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主論文

Early osteoinductive human bone marrow mesenchymal stromal/stem cells support an enhanced hematopoietic cell expansion with altered chemotaxis- and adhesion-related gene expression profiles

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Running title:

Hematopoiesis in early osteoinductive BM-MSCs

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Abbreviations:

BM, bone marrow; CC, C-C motif; CXC, C-X-C motif; e-MSC, early osteoinductive human bone marrow mesenchymal stromal/stem cell; Flt3-L, Flt3-ligand; G-CSF, granulocyte colony-stimulation factor; GO, gene ontology; GSEA, gene set enrichment analysis; Hb, hemoglobin; HSCT, hematopoietic stem and progenitor cell transplantation; HSPC, hematopoietic stem and progenitor cell; IL, interleukin; MNC, mononuclear cell; MSC, mesenchymal stromal/stem cell; OB, osteoblast; OICS, osteogenesis-inducing cocktails; PB, peripheral blood; qRT-PCR, quantitative reverse transcription PCR; SCF, stem cell factor; TPO, thrombopoietin; VCAM1, vascular cell adhesion molecule 1; VLA4, very late antigen 4; WBC, white blood cell.

Abstract

Bone marrow (BM) microenvironment has a crucial role in supporting hematopoiesis. Here, by using a microarray analysis, we demonstrate that human BM mesenchymal stromal/stem cells (MSCs) in an early osteoinductive stage (e-MSCs) are characterized by unique hematopoiesis-associated gene expression with an enhanced hematopoiesis-supportive ability. In comparison to BM-MSCs without osteoinductive treatment, gene expression in e-MSCs was significantly altered in terms of their cell adhesion- and chemotaxis-related profiles, as identified with Gene Ontology and Gene Set Enrichment Analysis. Noteworthy, expression of the hematopoiesis-associated molecules CXCL12 and vascular cell adhesion molecule 1 was remarkably decreased in e-MSCs. Furthermore, e-MSCs supported an enhanced expansion of $CD34^+$ hematopoietic stem and progenitor cells, and generation of myeloid lineage cells in vitro. In addition, short-term osteoinductive treatment favored in vivo hematopoietic recovery in lethally irradiated mice that underwent BM transplantation. e-MSCs exhibited the absence of decreased stemness-associated gene expression, increased osteogenesis-associated gene expression, and apparent mineralization, thus maintaining the ability to differentiate into adipogenic cells. Our findings demonstrate the unique biological characteristics of e-MSCs as hematopoiesis-regulatory stromal cells at differentiation stage between MSCs and osteoprogenitor cells and have significant

implications in developing new strategy for using pharmacological osteoinductive treatment to support hematopoiesis in hematopoietic stem and progenitor cell transplantation.

Keywords

bone marrow mesenchymal stromal/stem cells; hematopoiesis; CXCL12

1. Introduction

Human bone marrow (BM) mesenchymal/stromal stem cells (MSCs) are multipotent stromal cells that can differentiate into osteoblasts (OBs) and adipocytes [1], and have the ability to expand hematopoietic stem and progenitor cells (HSPCs) when co-cultured in vitro [2,3]. The ability of human BM-derived MSCs to support hematopoiesis has been validated in clinical trials in the setting of HSPC transplantation (HSCT) [4,5]. Therefore, further research into the potential of BM-MSCs in hematopoiesis would lead to an improved outcome of HSCT.

By the use of genetic mouse models, stromal cells that show similar characteristics to MSCs were demonstrated to be crucial for physiological hematopoiesis in BM microenvironments [6,7,8,9,10]. In addition, the pathological hematopoiesis of myelodysplasia results from a primary genetic abnormality in osteoprogenitor cells [11]. These findings imply that mouse osteoprogenitor cells are not simply in an intermediary differentiation stage between MSCs and OBs, but in a crucial functional stage for regulating hematopoiesis. However, the characteristics of such cells in humans are unknown.

The purpose of this study was to examine the hematopoiesis-supportive potential of early osteoinductive human BM-MSCs (e-MSCs) and to analyze the detailed gene

expression profiles of these cells using microarray analysis.

2. Material and Methods

2.1. Culture and osteogenic differentiation of human BM-MSCs

Normal human BM samples that were obtained from healthy adult volunteers with informed consent were purchased from AllCells (Emeryville, CA). Human BM-MSCs were isolated and cultured based on a previously published method [3,12,13,14,15]. In brief, a single-cell suspension of 1×10^6 BM mononuclear cells (MNCs) was seeded into a 15 cm culture dish. The primary culture of adherent cells was passaged to disperse the colony-forming cells (passage 1), and cells at passage 1–3 were used as BM-MSCs. Prior to experiments, the surface antigen profile of CD11b, CD19, CD34, CD45, CD73, CD90, and CD105 was examined by flow cytometric analysis to confirm that these cells expressed MSC markers, but did not express hematopoietic cell markers [16]. To induce osteogenic differentiation of BM-MSCs, osteogenesis-inducing cocktails (OICS) of 100 μM ascorbic acid (Wako Chemicals Industries, Osaka, Japan), 1.8 mM potassium dihydrogen phosphate, and 100 nM dexamethasone (both from Sigma-Aldrich, St. Louis, MO) were added to the culture media (osteoinductive medium). Mineralization was evaluated by 1% Alizarin Red S

staining. The study protocol was approved by the ethics committee of Kyoto University Hospital (#995).

2.2. Co-culture of human HSPCs and BM-MSCs

Human HSPCs were isolated from BM-MNCs using anti-CD34 immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the purity was confirmed by flow cytometric analysis using an antibody against human CD34. BM-MSCs (2×10^4 cells/well) were seeded in a 24-well culture plate. In some experiments, BM-MSCs were treated with OICS for osteoinduction prior to co-culture. HSPCs (0.6×10^3 cells/well) were then applied and the cells were co-cultured in StemSpan Serum Free Expansion Medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 100 ng/mL stem cell factor (SCF), 100 ng/mL Flt3-ligand (Flt3-L), 20 ng/mL interleukin (IL)-3 (all from Wako Chemicals Industries), and 50 ng/mL thrombopoietin (TPO) (Kyowa Hakko Kirin, Tokyo, Japan). After 10 days of co-culture, the number and surface marker expression of the expanded hematopoietic cells were examined by flow cytometric analysis. The antibodies used are listed in Supplementary Table 1.

2.3. Microarray analysis

Total RNA (1 μg/sample) from control unstimulated BM-MSCs (ctrl-MSCs) or BM-MSCs that were cultured in the osteoinductive medium for 2 days (e2-MSCs) or 5 days (e5-MSCs) was amplified using the Ambion Amino Allyl aRNA Kit (Ambion, Carlsbad, CA). Each sample of amplified RNA was labeled with Cy5, and labeled samples were co-hybridized with the 3D-Gene Human Oligo Chip 25k (Toray Industries, Tokyo, Japan) at 37°C for 16 hours. After washing the DNA chip, hybridization signals were scanned using a 3D-Gene Scanner 3000 (Toray Industries). All analyzed data were scaled by global normalization. Microarray data were analyzed using GeneSpring GX software (Tomy Digital Biology, Tokyo, Japan). All raw data were normalized and filtered based on the signal intensity values (20-100th percentile range). Hierarchical clustering and Gene ontology (GO) analysis was performed using GeneSpring GX software. Gene set enrichment analysis (GSEA) was performed using GSEA v2.0.14 software (http://www.broadinstitute.org/gsea/index.jsp). The gene set "Cell_Cell_Adhesion" was downloaded from the Molecular Signature Database (MSigDB; http://www.broadinstitute.org/gsea/msigdb/index.jsp). The genes included are listed in Supplementary Table 2. The analysis parameters were as follows: the number of permutations was 1,000, the permutation type was set to gene set, the gene

set size filters were set to min=15 and max=500, and the metric for ranking genes was Diff_of_Classes. The complete microarray data are available in the NCBI Gene Expression Omnibus (GEO). The microarray accession number is 74837.

