Effects of mouse fetal liver cell culture density on hematopoietic cell expansion in three-dimensional cocultures with stromal cells

Hirotoshi Miyoshi[1], Kenji Abo[1], Daiki Hosoya[1], Kazuyuki Matsuo[1], and Yoshio Utsumi[1]

Department of Biomedical Engineering, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

Corresponding author: Hirotoshi Miyoshi, PhD

Department of Biomedical Engineering, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

Tel.: +81 29 853 3210; fax: +81 29 853 3254.

E-mail address: hmiyoshi@md.tsukuba.ac.jp

Abstract

Objective: An effective *ex vivo* expansion system of primitive hematopoietic cells (HCs) is required for wider application of hematopoietic stem cell transplantation. In this study, we examined effects of culture density on mouse fetal liver cells (FLCs) used as an HC source for the expansion of primitive HCs in three-dimensional (3D) cocultures with two kinds of mouse stromal cell lines (OP9 or C3H10T1/2).

Materials and methods: FLCs were seeded at different densities (1, 2, and 10 x 10^7 cells/cm^3) into porous polymer scaffolds with or without stromal cell layers and HCs were expanded in the cultures for 2 weeks without exogenous cytokines.

Results: Differential effects of culture density on HC expansion were observed between cocultures and solitary FLC controls. In stromal cell cocultures, high expansion of HCs was achieved when FLCs were seeded at low densities. In contrast, the expansion in the controls was enhanced with increasing culture densities. With respect to expansion of primitive HCs existing in the FLCs, cocultures with C3H10T1/2 cells were superior to those with OP9 cells with a 29.3-fold expansion for c-kit^+ hematopoietic progenitor cells and 8.3-fold expansion for $CD34^+$ hematopoietic stem cells. In the controls, HC expansion was lower than in any cocultures, demonstrating the advantages of coculturing for HC expansion.

Conclusion: Stromal cell lines are useful in expanding primitive HCs derived from FLCs in 3D cocultures. Culture density is a pivotal factor for the effective expansion of primitive HCs and this effect differs by culture condition.

Keywords: Hematopoietic stem cell, fetal liver cell, stromal cell, ex vivo expansion, three-dimensional culture, coculture.

Introduction

For patients with fatal hematologic dysfunction such as acute leukemia or malignant lymphoma, hematopoietic stem cell (HSC) transplantation is a decisive treatment. Among the HSC transplantation options using bone marrow, peripheral blood or umbilical cord blood (UCB), the recent number of UCB transplantations has increased due to several advantages, including ease of collection and high tolerance for human leukocyte antigen (HLA) mismatches (1-5). However, UCB transplantation is generally limited to pediatric treatment since only small amounts of primitive hematopoietic cells (HCs; hematopoietic progenitor cells [HPCs] and HSCs) are present in a single unit of UCB. Thus, establishment of an effective *ex vivo* expansion method would contribute to a wider application of UCB transplantation to adult patients (3-6).

To expand HSCs in cultures, coculturing HCs with stromal cells is a useful approach and the importance of diverse soluble factors secreted by both stromal cells and HCs, plus the direct contact of HCs and stromal cells, has been reported (7-10). However, most of these investigations were performed in small-scale monolayer cultures. For clinical applications, an established three-dimensional (3D) HC expansion method is critical to expand the large amounts of primitive HCs required for HSC transplantation. Reports on these 3D cultures have mainly focused on the effects of 3D scaffold materials on HC expansion (11,12) and thus little is known about the influence of operational culture conditions on the expansion process.

Generally, since cellular growth and differentiation are strongly affected by culture density (13), it is plausible that HC expansion also depends on the factor. Therefore, to

optimize *ex vivo* expansion conditions for primitive HCs, the effects of culture density should first be determined (14). In a previous study, we performed 3D cocultures of HCs, derived from mouse bone marrow cells or mouse fetal liver cells (FLCs), with stromal cells by using porous polymer scaffolds (15-17). In 3D cocultures of HCs derived from FLCs with stromal cell lines, we achieved an over fifteen-fold expansion of primitive HCs without exogenous stimulating factors (17). However, the detailed effect of culture density on HC proliferation remains unclear and must be clarified to optimize expansion conditions.

