Expansion of mouse hematopoietic progenitor cells in three-dimensional cocultures on frozen-thawed stromal cell layers formed within porous scaffolds

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ABSTRACT: To establish a highly efficient method of ex vivo expansion of hematopoietic cells (HCs), three-dimensional (3D) cocultures of HCs and stromal cell lines were performed using porous polymer scaffolds. Hematopoietic cells derived from mouse fetal livers were expanded by two successive cultures without the use of exogenous cytokines, i.e., 3D cultures of stromal cells (DAS 104-8 cell line) to form stromal layers within the scaffolds and, subsequently, by cocultures of the HCs on the stromal cell layers for two weeks. To expand the HCs more conveniently, in some experiments the stromal layers formed within the scaffolds were frozen (3D freezing) before the cocultures, then stored, and applied to the cocultures after thawing. When the HCs were cocultured on the stromal layers of the DAS 104-8 cells, primitive HCs (c-kit$^+$ and CD34$^+$ cells) were expanded several-fold during the cocultures. In contrast, the expansion of these primitive HCs was remarkably enhanced in the cocultures using the 3D frozen-thawed DAS 104-8 stromal layers (c-kit$^+$ cells > 15-fold and CD34$^+$ cells > 30-fold), and these expansions were significantly higher than those without the 3D freezing. The expansions enhanced by cocultures on the 3D

frozen-thawed stromal layers were also observed in the cocultures with another stromal cell line (DAS 104-4). Because 3D frozen-thawed stromal cell lines are easy to handle, 3D coculture of HCs on frozen-thawed stromal cell lines may be an effective and convenient method for expanding primitive HCs.

Key words: hematopoietic stem cell; hematopoietic progenitor cell; stromal cell; tissue engineering; three-dimensional culture; cryopreservation

Introduction

To treat patients with potentially fatal hematologic diseases such as acute leukemia, hematopoietic stem cell (HSC) transplantation is the most effective life-saving therapy. However, widespread use of HSC transplantation has been prevented by the difficulties in finding major histocompatibility complex-matched donors, and establishment of effective methods of ex vivo expansion of hematopoietic stem/progenitor cells (HSPCs) obtained from bone marrow or human umbilical cord blood (hUCB) is well recognized as a promising approach for solving this serious issue [1,2].

For more than three decades, many investigations of ex vivo expansion of hematopoietic cells (HCs) sought to mimic the hematopoietic microenvironment of bone marrow, e.g., cocultures of HCs and stromal cells and addition of the soluble components secreted by these cells [1,3-7]. With respect to cocultures, HCs are generally seeded on stromal layers that are initially formed on culture dishes, and consequently, successive cultures to form a stromal layer and then expand HSPCs are

needed [3,6,7]. Although the effectiveness of these cocultures in expansion of HSPCs has been clearly demonstrated, the long culture period required for the successive cultures is a serious disadvantage of these methods. Moreover, the research studies on expansion of HCs were mostly performed with two-dimensional (2D) monolayer cultures, and little attention has been paid to the effects of three-dimensional (3D) cultures that also mimic the microenvironment of bone marrow [8-11].

From the viewpoint of tissue engineering, establishment of effective culture methods that allows safe, large-scale expansion of HSPCs is required for their clinical application, and 3D cultures of HCs seem to be essential for meeting this requirement. In our previous studies, HCs obtained from mouse bone marrow cells (BMCs) were cocultured with bone marrow-derived stromal cells (BMSCs) under 3D conditions using porous polymer scaffolds [12-14]. In those culture experiments, the stromal cell layers formed within the 3D scaffold were frozen (3D freezing) and then applied to the cocultures of HCs after thawing in order to shorten the culture period practically by separating the culture for formation of the stromal layers from that for expansion

of HSPCs [14]. Interestingly, we found that a greater expansion of HCs was achieved in the cultures using the stromal cells with 3D freezing than in those without freezing. Therefore, 3D cocultures with 3D frozen-thawed stromal cells seemed to be an attractive method for expansion of HCs with high efficiency.

As a next step toward the application of this coculture method to HSC transplantation, use of stromal cell lines instead of BMSCs and the choice of such cell lines suitable for HC expansion should be considered because stromal cell lines are easier to harvest and have greater homogeneity than BMSCs. In the present study, two stromal cell lines derived from the aorta-gonad-mesonephros (AGM) region were applied for the 3D cocultures, and HCs in the fetal liver cells (FLCs), which contain more primitive HCs than does bone marrow, were expanded in these cocultures as a model of HC expansion in hUCB [15]. The expansion of the HCs in these cultures was compared with that in the cocultures with BMSCs. The effects on the expansion of HCs of the 3D frozen-thawed stromal cell lines as well as those of the periods of stromal cell cultures prior to the cocultures were also investigated.

Methods

Cells and culture media

Two types of stromal cell lines, DAS 104-8 and DAS 104-4, both established from the AGM region of C57BL/6 mouse embryos (embryonic day [ED] 11), were provided by Professor Ohneda of the University of Tsukuba [16]. As in our previous studies [12-14], the BMCs used as the source of stromal cells in the control experiments were isolated from the tibiae and femora of 6-week-old C57BL/6N male mice (Charles River Laboratories Japan, Yokohama, Kanagawa, Japan). As a source of HCs, FLCs were harvested from C57BL/6NCrSlc mice (Japan SLC, Hamamatsu, Shizuoka, Japan) on ED 14 according to a previously reported method [17]. All mice were treated according to the University of Tsukuba Animal Experiment Committee Guidelines for the Care and Use of Laboratory Animals.

In the culture experiments using DAS 104-8 or DAS 104-4 cells, i.e., cultures of these stromal cell lines for formation of the stromal layer and subsequent

cocultures with FLCs, high-glucose Dulbecco's modified Eagle's medium (Gibco®, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Tokyo, Japan), 10^{-4} M MEM nonessential amino acids (Gibco), 2 x 10^{-3} M L-glutamine (Gibco), 10^{-4} M β-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), and 0.5% penicillin-streptomycin (Gibco) (HAVA medium) was used [16]. In the control experiments using BMSCs, BMSCs and FLCs reinoculated on the layers of the BMSCs were cultured in minimum essential medium-alpha (Gibco) containing 10% FBS, 10% horse serum (Stem Cell Technologies, Veritas Corporation, Tokyo, Japan), 10^{-7} M dexamethasone (Sigma-Aldrich Japan, Tokyo, Japan), 5 x 10^{-5} M 2-mercaptoethanol, and 0.2% antibiotics (Gibco) (αMEM), as in our previous studies [13,14].

