell assay. Colony-forming cell (CFC) assays were performed in 35-mm petri dishes (Becton-Dickinson) by incubating the cells in semisolid α-medium containing 0.8% methylcellulose (Shinetsu Chemicals Co., Tokyo, Japan), 30% FCS (Gibco), 1% BSA, 10^{-4} M 2-mercaptoethanol (2-ME) (Wako Pure Chemicals), 2 mM L-glutamine (Sigma), 10 ng/ml IL-3, 20 ng/ml SCF, 10 ng/ml G-CSF, 10 ng/ml GM-CSF and 2 U/ml Epo (Kirin Brewery) for 14 days at 37˚C in a humidified atmosphere flushed with 5% CO_2 in air (13). The colony-forming units (CFU)-GM, CFU-Mix and burst-forming units-erythroid (BFU-E) were identified by the ability to form granulocyte/macrophage (GM) colonies, mixed erythroid and myeloid (Mix) colonies and erythroid burst colonies, respectively, as described previously (16-18). High proliferative potential-colony-forming cells (HPP-CFC) were identified by their ability to form densely packed colonies that reached a size of >1 mm in diameter (19).

Transplantation into NOD/SCID mice (SCID-repopulating cell assay). CB $CD34^+CD38^-$ cells ($1x10^3$ cells/well in a 12-

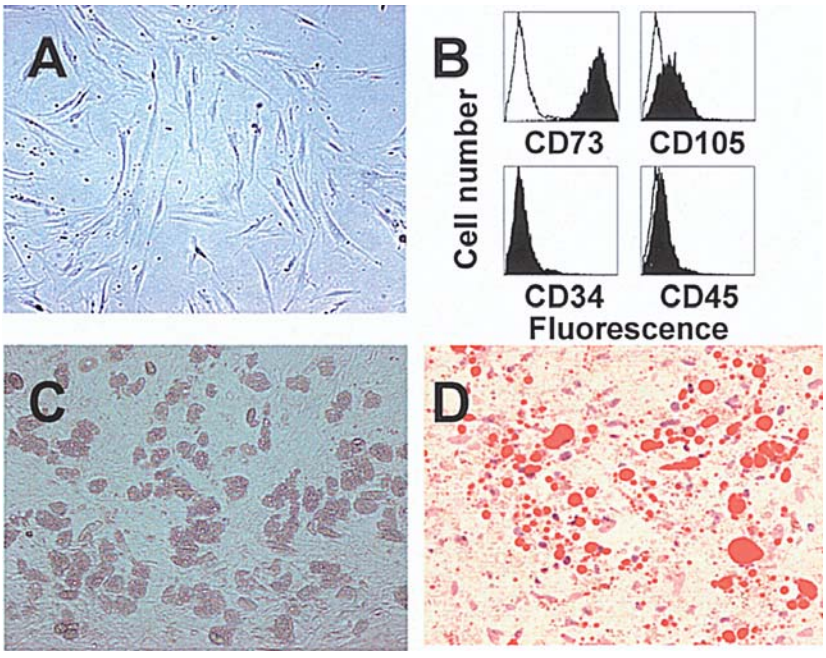

Figure 1. MSCs and differentiation into adipocytes. (A) Phase microscopy of human mesenchymal stem cells (x40). (B) Flow cytometric analysis of cell surface markers on MSCs. Adipocytic differentiation of MSC was confirmed by the presence of neural lipid vacuoles (C) (x40) and lipid positive for Oil red O staining (D) (x40).

well plate) were cultured on a layer of MSCs or adipocytes or without a stromal layer in the presence of SCF/TPO/FL for 2 weeks. The cells harvested from one well were injected into the tail veins of 10-week-old NOD/shi/SCID mice sublethally irradiated (3Gy X-ray). The mice were intraperitoneally injected with anti-asialo GM1 antibody (Wako Pure Chemical) to reduce the natural killer cell activity (20). The mice were sacrificed 8 weeks after the transplantation, DNA was extracted from BM cells using a SepaGene extraction kit (Sanko Pure Chemical Co., Tokyo, Japan) and was analyzed for the human-specific DNA 17α-satellite gene by quantitative real-time PCR (21). The primers used were sense 5'-ACGGGATAACTGCACCTAAC-3', and antisense 5'-CCATAGGAGGGTTCAACTCT-3'.