In the present study, we examined the effect of seeding densities on HC expansion in cocultures of FLCs with two kinds of stromal cell lines and solitary FLC cultures as controls. We found that culture density strongly affected expansion of primitive HCs and the effects differed between cocultures and control cultures.

MATERIALS AND METHODS

Cells and culture media

Fetal liver cells harvested from C57BL/6NCrSlc mice on embryonic day 14 (Japan SLC, Hamamatsu, Japan) were used as a source of HCs (17,18) since HCs are present in FLC populations in addition to mesenchymal stem cells, immature hepatocytes, and diverse other cell types. Two types of mouse stromal cell lines, OP9 and C3H10T1/2, were purchased from Riken BioResource Research Center (Tsukuba, Japan) (19,20). This study was approved by the University of Tsukuba Animal Experimental Committee (number 20-422). All mice were cared for according to guidelines developed by the committee.

In the coculture and control FLC culture experiments, Hava medium composed of high-glucose Dulbecco's modified Eagle's medium (Gibco, Life Technologies Japan, Tokyo,

Japan), 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 10^{-4} mmol/L minimum essential medium (MEM) nonessential amino acids (Gibco, Life Technologies Japan), 2 x 10^{-3} mmol/L L-glutamine (Gibco, Life Technologies Japan), 10^{-4} mmol/L 2-mercaptoethanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and 0.5% penicillin-streptomycin (Gibco, Life Technologies Japan) was used (17,18,21). To form stromal cell layers prior to coculture experiments, minimum essential medium-α (Gibco, Life Technologies Japan) containing 20% FBS and 0.5% penicillin-streptomycin or Basal Medium Eagle (Gibco, Life Technologies Japan) containing 10% FBS and 0.5% penicillin-streptomycin were used for culturing OP9 or C3H10T1/2 cells, respectively (19).

3D scaffolding, cell seeding, freezing of stromal cell layer, and cell harvesting

To generate 3D scaffolding, porous polyvinyl formal (PVF) resin (Aion, Osaka Japan) with a mean pore size of 130 μm was cut into cubes (2 x 2 x 2 mm^3), sterilized, and coated with type I collagen (Koken, Tokyo, Japan) (17,18). At culture/coculture initiation, stromal cells or FLCs were seeded into the scaffold cubes using the centrifugal cell immobilization method (17,22). Briefly, cell aliquots and 100 resin cubes were suspended in culture medium within a centrifugal bottle, and the bottle was centrifuged six times (300 x g, 1 min) to entrap the cells within the resin cubes.

The resin cubes containing stromal cell layers formed by 3D cultures (tissue-engineered constructs; TECs) were frozen to -80°C at -1°C/min in culture medium for each stromal cell line (OP9 or C3H10T1/2) supplemented with 10% dimethyl sulfoxide (17,18,23). These stromal-cell TECs were stored in liquid nitrogen and thawed just before the cocultures.

On days 1, 7, and 14, cells within scaffolds were harvested by vigorous pipetting in 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid)/0.2% collagenase solution after cutting the scaffold into small pieces (24).

Culture experiments

Hematopoietic cells were expanded by two successive cultures, *i.e.*, 3D cultures of stromal cells to construct stromal-cell TECs then 3D cocultures of HCs on the stromal layers within the TECs (15-18). In the stromal cell cultures, OP9 or C3H10T1/2 cells were seeded into resin cubes at a density of 5 x 10^5 cells/cm^3 and 20 cubes were put into a 35-mm culture dish. These cells were cultured with 2.5 mL of medium for 7 days.

In the cocultures, FLCs were seeded into freeze-thawed stromal-cell TECs at different densities of 1, 2, or 10 x 10^7 cells/cm^3 (D1, D2 and D10, respectively). The cells in 10 cubes were cocultured in a 35-mm dish with 2.5 mL of medium for 2 weeks. Controls, in the form of solitary 3D FLC cultures without stromal cells, were generated. Culture media were changed every other day for all culture experiments.