3D scaffold, cell seeding, and 3D freezing of stromal cells

In all culture experiments, a highly porous polyvinyl formal (PVF) resin (Aion, Osaka, Japan) was used as the 3D scaffold [12-14,18,19]. Resin with a mean pore

size of 130 μm was cut into cubes (2 x 2 x 2 mm^3), sterilized in an autoclave, and coated with type I collagen (Koken, Tokyo, Japan) [18].

At the initiation of the stromal cell cultures and cocultures of FLCs on the stromal layers, cells were seeded into the scaffolds by the centrifugal cell immobilization (CCI) method, as previously reported [20]. Briefly, a centrifuge bottle containing 100 PVF cubes and cell aliquots suspended in culture medium was centrifuged six times to entrap the cells within the scaffolds.

For the coculture experiments using 3D frozen-thawed stromal cells, the cubic scaffolds containing stromal layers formed by the stromal cell cultures were frozen to -80ºC at -1ºC/min in culture medium containing 10% dimethyl sulfoxide [14,21]. The frozen scaffolds were stored in liquid nitrogen and then thawed in a 37ºC water bath just before the cocultures.

Culture experiments

To expand the HCs present in the FLCs, stromal cells (DAS 104-8 or DAS 104-4, and

BMSCs as controls) were first cultured within the 3D scaffolds to form stromal layers, and successive cocultures of FLCs on these stromal layers were then performed [12-14]. The conditions of the stromal cell cultures, including the stromal cells used, culture media, duration of the cultures, and presence or absence of 3D freezing, are summarized in Table 1.

In the stromal cell cultures, the cells were seeded into cubic scaffolds at a density of 1 x 10^7 cells/cm^3 of scaffold. Next, 10 cubic scaffolds containing the cells were placed in a 35-mm culture dish, followed by an addition of 2 mL of culture medium. The cells were cultured for 3, 7, or 10 days (DAS 104-8), for 7 days (DAS 104-4) or for 14 days (BMSCs) prior to the cocultures (Table 1) [14]. At the beginning of the cocultures, freshly isolated FLCs at a density of 5 x 10^7 cells/cm^3 of scaffold were reinoculated by means of the CCI method into the scaffolds containing the stromal layers, and these cells were cocultured for 2 weeks under similar conditions to those used for the stromal cell cultures.

Cell counting, FACS analysis, and SEM observation

The number of total cells in the cultures using stromal cell lines (DAS 104-8 or DAS 104-4) was estimated by measuring the amount of DNA in the cells immobilized within the scaffolds using Hoechst 33342 solution [19]. In the control cultures using BMSCs, the total cell numbers were measured by a colorimetric MTT assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [22], as previously reported [13,14,18].

To examine the percentages of erythroid cells, B cells, hematopoietic progenitor cells (HPCs), and HSPCs among the cultured cells, fluorescence-activated cell sorter (FACS) analysis was performed with a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) [13,14]. As respective specific markers for these cells, anti-Ter119, anti-B220, anti-c-kit, and anti-CD34 monoclonal antibodies (all from PharMingen, San Diego, CA) were used. The numbers of specific-marker-positive cells were calculated from the numbers of total cells and the percentages of positive cells.

The morphology of the cultured cells within the scaffolds was observed by scanning electron microscopy (SEM; JSM-6320F; JEOL, Tokyo, Japan) [23].

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was carried out using the *t* test, and significance was set at $p < 0.05$.

Results

Growth of stromal cell lines

For formation of the stromal layers, both stromal cell lines proliferated well within the scaffolds (Fig. 1), and DAS 104-4 showed a higher growth rate than did DAS 104-8. To expand HCs, these cells cultured for 3, 7, and 10 days were applied to the coculture experiments just after the stromal cell cultures were completed or after the 3D freezing (Table 1).

Expansion of HCs in cocultures with DAS 104-8 cells

The changes in the densities of total cells and each HC type over time in the cocultures of FLCs with DAS 104-8 cells as well as in the control cultures with BMSCs are shown in Figure 2. No marked increases in the total cell densities from day 1 to day 14 were observed, probably owing to the high cell densities in these cocultures on day 1 (Fig. 2A). With respect to the expansion of HCs, the numbers of B cells, HPCs, and HSPCs increased in most of the cocultures (Fig. 2C-E). In particular, primitive HCs (HPCs and HSPCs) were well expanded in the cocultures with DAS 104-8 cells (DAS8/3, DAS8/7, DAS8/10) irrespective of the culture periods of the stromal cells (Fig. 2D, E), and these expansions were comparable to those of the cocultures with BMSCs (BM/14).

The percentages of HCs in these cocultures are summarized in Table 2. Cocultures in which significant changes in the percentages of the HCs were measured from day 1 to day 14 largely corresponded to those in which the numbers of these cells significantly changed (Fig. 2B-E). In the cocultures with DAS 104-8 cells, the

percentages of HPCs and HSPCs on day 14 were comparable to or slightly higher than those in the BM/14 coculture. From these results, it was revealed that DAS 104-8 cells have the ability to support ex vivo expansion of primitive HCs similar to that of BMSCs.

Expansion of HCs in cocultures with frozen-thawed DAS 104-8 cells

In the cocultures of FLCs on the frozen-thawed DAS 104-8 stromal layers (Fig. 3), total cell densities gradually increased with time in the cocultures using DAS 104-8 cells (fDAS8/7, fDAS8/10), whereas cell densities on day 14 in the control experiments (fBM/14) were almost the same as those on day 1 (Fig. 3A). With respect to the primitive HCs, the densities of HPCs and HSPCs significantly increased in the cocultures with DAS 104-8 cells (fDAS8/7, fDAS8/10) (Fig. 3D, E). These increases were far higher than those in the fBM/14 coculture and were also higher than those in the cocultures without the 3D freezing (Fig. 2D, E). Between the fDAS8/7 and fDAS8/10 cocultures, negligible differences in the expansion of each

HC type were detected.

As shown in Table 3, the percentages of the HPCs and HSPCs significantly increased with time in the fDAS8/7 and fDAS8/10 cocultures, which differed from the fBM/14 coculture. Notably, the percentages of the primitive HCs on day 14 in most cocultures with DAS 104-8 cells (fDAS8/7, fDAS8/10) were significantly higher than those in the fBM/14 coculture, although the percentages on day 1 were lower than in the fBM/14 coculture. These results revealed that the effects of 3D freezing of stromal cells on the expansion of primitive HCs differ considerably according to the stromal cells used.

Expansion of HCs in cocultures with DAS 104-4 cells

To examine the applicability of 3D freezing to other stromal cell lines, DAS 104-4 cells cultured for 7 days were used for the coculture experiments, in addition to DAS 104-8 cells. As shown in Figure 4, no significant changes in the densities of the HCs were detected in the cocultures without 3D freezing (DAS4/7) (Fig. 4B-E). In

contrast, the densities of B cells, HPCs, and HSPCs significantly increased in the cocultures with 3D freezing (fDAS4/7) (Fig. 4C-E), and the density of HPCs on day 14 was significantly higher in the fDAS4/7 coculture than in the DAS4/7 coculture (Fig. 4D). From the percentages of HCs in these cocultures (Table 4), significant increases in the percentages of the primitive HCs from day 1 to day 14 in the fDAS4/7 coculture contributed to the increases in the numbers of these cells (Fig. 4D, E).