2.4. BM transplantation

Specific pathogen-free 6–8-week-old female C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). BM nucleated cells (5×10^5/mouse) from C57BL/6 mice were transplanted into lethally irradiated (9 Gy) recipient C57BL/6 mice. OICS (dissolved in PBS) or PBS (control) was administered intraperitoneally to recipient mice on days 1–7 after BM transplantation. The survival of mice was observed each day until day 35 after BM transplantation. Peripheral blood (PB) was collected on days 0, 4, 7, 10, 13, 17, 21, 25, 28, and 35 after BM transplantation (n = 6) from the tail veins, and the number of white blood cells (WBCs) and the hemoglobin (Hb) levels were analyzed using an automated blood cell counter (Celltac α, Nihon Kohden, Tokyo, Japan). These studies were approved by the committee for animal research of the Kyoto University Graduate School of Medicine.

2.5. Statistical analysis

The unpaired Student's *t*-test was used for analysis, unless otherwise indicated. Data in bar graphs indicate the mean ± SD, and statistical significance is expressed as follows: *, $P < 0.05$; **, $P < 0.01$.

3. Results

3.1. Characteristics of early osteoinductive human BM-MSCs (e-MSCs)

First, we cultured human BM-MSCs in osteoinductive medium and examined their mineralization by Alizarin Red S staining. Mineralization was observed following 10 days of culture (Fig. 1A). Mineralization was not observed in BM-MSCs that were cultured in osteoinductive medium for just 2 or 5 days (Fig. 1A). When these early osteoinductive MSCs (e-MSCs) were cultured in adipogenesis-inducing medium for more than 2 weeks, significant fat deposition was observed (Fig. S1A–B), indicating their adipogenic differentiation ability. Thus, BM-MSCs cultured in osteoinductive medium for just 2 days (e2-MSCs) or 5 days (e5-MSCs) were considered to be immature cells that were in a differentiation stage between MSCs and osteoprogenitor cells. This was further supported by the observations that e2-MSCs and e5-MSCs did not show decreased expression of stemness-associated markers (Fig. S1C) or increased expression of osteogenesis-associated markers such as SP7 (also known as osterix),

BGLAP (also known as osteocalcin), and SPP1 (also known as osteopontin) (Fig. S1D) by microarray analysis.

3.2. e-MSCs support an enhanced expansion of hematopoietic cells

To investigate the ability of osteoinductive BM-MSCs to support hematopoiesis, in vitro co-culture experiments with these cells and human $CD34^+$ HSPCs were performed (Fig. 1B). When BM-MSCs were cultured in osteoinductive medium for 2 days (i.e., e2-MSCs) or 5 days (i.e., e5-MSCs) and then co-cultured with HSPCs for 10 days in the presence of SCF, Flt3-L, IL-3, and TPO, the expansion of $CD45^+$ cells and $CD34^+$ HSPCs was significantly enhanced in comparison to when they were co-cultured with control unstimulated BM-MSCs (ctrl-MSCs) (Fig. 1C–D). This enhanced hematopoietic expansion of these cells was not observed in co-cultures with BM-MSCs that were cultured in osteoinductive medium for 10 days (Fig. 1C–D). In addition, the proportions of $CD11b^+$ cells, $CD33^+$ cells, and $CD14^+$ cells were increased in co-culture with e2-MSCs in comparison to co-culture with ctrl-MSCs (Fig. 1E, S2A–B). These results suggested that e-MSCs, early osteoinductive BM-MSCs, are not simply in an intermediate differentiation stage between BM-MSCs and osteoprogenitor cells but in a crucial functional stage for regulating hematopoiesis.

3.3. Expression of adhesion-associated genes is decreased in e-MSCs

We next examined the gene expression profiles of e-MSCs by microarray analysis. Hierarchical clustering analysis demonstrated that e2-MSCs and e5-MSCs have similar gene expression patterns (Fig. S3). In comparison to control unstimulated BM-MSCs (ctrl-MSCs), 973 and 1331 genes were up- or down-regulated by more than 2-fold in e2-MSCs (up-regulated, 500 genes; and down-regulated, 473 genes) and e5-MSCs (up-regulated, 670 genes; and down-regulated, 661 genes), respectively (Fig. 1F). Because hematopoiesis is regulated by various types of soluble factors, we focused on cytokines that are related to hematopoiesis and analyzed the change in their expression. There was no apparent change in the expression of granulocyte macrophage colony-stimulation factor (also known as CSF2), granulocyte colony-stimulation factor (G-CSF, also known as CSF3), SCF (also known as KITLG), IL-3, FLT3LG, or EPO in e2- and e5-MSCs (Fig. 1G).

We found that 358 genes were commonly down-regulated in e2- and e5-MSCs (Fig. 2A). GO analysis showed that these commonly down-regulated genes were significantly included in the GO term of “Cell adhesion” (accession number 0007155, Fig. 2B). GSEA also revealed that the gene signature of Cell_Cell_Adhesion

was negatively enriched in e2- and e5-MSCs (Fig. 2C). We focused on cell adhesion-related genes that contribute to hematopoiesis and found that expression of most of them, especially vascular cell adhesion molecule 1 (VCAM1), was decreased in e2- and e5-MSCs (Fig. 2D). Quantitative reverse transcription PCR (qRT-PCR) analysis validated the decrease in mRNA expression of VCAM1 (Fig. 2E). This was further confirmed in e2- and e5-MSCs derived from different BM-MSC lots (Fig. S4A). Thus, short-term osteogenic induction inhibits the contribution of the cell adhesion-associated mechanism by MSCs to hematopoietic regulation.

3.4. Expression of chemotaxis-associated genes is increased in e-MSCs

With regard to commonly up-regulated genes in e2- and e5-MSCs, 363 genes were extracted (Fig. 3A). GO analysis showed that commonly up-regulated genes were significantly included in the GO term of “Cell chemotaxis” (accession number 0060326, Fig. 3B). Because chemotaxis is regulated by various chemokines, the expression change in respective chemokines was examined. Expression of C-X-C motif (CXC) chemokine family members such as CXCL1, CXCL2, CXCL5, and CXCL6 was greatly increased. On the other hand, the expression of CXCL12 was greatly decreased among CXC chemokine family members (Fig. 3C). qRT-PCR analysis validated the decrease in

mRNA expression of CXCL12 (Fig. 3D). This was further confirmed in e2- and e5-MSCs derived from different BM-MSC lots (Fig. S4B). In contrast to the remarkable change in expression of CXC chemokine family members, the expression of C-C motif (CC) chemokine family members did not apparently change (Fig. 3E).

3.5. Osteoinductive stimulation favors in vivo hematopoietic recovery after BM transplantation

Our in vitro data suggested that e-MSCs enhance the expansion of hematopoietic cells; therefore, we hypothesized that osteoinductive stimulation could support hematopoietic recovery after chemotherapy, radiotherapy, and BM transplantation. To investigate the possibility of clinical application, we administered OICS or vehicle to lethally irradiated (9 Gy) C57BL/6 mice for 7 days after BM transplantation (Fig. 4A). Although two of six vehicle-treated mice died at day 10 or 12 after transplantation, all OICS-treated mice survived for more than 1 month. The WBC number and Hb levels at 10 days after BM transplantation were significantly higher in OICS-treated mice than in vehicle-treated mice, implying that OICS rescued the recipient mice from death due to cytopenia-related complications (Fig. 4B–C).

4. Discussion

Raaijmakers et al. [11] reported that myelodysplasia resulted from a primary abnormality not in hematopoietic cells but in osterix^{+} mouse osteoprogenitor cells. This implied that mouse osteoprogenitor cells differentiated from MSCs are critical for regulating hematopoiesis. In the current study, MSCs in the early osteoinductive stage (e-MSCs) did not show increased expression of osterix, retained expression of pluripotency-associated genes, and displayed the capacity to differentiate into adipocytes. These findings demonstrate that e-MSCs are distinctive hematopoiesis-regulatory stromal cells at differentiation stage between MSCs and osteoprogenitor cells in human and may be a functional counterpart of osteoprogenitor cells in mice. However, further studies are needed to elucidate their similarity in hematopoiesis-associated function between e-MSCs in humans and osteoprogenitor cells in mice.