Statistical analysis. The data are presented as the mean ± standard deviation (SD). The Student's t-test was used. P values of <0.05 were accepted as significant.

Results

Differentiation of MSCs into adipocyte lineage. Cultivation of bone marrow mononuclear cells from normal subjects in a mesenchymal stem cell growth medium (MSCGM) resulted in gradual proliferation of adherent MSCs. These cells were fibroblast-like in morphology (Fig. 1A), positive for CD73, CD105 (Fig. 1B), CD49, CD54, CD90 and CD106 (data not shown), and negative for CD34 and CD45 (Fig. 1B). Differentiation of MSCs into adipocytes was determined by the presence of neural lipid vacuoles (Fig. 1C) and lipid detectable by Oil red O stain (Fig. 1D). MSCs were also capable of undergoing osteogeneic and chondrogeneic differentiation under specific conditions (data not shown).

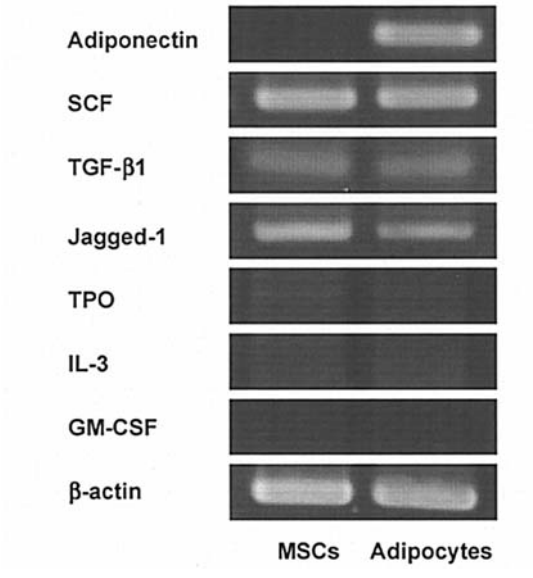

Figure 2. RT-PCR analysis of cytokine mRNAs. Total RNA was extracted from MSCs and adipocytes and analyzed for various cytokine mRNAs by RT-PCR.

The expression of cytokines and growth-related molecules in MSCs and adipocytes. We performed RT-PCR analysis of the mRNA of cytokines and growth-related molecules in MSCs and adipocytes. MSCs expressed SCF, TGF-ß1 and Jagged-1 but not TPO, IL-3 and GM-CSF (Fig. 2). The expression of SCF, TGF-ß1 and Jagged-1 was maintained after adipocyte differentiation. The message of adiponectin, an adipocyte-specific secretory protein (22), was not detected in MSCs but was expressed in adipocytes (Fig. 2).