Comparison of HC expansion in coculture experiments

The expansions of HCs in all coculture experiments are summarized in Figure 5. In most cultures, the HCs other than erythroid cells were expanded (cell expansion > 1) during the 2-week coculture experiments. In the cocultures with 3D frozen-thawed DAS 104-8 cells, except those of HPCs in the fDAS8/10 coculture, significantly higher expansions of these HCs than of those without 3D freezing were obtained (Fig. 5B-D). High expansions of primitive HCs (HPCs >15-fold, HSPCs > 30-fold) were

also achieved in the fDAS8/7 and fDAS8/10 cocultures (Fig. 5C, D), and these expansions were significantly higher than those in the fBM/14 coculture.

With respect to the cocultures with DAS 104-4 cells, similar to the cocultures with DAS 104-8 cells, the expansion of the HCs in the fDAS4/7 coculture was higher than in the DAS4/7 coculture (Fig. 5B, C). Compared with the fDAS8/7 coculture, expansion of HSPCs in fDAS4/7 was significantly lower (Fig. 5D), suggesting that DAS 104-8 cells are preferable to DAS 104-4 cells for expanding HSPCs in 3D cocultures.

From these results, it was clearly shown that 3D freezing of DAS 104-8 and DAS 104-4 cells strongly enhanced the expansion of primitive HCs in addition to B cells, whereas no beneficial effects of 3D freezing were observed in the cocultures with BMSCs.

SEM observation

Figure 6 shows representative SEM images of the 3D cocultured cells. When FLCs

were cocultured with BMSCs, both spherical cells that seemed to be HCs and flattened cells like stromal cells were observed (Fig. 6A, B). The flattened cells contacted with the spherical cells either by overlying (Fig. 6A) or surrounding (Fig. 6B) a cluster of spherical cells on the 3D scaffolds. In the cocultures with DAS 104-8 cells, a large amount of spindle-shaped cells covered the scaffold surfaces (Fig. 6C), and only a limited number of spherical cells existed on the surfaces (Fig. 6D).

Discussion

Umbilical cord blood cells have several advantages in HSC transplantation over BMCs; for example, they have a higher proportion of primitive HCs and are easier to harvest. By contrast, the small number of HSCs in each hUCB unit is a major obstacle to UCB transplantation into adult patients, so a several-fold expansion of HSCs in ex vivo cultures is expected to facilitate wider applicability of UCB transplantation [1,24]. To achieve ex vivo expansion of hUCB-derived HSCs, many investigations have been reported in which the in vivo hematopoietic

microenvironment was mimicked, e.g., cocultures with stromal cells [7,25], HSC cultures under hypoxic conditions [4,26], and cultures on acellular matrices [26]. These efforts have contributed to the sufficient expansion of primitive HCs dozens of times; however, exogenous cytokines such as stem cell factor (SCF) and thrombopoietin (TPO) were used to expand HCs in most of those cultures [1]. Because the added cytokines increase both the risks for inducing unwanted differentiation of HSPCs and the costs for clinical application, development of an effective expansion method without using exogenous cytokines is expected [27].

In addition to the culture methods that mimic the hematopoietic microenvironment as described above, the importance of 3D architecture in expanding HSPCs has been recognized in recent years [28,29], and expansion of primitive HCs was achieved in 3D cultures using porous scaffolds constructed from biological products [11] and polymers [10,27,30]. Similarly to our previous studies [12-14,17], in the present study, a porous PVF scaffold was applied for the expansion of HCs to establish an effective method of expansion of primitive HCs that can be

applied to UCB transplantation. Instead of hUCB, mouse FLCs (ED 14) were used as a source of HCs, because the mouse fetal liver is a major hematopoietic organ after ED 11.5 and, similar to hUCB, contains more primitive HCs than does the bone marrow. As stromal cells, stromal cell lines were applied that were established from ED 11 mice and were close in origin to the FLCs. Consequently, more than 15-fold expansions of primitive HCs were achieved without addition of cytokines when the HCs were cocultured with 3D frozen-thawed stromal cell lines (Fig. 5). Although the details of the 3D culture conditions differed from those of previous reports, these expansion values seem to be comparable to those of previous reports and to be satisfactory for clinical application [24,26,27,30].

In the previous study using the same stromal cell lines, it was reported that DAS 104-4 was preferable to DAS 104-8 for expansion of primitive HCs without differentiation and that DAS 104-8 showed scarce ability to support the expansion [16]. These results were inconsistent with those obtained in this study (Fig. 5C, D), and the difference in culture conditions between these studies seems to be one of the

major causes for the inconsistency. In this study, FLCs were cocultured with the stromal cells instead of purified HSPCs that were applied in the previous report, and several types of cells included in the FLCs might affect the expansion of the primitive HCs. To examine the effects of HC sources on expansion of primitive HCs, expansion experiments of purified HSPCs using the present 3D cocultures will be needed.

With respect to the differences between 2D and 3D cultures, formation of cell aggregates in the 3D culture might have enhanced cell-cell interaction (Fig. 6), compared with the 2D culture. Cells cultured under 3D conditions are also known to exhibit activities different from those in 2D cultures [13,17-19,23]. Thus, it is plausible that the types and amount of surface markers expressed and of factors secreted by the stromal cell lines differed between the 2D and 3D cultures. Identification of these markers and factors will contribute to elucidation of the mechanism and the effective factors of HSC expansion.

Both the DAS 104-8 and the DAS 104-4 stromal cell lines used in this study

are $CD34^+$ cells [16]; therefore, the possibility cannot be excluded that the remarkable increases in $CD34^+$ cells in this study (Figs. 2-5) were caused by the proliferation of these stromal cell lines. However, this possibility seems to be negligible owing to the following facts: (1) The stromal cell lines showed poor proliferation after the processes of 3D freezing and thawing (data not shown), similar to the BMSCs used in our previous study [14]. (2) The cocultures with the stromal cell lines without 3D freezing resulted in considerably lower expansion of the $CD34^+$ cells than of those with 3D freezing under all culture conditions (Fig. 5D), although the proliferating ability of the stromal cell lines without freezing should be higher than those with 3D freezing. Therefore, the high expansion of the $CD34^+$ cells in the cocultures of HCs with DAS 104-8 or DAS 104-4 cells was almost certainly induced by that of primitive HCs.