We clarified that the adhesion-associated gene signature was negatively enriched and the chemotaxis-associated gene signature was positively enriched in e-MSCs. Importantly, expression of VCAM1 and CXCL12 was remarkably decreased in e-MSCs. VCAM1 is an adhesion molecule that is expressed on BM-MSCs [1] and is a ligand for very late antigen 4 (VLA4). CXCL12 is a chemokine that is produced by

stromal cells including MSCs [6,7]. CXCL12 binds to its receptor CXCR4 and activates downstream signaling pathways [17]. Both VLA4 and CXCR4 are expressed on the surface of HSPCs and interact with VCAM1 and CXCL12, respectively [18,19]. In addition, when CXCR4 signaling is activated by CXCL12, VCAM1-VLA4 binding is significantly increased [20]. Many lines of evidence have firmly demonstrated that these interactions are essential for regulating the maintenance of HSPCs [17,18,19,21]. Therefore, the decrease in expression of VCAM1 and CXCL12 in e-MSCs could lead to the release of HSPCs captured by stromal cells and alteration of the status of HSPCs. Actually, we found the enhanced expansion of HSPCs and generation of $CD11b^+$ differentiated myeloid cells from HSPCs in co-cultures with e-MSCs. This enhanced generation of $CD11b^+$ cells was also supported by our observation of up-regulated expression of members of the neutrophil chemotactic CXC chemokine family in e-MSCs. Clinically, G-CSF is used for the mobilization of HSPCs from BM to PB and for the acceleration of neutrophil production, which is mediated by down-regulation of VLA4 on HSPCs [22]. G-CSF also modulates CXCL12-CXCR4 interactions [23]. Furthermore, recent clinical studies have shown that the interruption of CXCL12-CXCR4 [21] or VCAM1-VLA4 [21,24] binding using small molecule inhibitors leads to the rapid mobilization of HSPCs into circulating PB. These clinical

observations endorse the determinant roles of the VCAM1-VLA4- and CXCL12-CXCR4-mediated mutual interaction between MSCs and HSPCs for HSPC regulation.

In summary, e-MSCs have unique hematopoiesis-supportive characteristics with altered chemotaxis- and adhesion-related gene expression profiles. The decrease in expression of VCAM1 and CXCL12 on e-MSCs is considered to be associated with an enhanced expansion of HSPCs and generation of myeloid lineage cells. Pharmacological stimulation of BM-MSCs could modify the BM microenvironment via changing the biological potency of BM-MSCs and could be applied in the clinical setting of HSCT.

Conflicts of interest

There is no conflict of interest.

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Supplemental data

Supplemental information can be found in the online version of this article.

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Figure Legends

Figure 1. Early osteoinductive human BM-MSCs (e-MSCs) support an enhanced expansion of hematopoietic cells. (A) Alizarin Red S staining of human BM-MSCs cultured in osteoinductive medium containing OICS for 2, 5, and 10 days. Representative images are shown. Original magnification, 200×. Bars, 250 μm. ctrl-MSC at day 0 represent untreated BM-MSCs. (B) Schema of the experimental procedure for co-culture of OICS-treated BM-MSCs and $CD34^+$ HSPCs. (C, D) The number of $CD45^+$ hematopoietic cells (C) and $CD34^+$ HSPCs (D) expanded after 10 days of culture alone (no MSC), co-culture with BM-MSCs without OICS treatment (ctrl-MSC) or co-culture with BM-MSCs that were pretreated with OICS for 2, 5, 10 days (OICS-treated MSC), as determined by flow cytometric analysis (n = 5). (E) Flow cytometric analysis of CD11b expression on expanded $CD45^+$ cells after 10 days of culture. The filled histogram indicates CD11b expression level on cells cultured alone (no MSC). The open histogram indicates CD11b expression level on cells cultured with untreated BM-MSCs (ctrl-MSC, blue line) or with BM-MSCs that were treated with OICS for 2 days (e2-MSC, red line). The bar graph shows the proportions of $CD11b^+$ cells indicated by the horizontal bar. (F) Scatter plots of genes differentially expressed in e2-MSCs (left) and e5-MSCs (right) compared with control BM-MSCs (ctrl-MSCs).

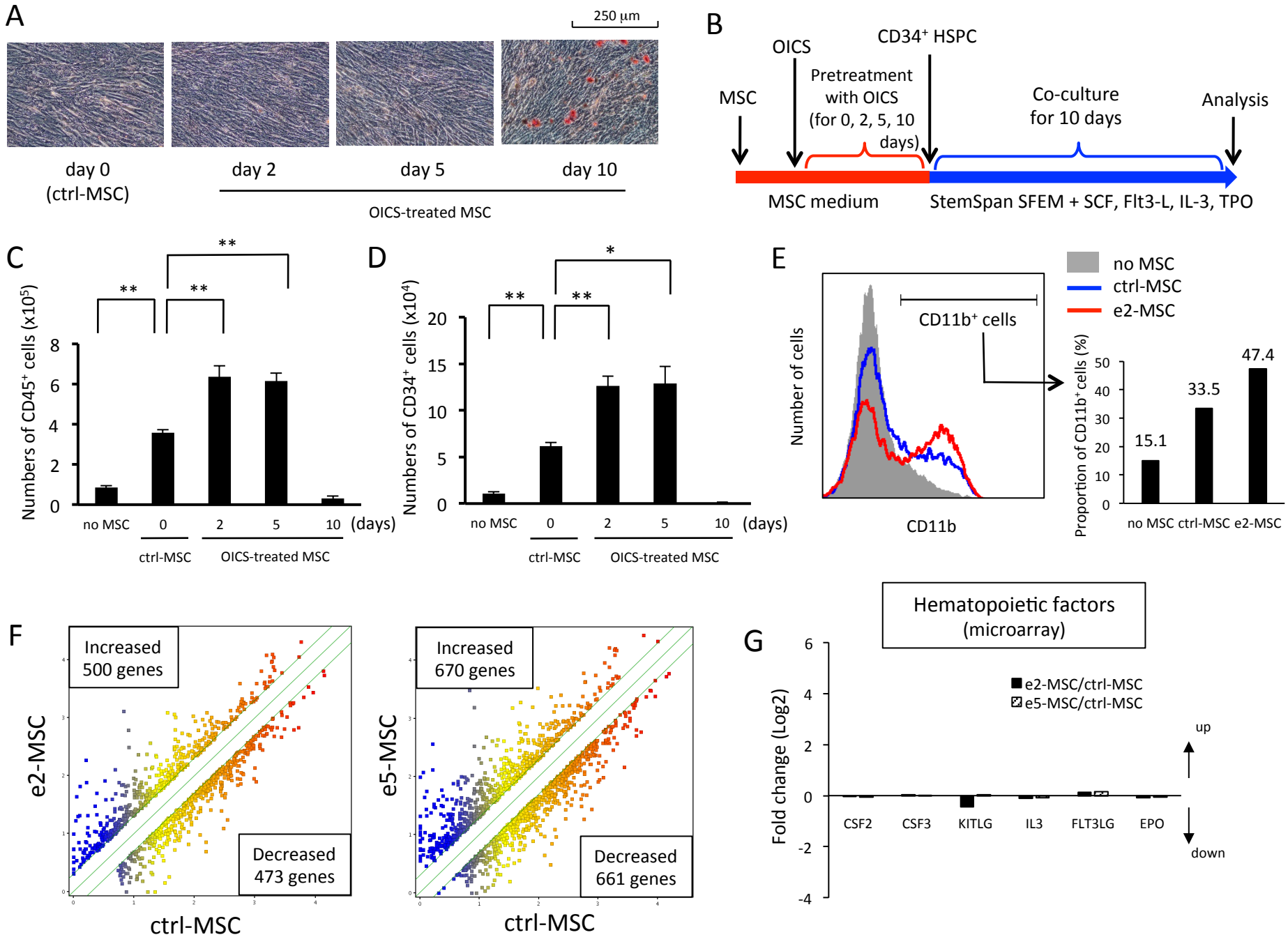
Genes whose expression was changed by more than 2-fold are plotted in log10 raw intensity values. (G) Comparison of the gene expression of hematopoietic factors, as determined by microarray analysis. The fold changes in e2-MSCs and e5-MSCs versus control BM-MSCs are shown on a log2 scale. Upward bars indicate increased expression; downward bars indicate decreased expression. *, $P < 0.05$; **, $P < 0.01$.