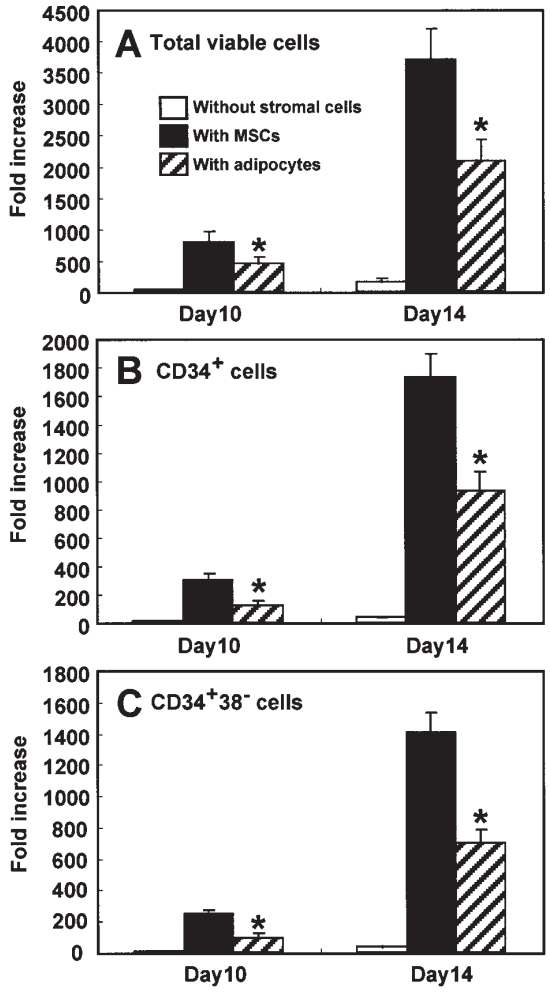

Figure 3. Effect of adipocytes on the proliferation of CB34$^+$38$^-$ cells. CB CD34$^+$CD38$^-$ cells (1x10^3) were cultured without stromal cells, or on MSCs or adipocytes in the presence of SCF, TPO and FL. On day 10 and 14, cells were collected and analyzed for the number of total viable cells (A) CD34$^+$ cells (B) and CD34$^+$38$^-$ cells (C). Data are the mean ± SD of fold increases over the values on day 0 of four separate experiments. *P<0.05 in comparison to the MSC culture.

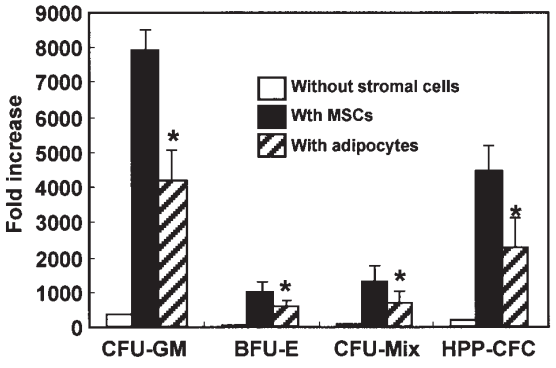

Figure 4. Effect of adipocytes on colony formation of CB34$^+$38$^-$ cells. CB CD34$^+$CD38$^-$ cells (1x10^3) were cultured on MSCs or adipocytes or without stromal cells in the presence of SCF, TPO and FL. On day 10 and 14, the cells were collected and analyzed for the number of CFU-GM, BFU-E, CFU-Mix and HPP-CFC by CFC assays. Data are the mean ± SD of fold increases over the values on day 0 of four separate experiments. *P<0.05 compared with the MSC culture.

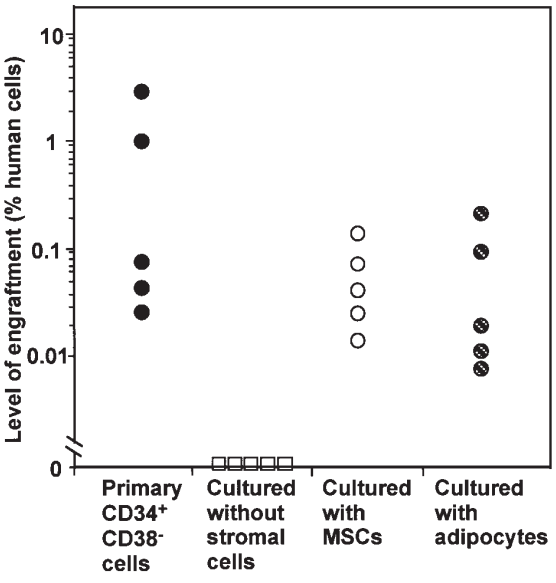

Figure 5. Analysis of engraftment of human SCID-repopulating cells after co-culture with MSCs or adipocytes. All the progeny of CD34$^+$CD38$^-$ cells (1x10^3) cultured on MSC or adipocytes or without stromal cells in the presence of SCF/TPO/FL for 14 days, were transplanted into sublethally irradiated NOD/SCID mice. Eight weeks later, the engraftment level in the bone marrow was assessed by analyzing the human-specific 17α-satellite DNA gene using real-time PCR. Each symbol represents a single chimeric mouse.

Effect of MSCs or adipocytes on proliferation of CB CD34$^+$CD38$^-$ cells. We examined the ability of MSCs and adipocytes to support proliferation of CB CD34$^+$CD38$^-$ cells by culturing CB CD34$^+$CD38$^-$ cells on a layer of MSCs or adipocytes in the presence of SCF, TPO and FL. Analysis of total viable cells on day 10 and 14 showed that these cells proliferated more abundantly on the MSC layer than on the adipocyte layer (P<0.05) (Fig. 3A). Similarly, proliferation of CD34$^+$ cells (Fig. 3B) and CD34$^+$CD38$^-$ cells (Fig. 3C) was more pronounced on MSCs than on adipocytes (P<0.05). Colony-forming cell assay showed that CFU-GM, BFU-E, CFU-Mix and HPP-CFC were also higher in CD34$^+$CD38$^-$ cells cultured on MSCs than on adipocytes (P<0.05) (Fig. 4). These results showed that the ability of MSCs to support proliferation of human HPC and colony-forming cells decreased after differentiation into adipocytes.