With respect to the supportive mechanism of the stromal cells in HC expansion, several components might be involved, e.g., soluble factors secreted by the stromal cells, extracellular matrices accumulated around the stromal cells, and cell-cell

interactions between stromal cells and HCs [5,26]. In the present study, the 3D frozen-thawed stromal cell lines, in which the metabolic activities had probably deteriorated significantly as mentioned above, enhanced the expansion of HCs. These results strongly suggest that the soluble factors secreted by the stromal cell lines were not predominant in expanding the HCs when compared with the direct contact between the HCs and stromal cell lines or extracellular matrices in the 3D cultures [16,25]. Recently, it is increasingly recognized that stromal cells and differentiated HCs secrete a certain amount of factors that inhibit expansion of undifferentiated HCs [2,26], suggesting that decreased secretion of these inhibitory factors from the stromal cell lines caused by the damage from 3D freezing partly contributed to the high expansion of the primitive HCs.

In the present study, the effectiveness of 3D frozen-thawed stromal cells on HSC expansion was demonstrated by using AGM-region-derived stromal cell lines. Generally, growth of stromal cells is suppressed by gamma-irradiation or treatment with an inhibitor of DNA synthesis before the coculture with HCs for efficient

expansion of HCs [31-34]. Compared with other methods to suppress stromal cell growth, 3D freezing is very easy to perform and requires no special equipment or reagents. This method is also suited to treating large numbers of stromal cells. Therefore, 3D freezing is expected to be a novel means to treat stromal cells for the ex vivo expansion of primitive HCs.

To apply this method to expansion of hUCB-derived HSPCs clinically, selection of stromal cells suited to the expansion is needed [15]. Mouse and human stromal cells derived from the bone marrow, AGM region and fetal liver seem to be potential candidates [15], and the abilities of these stromal cells to expand hUCB-derived HSPCs should be compared under the 3D coculture conditions used in this study. In our preliminary study, bone marrow-derived stromal cell lines supported expansions of mouse HSPCs, similar to the cell lines derived from the AGM region shown in this study, and we plan to apply these stromal cells for expansion of HSPCs derived from hUCB. In addition to the selection of the stromal cells, detailed analysis of the expanded cells is also required. By using colony assay

and long-term bone marrow reconstitution activity by transplantation experiments of the expanded cells into lethally irradiated mice, the performances of expanded HCs should be evaluated accurately [12,16,18,35].

In conclusion, sufficient expansion of primitive HCs was achieved without addition of exogenous cytokines when the cells were cocultured with 3D frozen-thawed stromal cell lines. The coculture method is easy to perform, requires no special equipment or reagents, and offers a practical way to shorten the culture period for HC expansion. Therefore, this method is expected to be useful for expansion of HCs.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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Figure legends

Figure 1. Growth of stromal cell lines in 3D cultures. Mean ± standard deviation (SD). Open circles, DAS 104-8 cells (n = 5); open squares, DAS 104-4 cells (n = 3).

Figure 2. Changes over time in the numbers of total cells and hematopoietic cells (HCs) after reinoculation in 3D coculture experiments of fetal liver cells (FLCs) with DAS 104-8 stromal cells. Mean ± SD. Open triangles, 3D coculture with DAS 104-8 cells cultured for 3 days prior to the coculture (DAS8/3) (n = 4); open circles, 3D coculture with DAS 104-8 cells cultured for 7 days prior to the coculture (DAS8/7) (n = 3); open squares, 3D coculture with DAS 104-8 cells cultured for 10 days prior to the coculture (DAS8/10) (n = 3); open diamonds, 3D coculture with bone marrow-derived stromal cells (BMSCs) cultured for 14 days prior to the coculture (BM/14) (n = 3). Numbers of (A) total cells, (B) erythroid cells, (C) B cells, (D) hematopoietic progenitor cells (HPCs), and (E) hematopoietic stem/progenitor cells (HSPCs). $^{*}p < 0.05$ vs. day 1; $^{\dagger}p < 0.05$ vs. DAS8/3.

Figure 3. Changes over time in the numbers of total cells and HCs after reinoculation in 3D coculture experiments of FLCs with frozen-thawed DAS 104-8 stromal cells. Mean ± SD. Closed circles, 3D coculture with frozen-thawed DAS 104-8 cells cultured for 7 days prior to 3D freezing (fDAS8/7) (n = 3); closed squares, 3D coculture with frozen-thawed DAS 104-8 cells cultured for 10 days prior to 3D freezing (fDAS8/10) (n = 3); closed diamonds, 3D coculture with frozen-thawed BMSCs cultured for 14 days prior to 3D freezing (fBM/14) (n = 3). Numbers of (A) total cells, (B) erythroid cells, (C) B cells, (D) HPCs, and (E) HSPCs. *$p < 0.05$ vs. day 1.

Figure 4. Changes over time in the numbers of total cells and HCs after reinoculation in 3D coculture experiments of FLCs with DAS 104-4 stromal cells with or without 3D freezing. Mean ± SD. Open circles, 3D coculture with DAS 104-4 cells cultured for 7 days prior to the coculture (DAS4/7); closed circles, 3D coculture

with frozen-thawed DAS 104-4 cells cultured for 7 days prior to the 3D freezing (fDAS4/7). Numbers of (A) total cells (n = 4), (B) erythroid cells (n = 4), (C) B cells (n = 3), (D) HPCs (n = 3-4), and (E) HSPCs (n = 3-4). *$p < 0.05$ vs. day 1; $^{\#}p < 0.05$ vs. cocultures without 3D freezing.

Figure 5. Expansions of HCs in 3D coculture experiments of FLCs with stromal cells with or without 3D freezing. Mean ± SD. Gray bars, 3D cocultures without 3D freezing (n = 3-4); closed bars, 3D cocultures with frozen-thawed stromal cells (n = 3-4). Expansions of (A) erythroid cells, (B) B cells, (C) HPCs, and (D) HSPCs. $^{\#}p < 0.05$ vs. cocultures without 3D freezing; **$p < 0.05$ vs. cocultures with BMSCs (BM/14 or fBM/14); ***$p < 0.05$ vs. DAS 104-8 cells (DAS8/7 or fDAS8/7). ND, not done.

Figure 6. Scanning electron micrographs of FLCs cocultured with stromal cells within 3D scaffolds. (A) FLCs cocultured with frozen-thawed BMSCs (fBM/14, day

14). Bar = 100 μm. (B) Cluster of spherical cells surrounded by flattened cells (fBM/14, day 14). Bar = 10 μm. (C) FLCs cocultured with DAS 104-8 stromal cells (DAS8/7, day 14). Bar = 100 μm. (D) Higher magnification of Figure 6C. Bar = 10 μm. *PVF scaffold; arrow: flattened cells overlying a cluster of spherical cells.