Figure 2. Expression of cell adhesion-associated genes is decreased in e-MSCs. (A) Venn diagram of the number of genes whose expression levels were decreased by more than 2-fold in e2-MSCs and e5-MSCs in comparison to control BM-MSCs. (B) GO analysis of 358 genes commonly down-regulated in e2- and e5-MSCs was performed, and the GO term of "Cell adhesion" was enriched. (C) The Cell_Cell_Adhesion gene set signature in e2- and e5-MSCs by GSEA. The normalized enrichment score (NES) and false discovery rate (FDR) are described. The gene set is listed in Supplementary Table 2. (D) Comparison of the expression of genes associated with cell adhesion, as determined by microarray analysis. The fold changes in e2- and e5-MSCs versus control BM-MSCs are shown on a log2 scale. Upward bars indicate increased expression; downward bars indicate decreased expression. (E) The relative mRNA expression of VCAM1 in e2- and e5-MSCs, as determined by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$.

Figure 3. Expression changes in chemotaxis-associated genes in e-MSCs. (A) Venn diagram of the number of genes whose expression levels were increased by more than 2-fold in e2-MSCs and e5-MSCs in comparison to control BM-MSCs. (B) GO analysis of 363 genes commonly up-regulated in e2- and e5-MSCs was performed, and the GO term of "Cell chemotaxis" was enriched. (C, E) Comparisons of the expression of CXC chemokine family (C) and CC chemokine family (E) members, as determined by microarray analysis. The fold changes in e2- and e5-MSCs versus control BM-MSCs are shown on a log2 scale. Upward bars indicate increased expression; downward bars indicate decreased expression. (D) The relative mRNA expression of CXCL12 in e2- and e5-MSCs, as determined by qRT-PCR. **, $P < 0.01$.

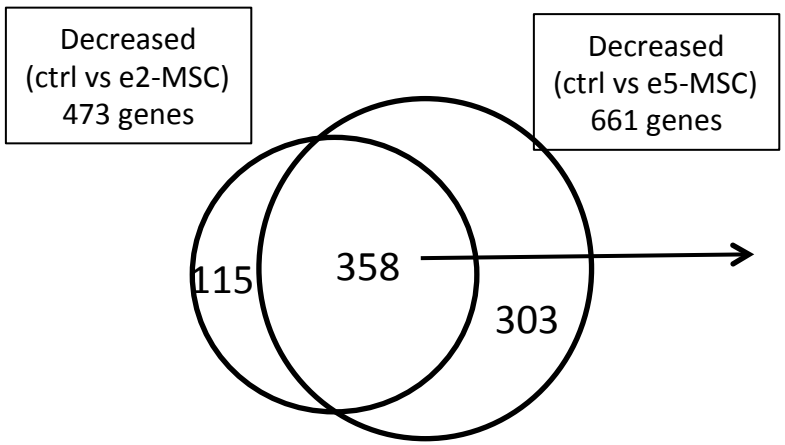
Figure 4. Osteoinductive treatment favors in vivo hematopoietic recovery after BM transplantation. (A) Schema of the in vivo experiment. Lethally irradiated (9 Gy) C57BL/6 mice were treated with OICS (n = 6) or PBS (n = 6) for 7 days after BM transplantation. (B, C) The number of WBCs (B) and the Hb levels (C) in PB of mice treated with OICS (red line) or PBS (blue line) during the 35 day follow-up after BM

transplantation. Crosses indicate the death of control mice on days 10 and 12 (n = 2). *, $P < 0.05$; **, $P < 0.01$.



Sugino N, et al. Fig.1

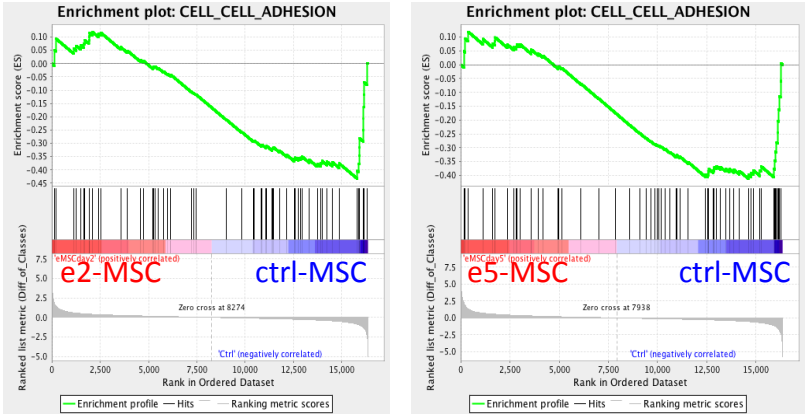
A



B

GO accession number	GO Term	Included genes	Corrected p-value
0007155	Cell adhesion	LAMA4, AMIGO2, COL5A1, RGMB, CADM1, C20orf100, WWTR1, CCL2, KAL1, DCBLD2, LPXN, CLDN14, ITGA8, OPCML, HNT, VCAM1, AEBP1, TNFAIP6, HMCN1, DDR2, COL14A1, ISLR, CD300A, FBLN7, LOXL2, EFNB1, PLXDC1, CLDN2	0.0192

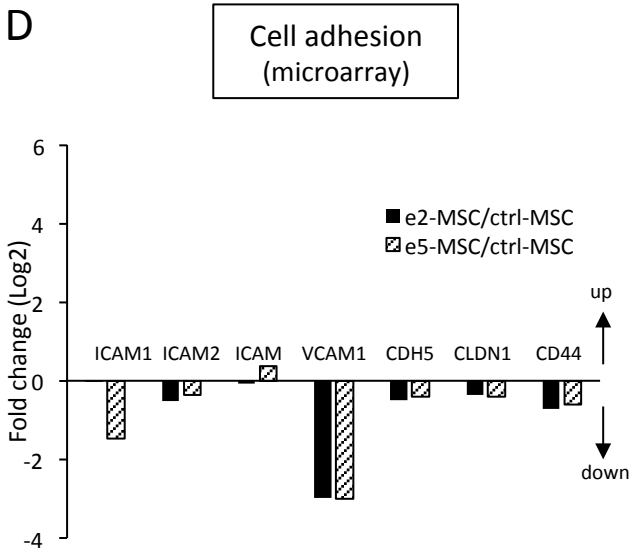
C



NES -1.488
FDR-q value 0.021

NES -1.429
FDR-q value 0.043

D



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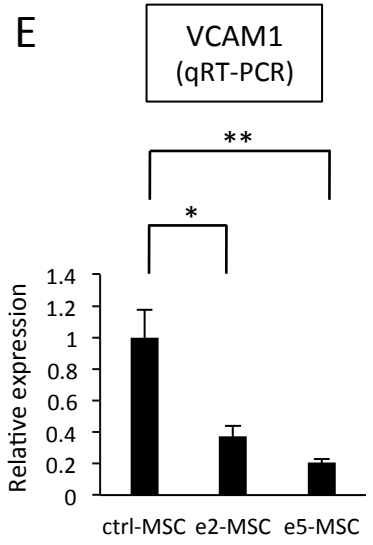
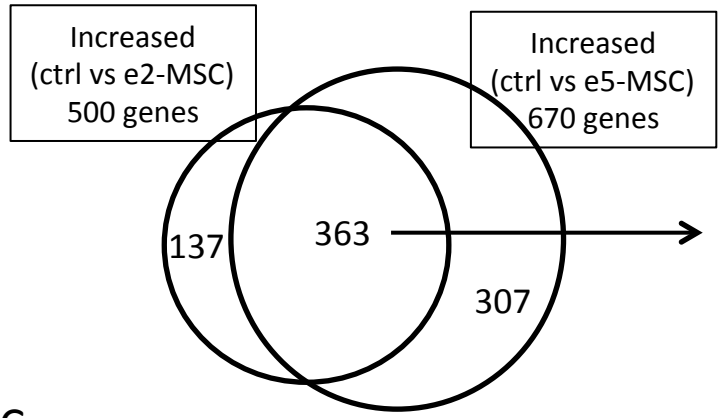