Effect of MSCs or adipocytes on SCID-repopulating cells. We next investigated the ability of MSCs or adipocytes to maintain transplantable primitive progenitor cells by performing SCID-repopulating cell (SRC) assays. All the progeny of CD34$^+$CD38$^-$ cells (1x10^3) cultured on MSCs or adipocytes in the presence of SCF/TPO/FL for 14 days were transplanted into NOD/SCID mice through the tail vein, and 8 weeks later, the mice were analyzed for human hematopoietic cells in the bone marrow by identifying the human-specific 17α-satellite DNA using real-time PCR. All five mice transplanted with CB CD34$^+$CD38$^-$ cells cultured on MSCs were positive for human DNA, indicating that MSCs maintain SRCs (Fig. 5). All five mice transplanted with CB CD34$^+$CD38$^-$ cells cultured on adipocytes were also positive

1 DNA, thus indicating that adipocytes are also able SCID-repopulating cells. Control mice, transplanted with CB $CD34^+CD38^-$ cells cultured without stromal cells, were negative for the human DNA, indicating the loss of the SCID-repopulating capacity during liquid culture >9 days (23). These results show that adipocytes provide a milieu that maintains transplantable primitive hematopoietic progenitors.

Discussion

Adipocytes are the most abundant stromal cell type in adult human bone marrow, particularly in the long bone (4). In aplastic anemia patients, the bone marrow contains a reduced number of hematopoietic cells with abundant adipocytes. The increase in marrow fat in aplastic anemia is known to be caused by an increase in the size and number of individual fat cells. However, the precise role of adipocytes in the bone marrow is not fully understood. In the present study, we analyzed the hematopoietic-supporting capacity of adipocytes.

MSCs have the potential to differentiate into osteoblasts, adipocytes and stromal fibroblasts which constitute bone marrow stromal cells. MSCs have been shown to support hematopoiesis (10,11), but whether human marrow adipocytes possess a hematopoietic-supporting capacity remains controversial (6,8). It is also not clear whether marrow adipocytes are able to support SCID-repopulating cells, which are immature transplantable hematopoietic stem cells. Our results showed that bone marrow adipocytes were inferior to MSCs in supporting hematopoietic progenitors (Figs. 3 and 4). However, adipocytes were found to be similar to MSCs in maintaining SCID-repopulating cells (Fig. 5). Considering that only a small population of the $CD34^+CD38^-$ cells were SCID-repopulating cells, it is possible that adipocytes provided a niche to attach SCID-repopulating cells, resulting in their selective preservation.

The mechanism by which adipocytes regulate hematopoiesis remains unknown. When MSCs differentiated into adipocytes, adiponectin, an adipocyte-specific molecule, was expressed (Fig. 2). Adiponectin suppressed mature hematopoietic progenitors, such as CFU-GM, CFU-M and CFU-G, but not relatively immature progenitors, such as BFU-E and CFU-Mix (22). We observed that adipocytes, like MSCs, expressed mRNA of SCF, TGF-ß1 and Jagged-1 (Fig. 2). Notch-1 was expressed on $CD34^+$ hematopoietic cells (24) and its ligand, Jagged-1, was expressed on stromal cells (25). Jagged-1 promoted hematopoiesis (25) and was able to expand SCID-repopulating cells (26). As adipocytes express such growth-related molecules, they may be able to provide an environment favorable for the maintenance of SCID-repopulating cells.

Our finding that adipocytes do not efficiently support mature hematopoietic progenitors may be consistent with the fact that mature blood cells, such as erythrocytes, WBC and platelets decrease in number in hypoplastic adipocyte-rich marrow in aplastic anemia. However, the hemopoietic microenvironment in adipocyte-rich marrow may still be able to preserve immature hematopoietic progenitor cells. Thus, adipocytes in the bone marrow may not be simple replacements of the marrow cavity but may positively influence immature hematopoietic progenitor cells.

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