Table 1. Conditions of stromal cell cultures prior to the cocultures for expanding hematopoietic cells.

Culture condition	Stromal cell	Culture medium	Duration of stromal cell culture [days]	3D freezing
DAS8/3	DAS 104-8	HAVA medium	3	-
DAS8/7	DAS 104-8	HAVA medium	7	-
DAS8/10	DAS 104-8	HAVA medium	10	-
fDAS8/7	DAS 104-8	HAVA medium	7	+
fDAS8/10	DAS 104-8	HAVA medium	10	+
DAS4/7	DAS 104-4	HAVA medium	7	-
fDAS4/7	DAS 104-4	HAVA medium	7	+
BM/14	BMSCs	αMEM	14	-
fBM/14	BMSCs	αMEM	14	+

Table 2. Percentages of Ter119^{+} erythroid cells, B220^{+} B cells, c-kit^{+} hematopoietic progenitor cells (HPCs), and CD34^{+} hematopoietic stem/progenitor cells (HSPCs) in 3D cocultures with DAS 104-8 stromal cells or bone marrow-derived stromal cells (BMSCs).

Marker	Culture condition	% of positive cells		Marker	Culture condition	% of positive cells	
		Day 1	Day 14			Day 1	Day 14
Ter119	DAS8/3	87.0 ± 1.6	18.7 ± 10.8*,**	B220	DAS8/3	3.6 ± 0.5	6.3 ± 4.1**
	DAS8/7	79.2 ± 6.9	21.1 ± 5.9*,**		DAS8/7	6.8 ± 4.6	8.5 ± 3.9**
	DAS8/10	76.6 ± 14.1	25.1 ± 10.3*		DAS8/10	3.1 ± 1.4	10.4 ± 1.5*,**
	BM/14	84.5 ± 4.9	41.8 ± 5.9*		BM/14	3.5 ± 2.9	17.6 ± 2.0*
c-kit	DAS8/3	4.6 ± 2.4**	22.7 ± 12.2*	CD34	DAS8/3	4.4 ± 1.3	27.3 ± 11.8*
	DAS8/7	10.6 ± 5.5	33.5 ± 13.3		DAS8/7	9.7 ± 2.7†,**	40.3 ± 16.0*
	DAS8/10	7.4 ± 6.1	35.1 ± 16.6		DAS8/10	6.2 ± 4.9	36.7 ± 11.3*
	BM/14	9.0 ± 1.9	21.3 ± 1.7*		BM/14	3.1 ± 1.6	22.0 ± 15.9

The number of experiments is the same as in Figure 2.

*$p < 0.05$ vs. day 1; $^{\dagger}p < 0.05$ vs. DAS8/3; **$p < 0.05$ vs. BM/14.

Table 3. Percentages of Ter119^{+} erythroid cells, B220^{+} B cells, c-kit^{+} HPCs, and CD34^{+} HSPCs in 3D cocultures with frozen-thawed DAS 104-8 stromal cells or frozen-thawed BMSCs.

Marker	Culture condition	% of positive cells		Marker	Culture condition	% of positive cells	
		Day 1	Day 14			Day 1	Day 14
Ter119	fDAS8/7	90.7 ± 2.2**	21.0 ± 8.1*	B220	fDAS8/7	1.8 ± 0.5	11.7 ± 6.6
	fDAS8/10	88.3 ± 1.9**	22.6 ± 5.8*		fDAS8/10	1.1 ± 0.4	10.6 ± 1.0*
	fBM/14	78.9 ± 4.2	28.8 ± 7.9*		fBM/14	3.4 ± 2.2	20.7 ± 10.7
c-kit	fDAS8/7	3.4 ± 0.9**	34.5 ± 16.9*	CD34	fDAS8/7	2.2 ± 0.4	41.8 ± 7.9*,**
	fDAS8/10	3.0 ± 0.8**	29.1 ± 3.7*,**		fDAS8/10	1.7 ± 0.7	42.6 ± 10.9*,**
	fBM/14	10.5 ± 2.8	13.6 ± 7.2		fBM/14	4.6 ± 1.8	16.9 ± 9.6

The number of experiments is the same as in Figure 3.

*p < 0.05 vs. day 1; **p < 0.05 vs. fBM/14.

Table 4. Percentages of Ter119^{+} erythroid cells, B220^{+} B cells, c-kit^{+} HPCs, and CD34^{+} HSPCs in 3D cocultures with DAS 104-4 stromal cells with or without 3D freezing.

Marker	Culture condition	% of positive cells		Marker	Culture condition	% of positive cells	
		Day 1	Day 14			Day 1	Day 14
Ter119	DAS4/7	26.3 ± 9.0**,***	21.4 ± 12.5**	B220	DAS4/7	8.0 ± 0.5	4.0 ± 3.4**
	fDAS4/7	29.5 ± 5.6**,***	25.8 ± 5.5		fDAS4/7	3.3 ± 1.4	9.1 ± 5.5
c-kit	DAS4/7	2.1 ± 0.9**,***	9.7 ± 7.9***	CD34	DAS4/7	5.9 ± 3.5	9.9 ± 6.9***
	fDAS4/7	1.2 ± 0.4**,***	25.2 ± 2.9*,**		fDAS4/7	4.0 ± 0.5***	14.1 ± 6.2*,***

The number of experiments is the same as in Figure 4.

*p < 0.05 vs. day 1; **p < 0.05 vs. cocultures with BMSCs (BM/14 or fBM/14); ***p < 0.05 vs. cocultures with DAS 104-8 cells (DAS8/7 or fDAS8/7).