Sugino N, et al. Fig.2

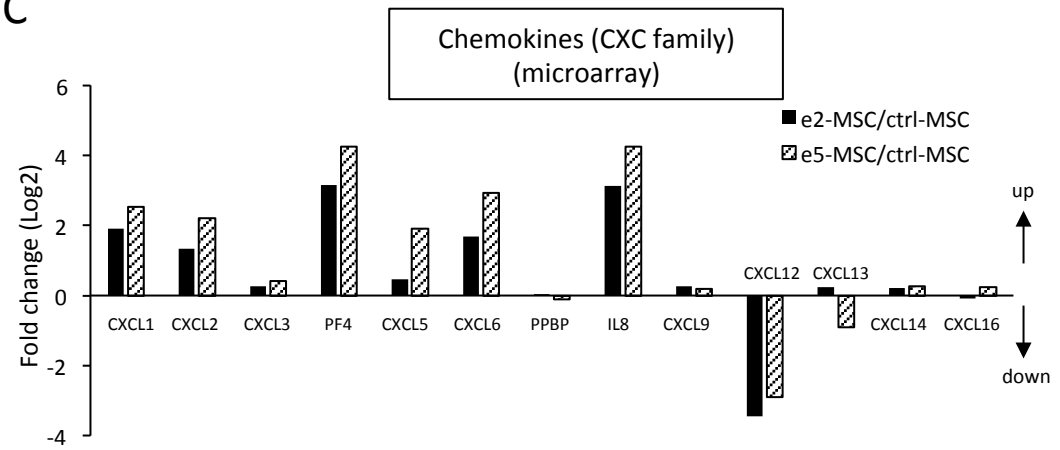
A



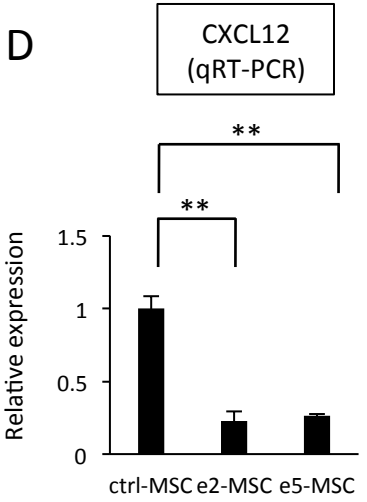
B

GO accession number	GO Term	Included genes	Corrected p-value
0060326	Cell chemotaxis	SCG2, CXCL6, CXCL1, CXCL2, IL8, SAA1, PF4, EDNRB	0.0183

C



D



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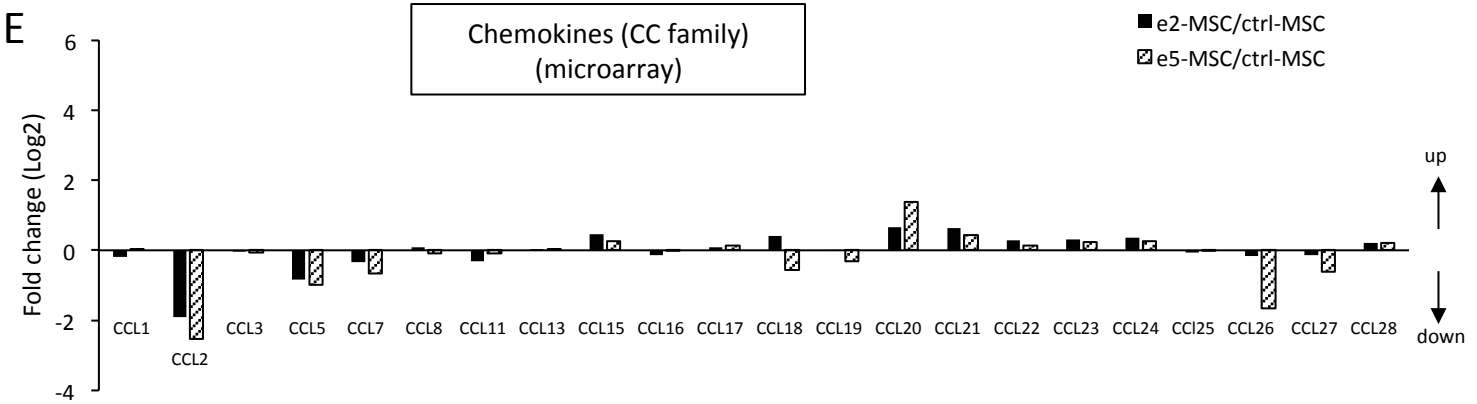
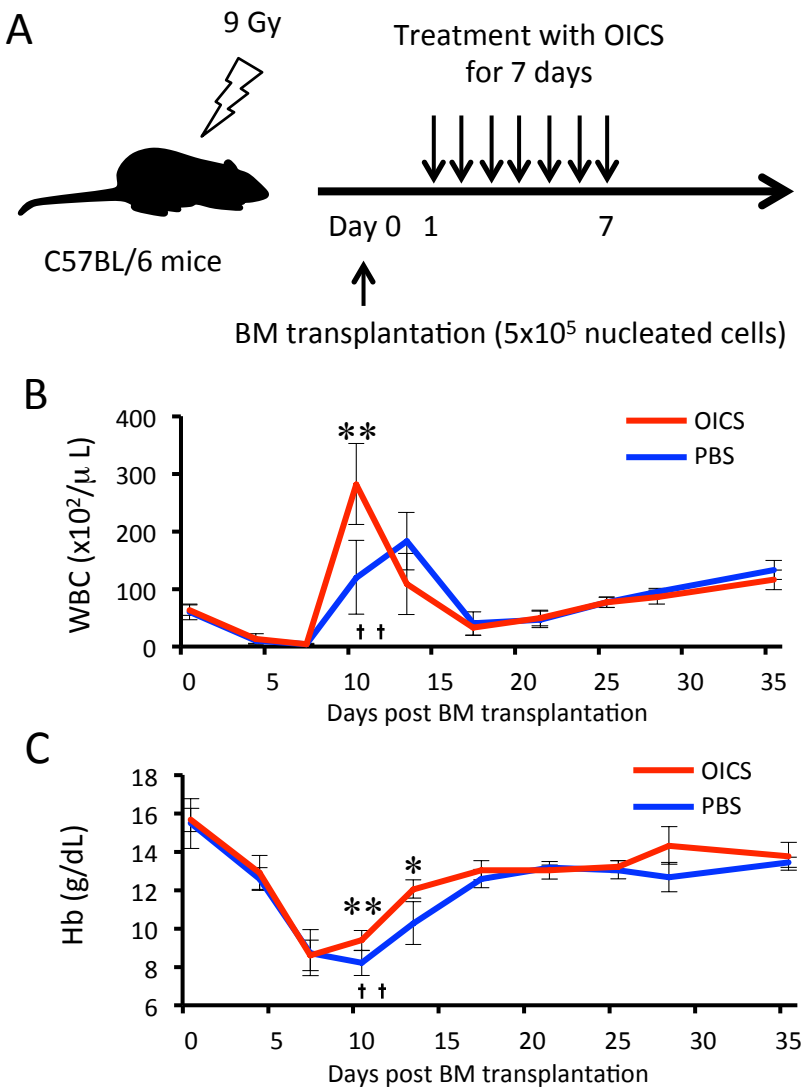
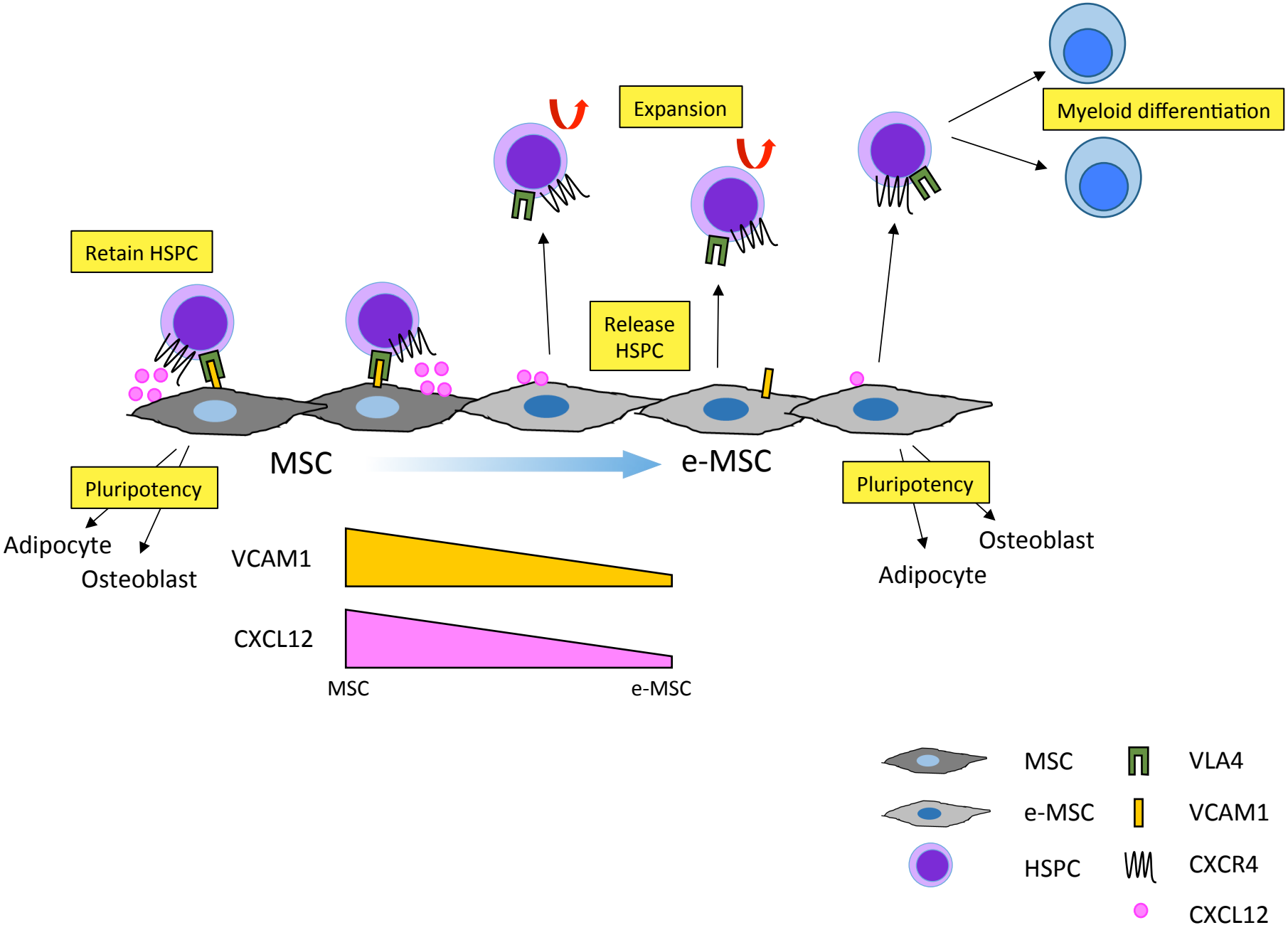