Figure 1

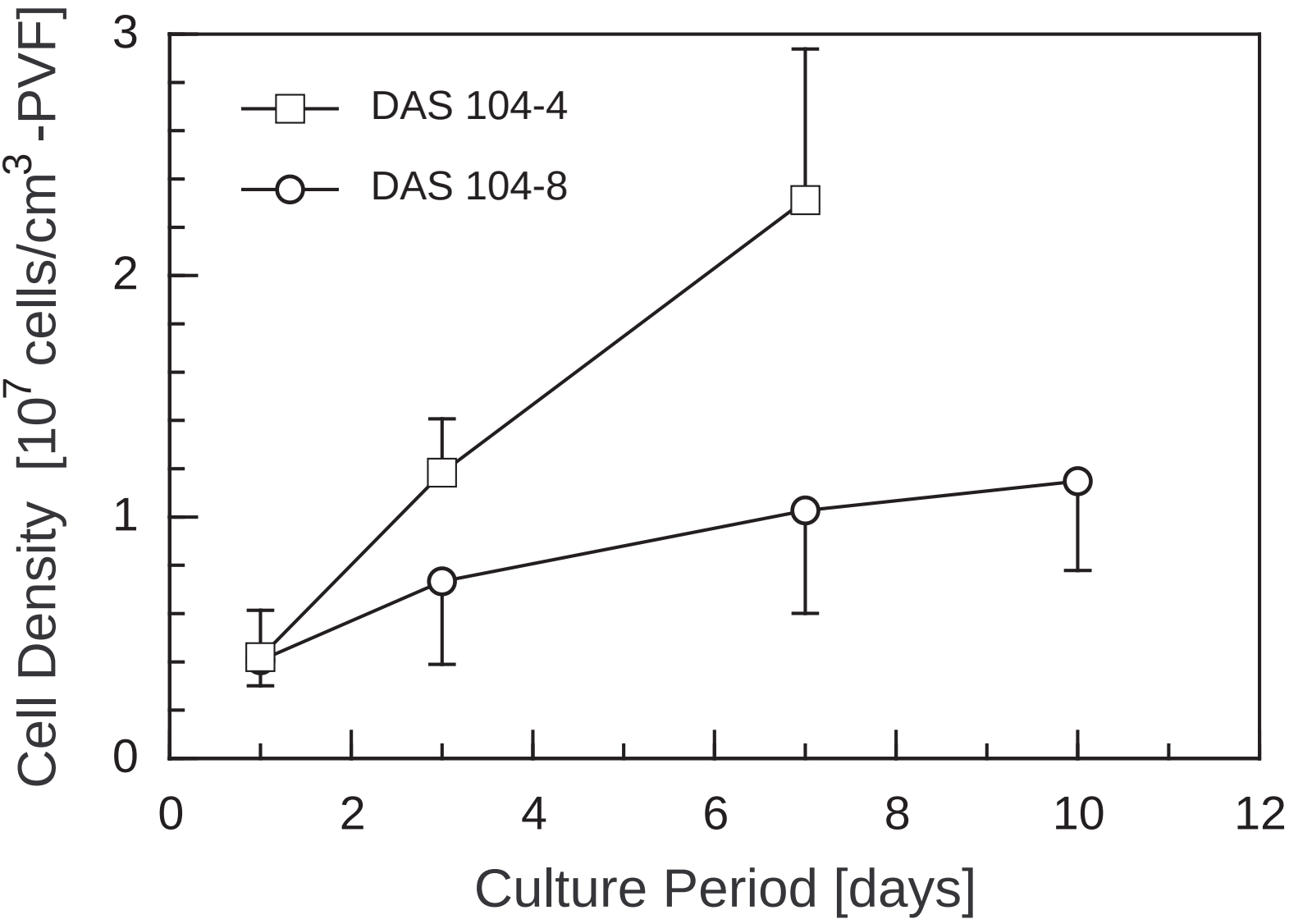

Cell Density [10^7 cells/cm^3-PVF]
3
2
1
0
DAS 104-4
DAS 104-8
0
2
4
6
8
10
12
Culture Period [days]

Figure 2

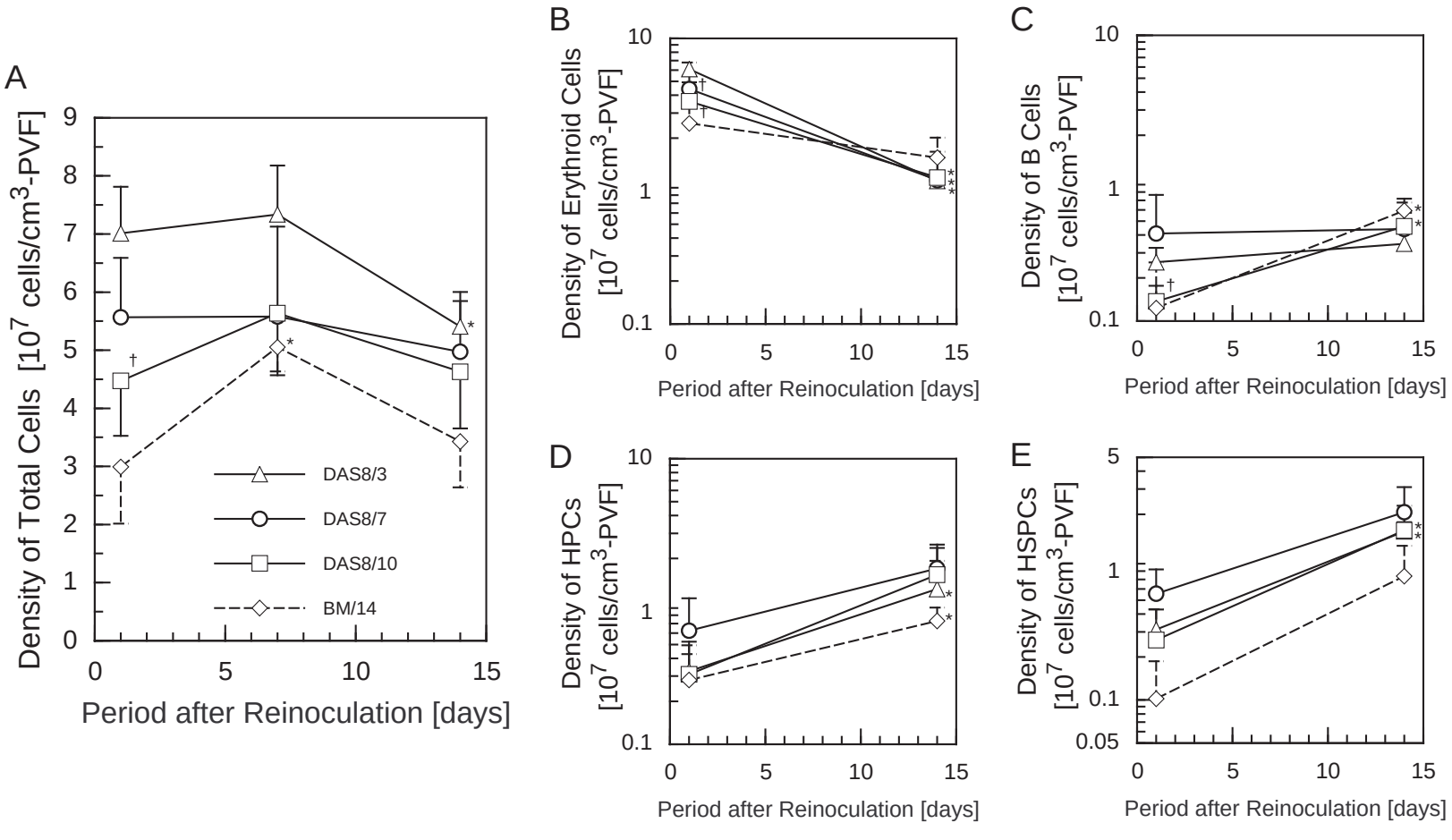
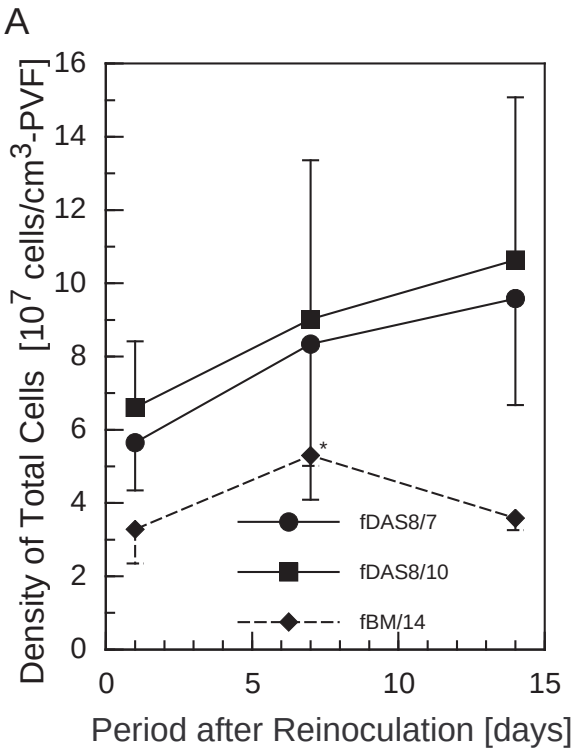
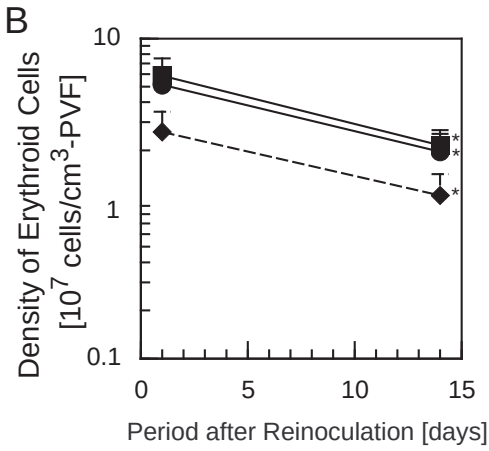