Sugino N, et al. Fig.3



Sugino N, et al. Fig.4

Sugino N, et al. Graphical abstract



Supplementary Information

Early osteoinductive human bone marrow mesenchymal stromal/stem cells support an enhanced hematopoietic cell expansion with altered chemotaxis- and adhesion-related gene expression profiles

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Supplementary Materials and Methods

Adipogenic differentiation assay of BM-MSCs

To induce adipogenic differentiation of BM-MSCs, 0.5 mM isobutyl-methylxanthine, 60 μM indomethacin, 0.5 μM hydrocortisone, and 10 μg/mL insulin (all from Sigma-Aldrich) were added to the culture media. Oil Red O staining was used to assess lipid-laden fat cells. The number of Oil Red O^+ cells was quantitated, as previously described [12]. Images were acquired using a Biozero BZ-8100 microscope and BZ Viewer software (both from Keyence, Osaka, Japan).

qRT-PCR

Total RNA was extracted using the QIAamp RNA Blood Mini Kit (Qiagen Japan, Tokyo, Japan) and subjected to reverse transcription. The 10 μL PCR mixture contained Taqman Fast Universal PCR master mix (Applied Biosystems, Carlsbad, CA), cDNA, primer pairs, and the Taqman probe (Universal Probe Library). cDNA was amplified with the StepOne Plus Real-Time PCR system (Applied Biosystems) using the following parameters: 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize any loading differences. The

primer sets and universal probes used are listed in Supplementary Table 3.

Figure Legends for Supplementary Figures

Supplementary Fig. 1. Characteristics of e-MSCs. (A, B) Adipogenic differentiation ability of e2- and e5-MSCs, as assessed by Oil Red O staining. Representative images are shown. Original magnification, 100×. Bars, 100 μm (A). Quantitative measurement of the number of Oil Red O^+ cells in five different fields viewed at 200× magnification (B). (C, D) Comparison of the expression of stemness-associated genes (C) and osteogenesis-associated genes (D) determined by microarray analysis. The fold changes in e2- and e5-MSCs versus control BM-MSCs (ctrl-MSC) are shown on a log2 scale. Upward bars indicate increased expression; downward bars indicate decreased expression.

Supplementary Fig. 2. e-MSCs support the generation of myeloid lineage cells. (A, B) Flow cytometric analysis of CD33 (A) and CD14 (B) expression on expanded $CD45^+$ cells after 10 days of culture. The filled histogram indicates the expression levels of the respective markers on cells cultured alone (no MSC). The open histogram indicates the expression levels of the respective markers on cells cultured with untreated

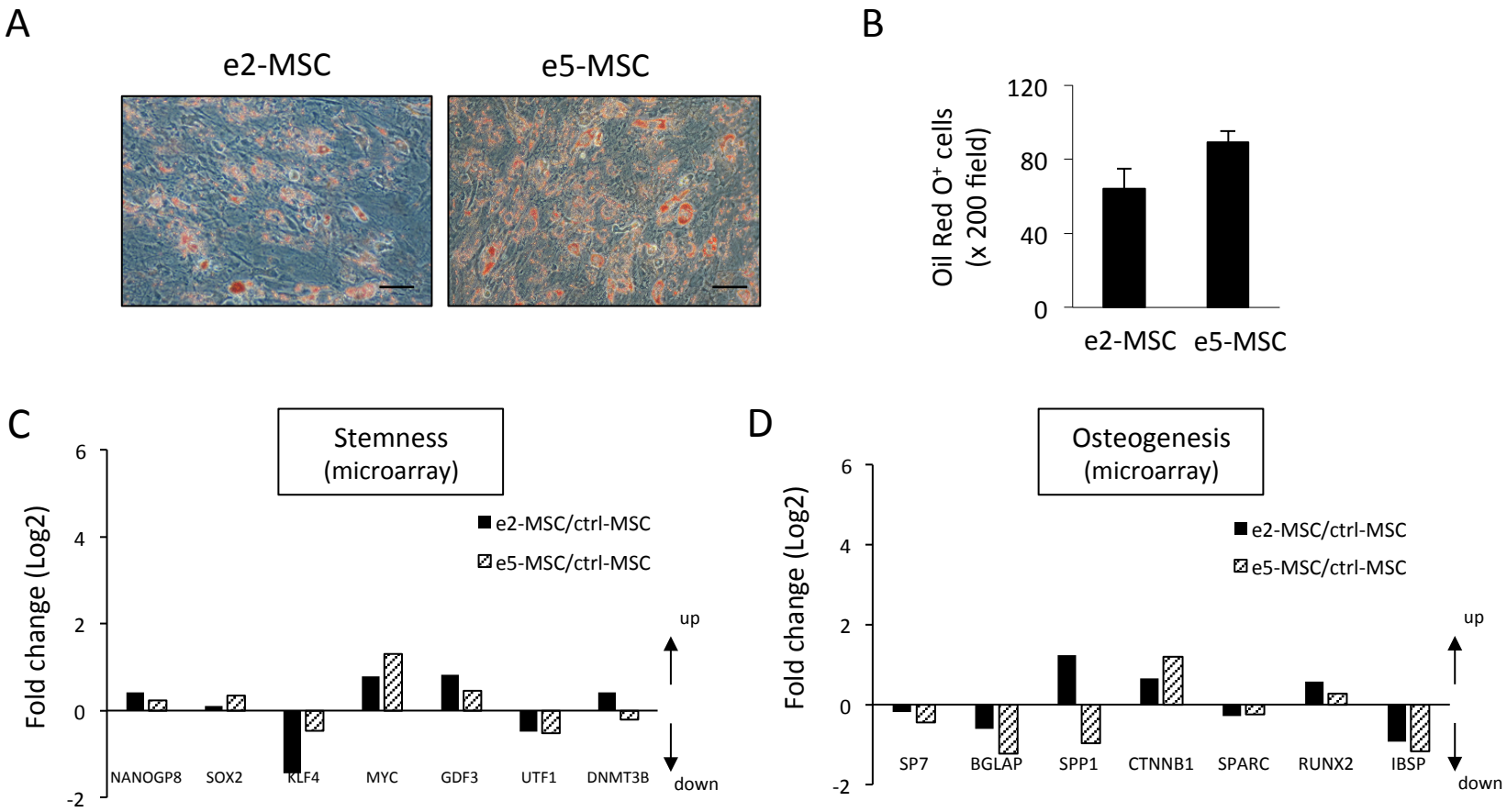
BM-MSCs (ctrl-MSC, blue line) or with BM-MSCs that were treated with OICS for 2 days (e2-MSCs, red line). The bar graph shows the proportions of $CD33^+$ or $CD14^+$ cells indicated by the horizontal bar.

Supplementary Fig. 3. Similar gene expression profiles in e2-MSCs and e5-MSCs. Gene expression profiles of three samples (ctrl-MSC, e2-MSC and e5-MSC) were performed using microarray analysis. Heat map of gene expression and hierarchical clustering are shown. The hierarchical clustering of individual genes with respect to the expression levels is represented by the dendrogram to the left. The hierarchical clustering of samples with respect to their similarity in gene expression patterns is represented by the dendrogram at the top.

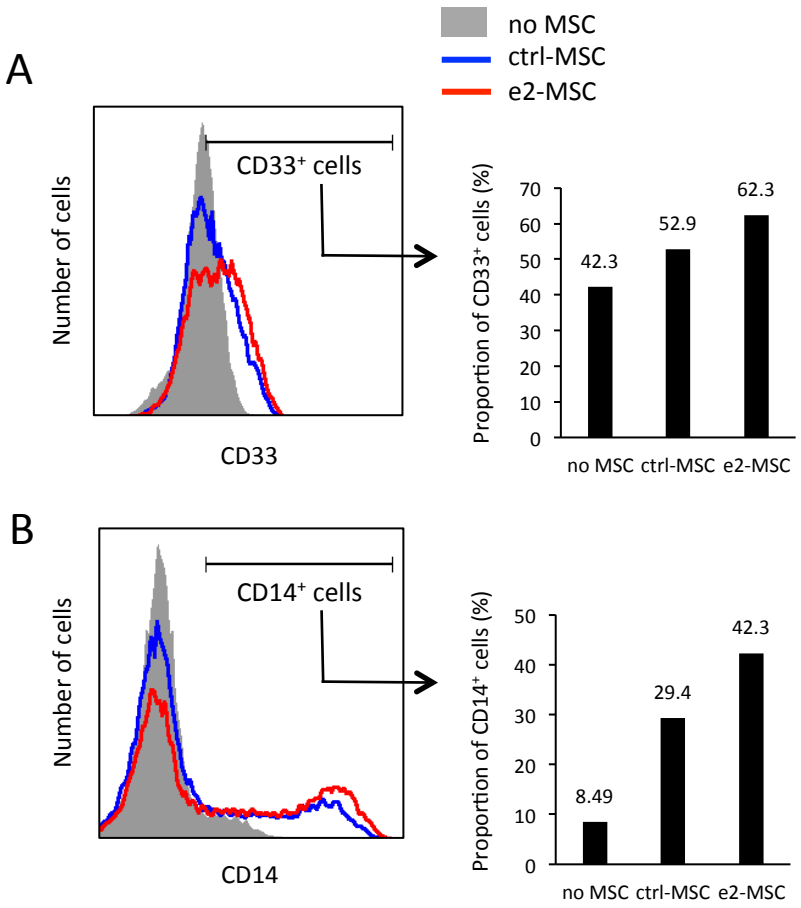
Supplementary Fig. 4. mRNA expression of VCAM1 and CXCL12 in e-MSCs derived from different BM-MSC lots. (A, B) BM-MSCs were isolated from different individuals and cultured in osteoinductive medium for 2 days (e2-MSCs) and 5 days (e5-MSCs). The mRNA expression of VCAM1 (A) and CXCL12 (B) in these cells was examined by qRT-PCR (n=3). **, $P < 0.01$.

Acknowledgments

Microarray analysis using GeneSpring GX software was performed at the Medical Research Support Center, Graduate School of Medicine, Kyoto University, which was supported by the Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan.