A
Density of Total Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
DAS8/3
DAS8/7
DAS8/10
BM/14
B
Density of Erythroid Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
C
Density of B Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
D
Density of HPCs [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
E
Density of HSPCs [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]

Figure 3


A
Density of Total Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
fDAS8/7
fDAS8/10
fBM/14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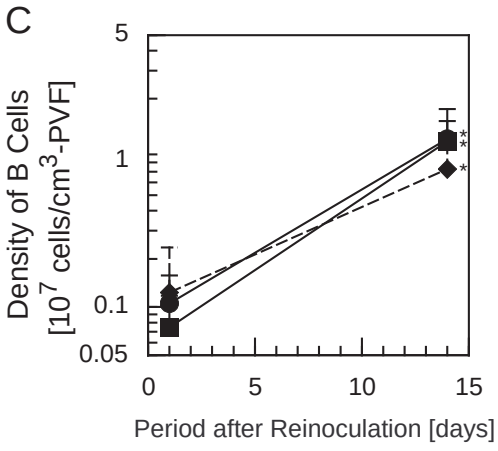
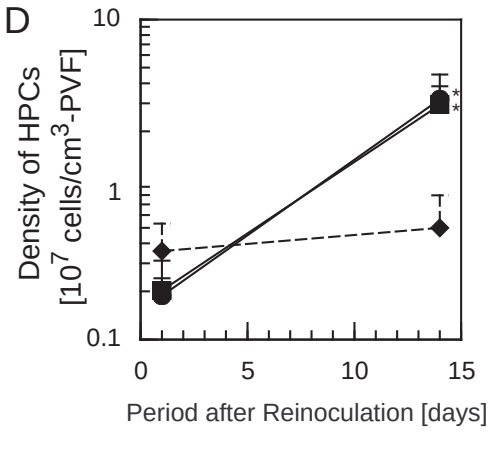
Density of Erythroid Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]


C
Density of B Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]


D
Density of HPCs [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]

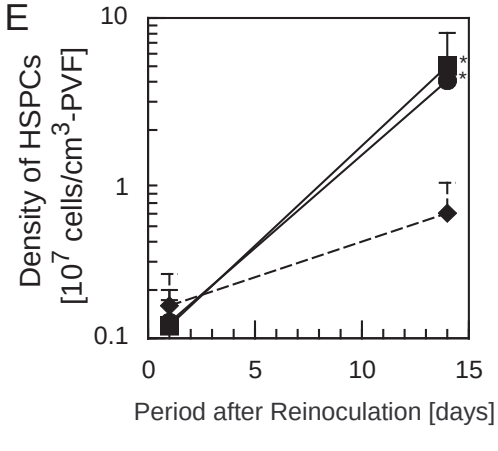

E
Density of HSPCs [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]

Figure 4

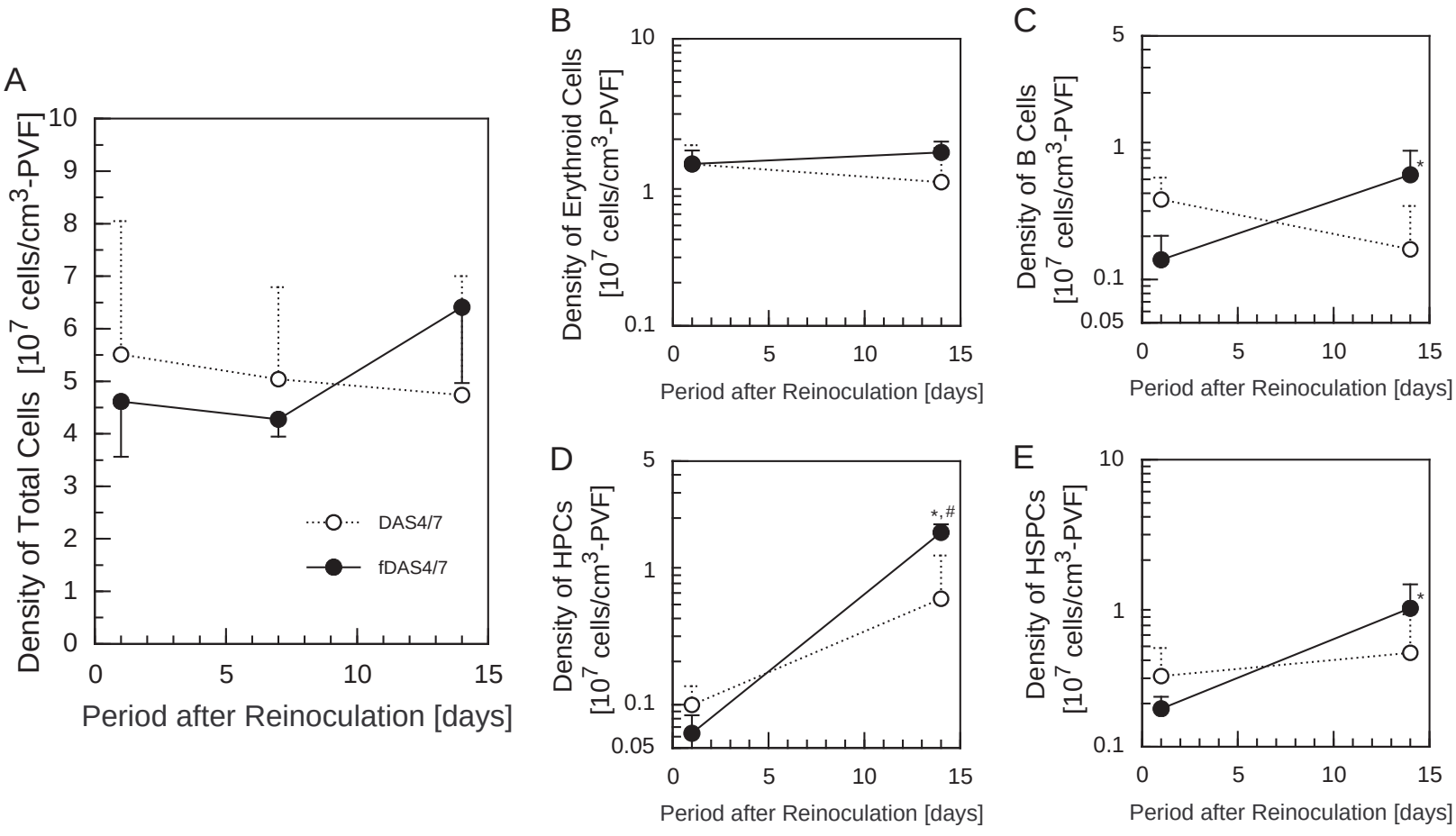

A
Density of Total Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
DAS4/7
fDAS4/7
B
Density of Erythroid Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
C
Density of B Cells [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
D
Density of HPCs [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]
E
Density of HSPCs [10^7 cells/cm^3-PVF]
Period after Reinoculation [days]

Figure 5

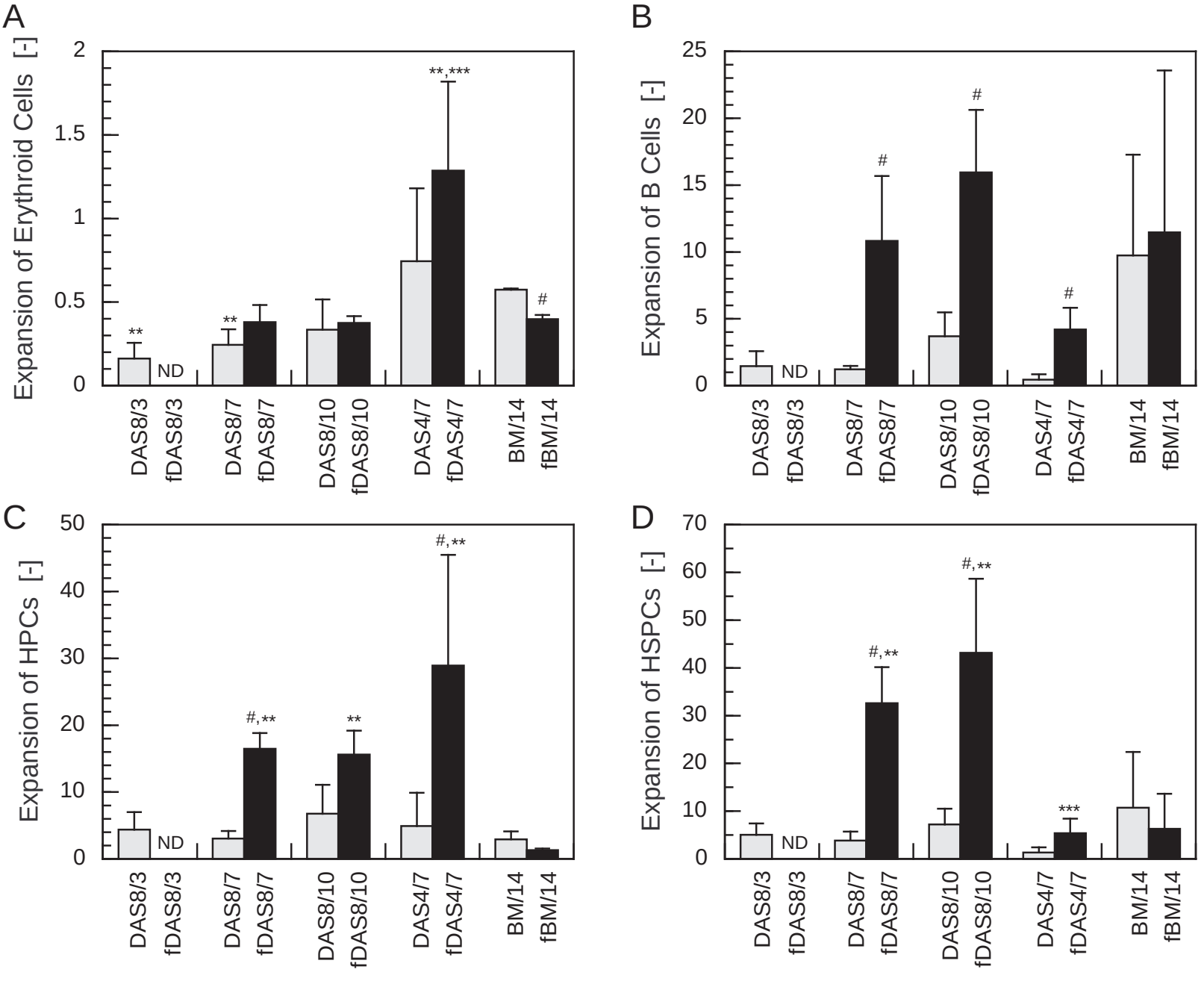

A
Expansion of Erythroid Cells [-]
2
1.5
1
0.5
0
**
ND
**
,*
#
DAS8/3
fDAS8/3
DAS8/7
fDAS8/7
DAS8/10
fDAS8/10
DAS4/7
fDAS4/7
BM/14
fBM/14
B
Expansion of B Cells [-]
25
20
15
10
5
0
ND
#
#
#
C
Expansion of HPCs [-]
50
40
30
20
10
0
ND
#,**
**
#,**
D
Expansion of HSPCs [-]
70
60
50
40
30
20
10
0
ND
#,**
#,**
***

Figure 6

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