Sugino N, et al. Fig.S1



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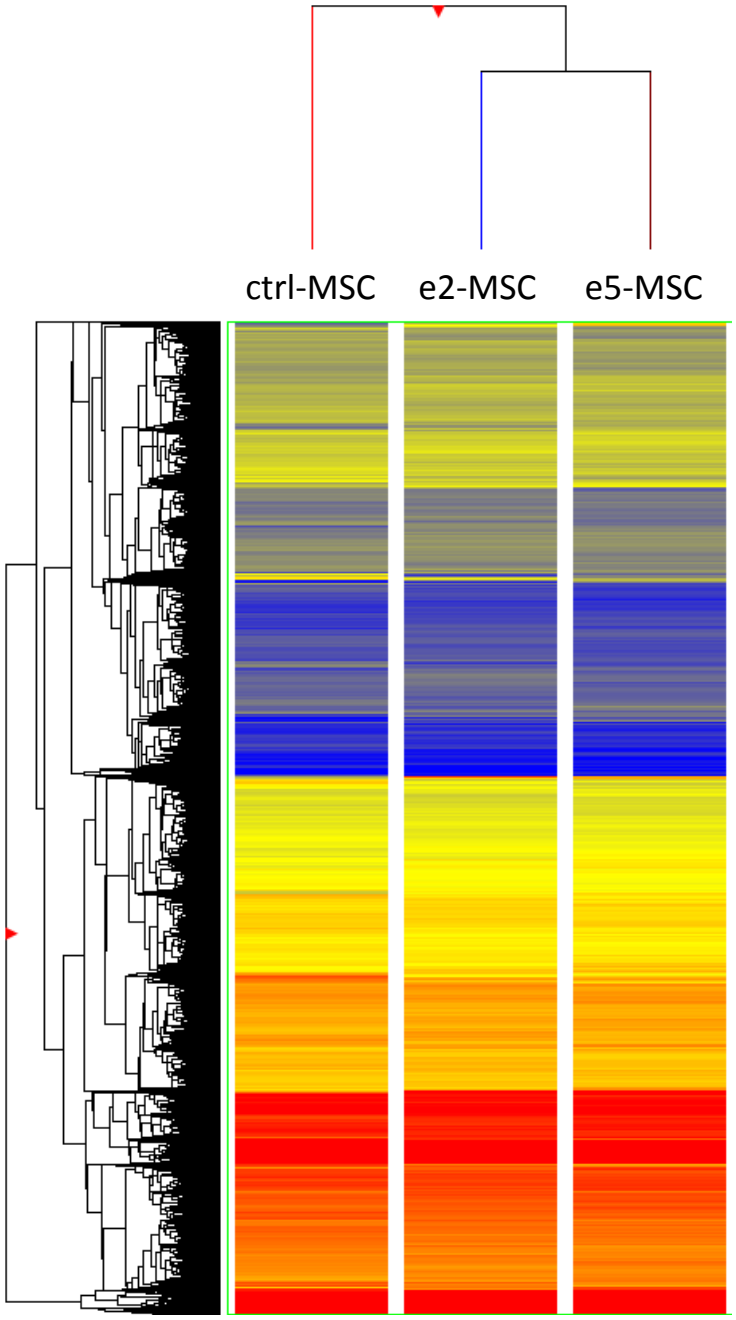

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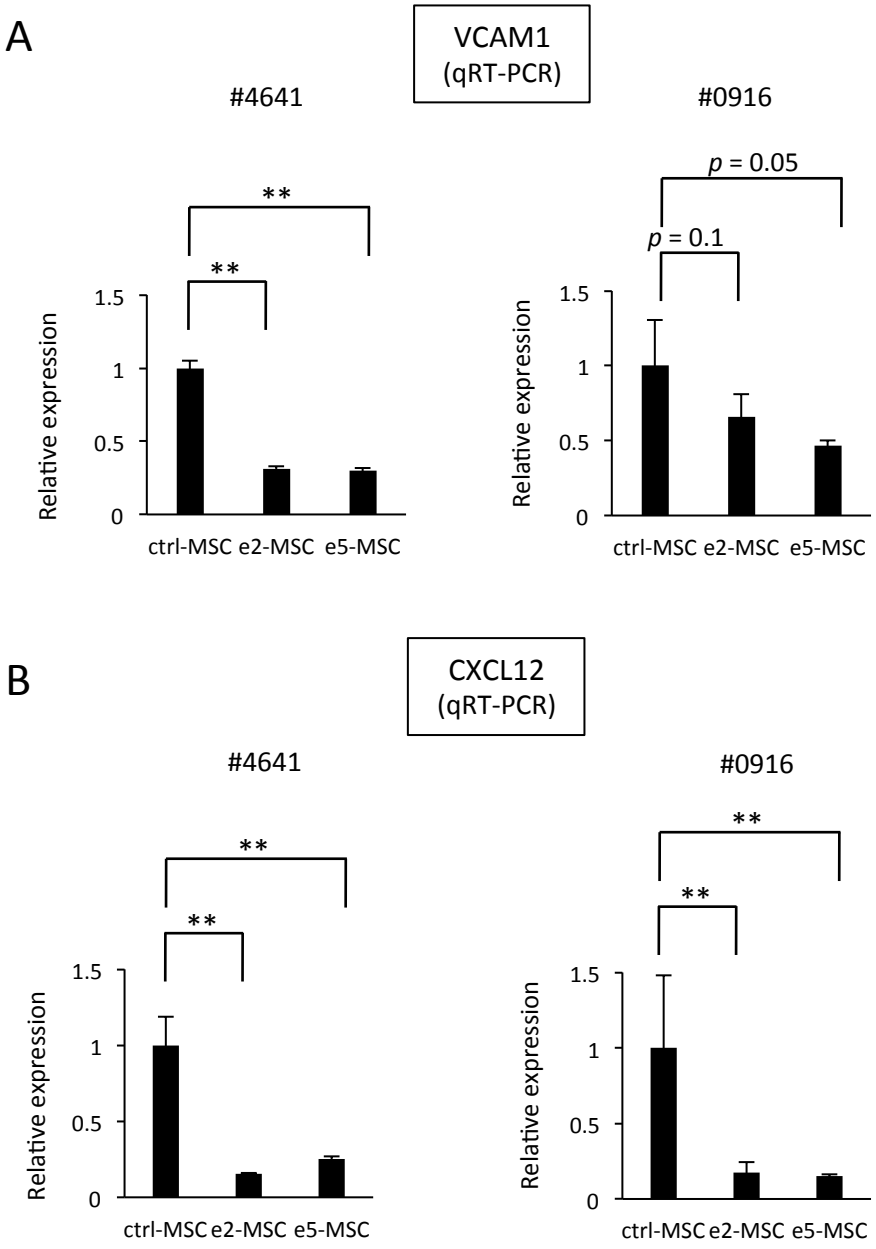

Sugino N, et al. Fig.S4

Supplementary Table 1. List of antibodies. PE: Phycoerythrin, APC: Allophycocyanin, FITC: Fluorescein isothiocyanate.

Antigen	Vendor	Clone/Product #	Fluorochrome
CD11b	eBioscience	ICRF44	PE
CD14	eBioscience	61D3	PE
CD19	eBioscience	SJ25C1	PE
CD33	eBioscience	WM-53	PE
CD34	BD Pharmingen	4H11	APC
CD34	BD Pharmingen	563	PE
CD45	BD Pharmingen	HI30	FITC
CD73	eBioscience	AD2	PE
CD90	eBioscience	5E10	PE
CD105	eBioscience	SN6	PE
Isotype control	BD Pharmingen	MOPC-21	FITC or PE
Isotype control	eBioscience	P3.6.2.8.1	APC

Supplementary Table 2. Gene set used for GSEA.

Gene set; CELL_CELL_ADHESION			
ACVRL1	CDK5R1	CLDN6	NF2
ALX1	CDKN2A	CLDN7	NINJ2
AMIGO1	CDSN	CLDN8	NLGN1
AMIGO2	CELSR1	CLDN9	NPTN
AMIGO3	CERCAM	CNTN4	PKD1
ANXA9	CLDN1	COL11A1	PKHD1
APOA4	CLDN10	COL13A1	PTEN
ATP2C1	CLDN11	CRNN	PVRL1
B4GALNT2	CLDN12	CTNNA3	PVRL2
BCL10	CLDN14	CX3CL1	PVRL3
BMP1	CLDN15	CYFIP2	RASA1
CADM1	CLDN16	DLG1	REG3A
CADM3	CLDN17	EGFR	ROBO1
CALCA	CLDN18	EMCN	ROBO2
CD164	CLDN19	GTPBP4	SIRPG
CD209	CLDN2	ITGB1	SYK
CD34	CLDN20	ITGB2	THY1
CD47	CLDN22	LGALS7	TNF
CD84	CLDN23	LMO4	TRO
CD93	CLDN3	MGP	VANGL2
CDH13	CLDN4	MPZL2	
CDH5	CLDN5	NCAM2	

Supplementary Table 3. List of primer sets and universal probes for qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Universal Probe (#)
CXCL12	ccaaactgtgcccttcagat	tggctgttgtgcttacttgttt	80
VCAM1	tggacataagaaactggaaaagg	ccactcatctcgatttctgga	39
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc	60