Measurements of numbers of total cells and HCs

Total cell numbers within 3D scaffolds were measured by MTT assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (25) as previously reported (15-17).

To determine the percentages of HCs within 3D cultures, cells detached from scaffolds were filtered through a 70 μm mesh to remove cell aggregates and small pieces of the scaffolds before analysis by fluorescence-activated cell sorting (BD FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) (24). Monoclonal antibodies of anti-Ter119 labeled with

phycoerythrin (PE) (erythroid cells), anti-B220 labeled with fluorescein isothiocyanate (FITC) (B cells), and anti-c-kit labeled with PE (HPCs) were used as specific markers for HCs (all from PharMingen, San Diego, CA) (17,18). In addition, $CD34^+$ was labeled as a biomarker for HSCs using anti-CD34 monoclonal antibody labeled with FITC (PharMingen). The numbers of HCs were calculated as percentages from total cell numbers.

Statistical analysis

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

RESULTS

Control experiments

Figure 1 shows a representative result of a flow cytometric analysis of HCs in a control culture on day 1. Most cells expressed Ter119 since whole FLCs were seeded and recognizable percentages of primitive HCs (HPCs and HSCs) were also detected.

The changes in total cell and HC densities over time when FLCs were solely cultured under different seeding density conditions (control cultures) are shown in Figure 2. In the high-density cultures (D10), total cell density increased gradually whereas significant decreases in density were detected in the D1 and D2 low-density cultures (Figure 2(a)).

With respect to HCs, cell densities of all HCs measured decreased in the low-density cultures (Figure 2(b)-(e)). On the contrary, the HC density was maintained stably and significant increases in the densities of B cells and HPCs were observed in the high-density

cultures (D10) (Figure 2(c), (d)). Thus, it was found that cultures with high cell densities are indispensable to expand HCs in solitary FLC cultures.

Cocultures of FLCs with OP9 stromal cells

In the cocultures of FLCs with OP9 stromal cells, total cell densities increased under all seeding density conditions (Figure 3(a)). Each HC was maintained rather stably under low-density conditions, which differed from control cultures (D1, D2; Figure 3(b)-(e)), and increases in primitive HCs were detected under these conditions (Figure 3(d), (e)). Compared with controls, the density of each HC in D10 was lower, probably due to lower total cell density (Figure 3(a)).

Cocultures of FLCs with C3H10T1/2 stromal cells

Total cell counts in the cocultures of FLCs and C3H10T1/2 stromal cells sharply increased up to day 7 before plateauing (Figure 4(a)). These increases were significant, especially in the low-density cultures (D1, D2), and the densities on day 14 were also significantly higher than control and OP9 cocultures.

Densities of all HCs were maintained or increased and no notable decreases were observed under all density conditions (Figure 4(b)-(e)). In the low-density cultures (D1, D2), densities of all HCs were significantly higher than controls. With respect to primitive HCs, the cell densities of D1 and D2 increased significantly until day 7 (Figure 4(d), (e)) and the HSC densities on day 14 were also higher than those in OP9 cocultures.

Comparison of HC expansion under each culture condition

The expansion of each HC, as demonstrated by the density ratios on day 14 and 1, are summarized in Figure 5. In the controls, cell expansions > 1 were achieved only in the high-density cultures (D10) (Figure 5(a)-(d)). On the contrary, HC expansions were higher in low-density cultures (D1, D2) compared with high-density cultures (D10) in both OP9 and C3H10T1/2 cocultures. Thus, culture density was negatively correlated with the expansion of HCs in the solitary FLC cultures and cocultures of FLCs with stromal cell lines.

Concerning primitive HCs, maximum expansion of HPCs in the OP9 or C3H10T1/2 cocultures were 6.8-fold (OP9) and 29.3-fold (C3H10T1/2) (Figure 5(c)) while that of HSCs were 13.9-fold (OP9) and 8.3-fold (C3H10T1/2) (Figure 5(d)). On the other hand, maximum expansion of these cells in the control cultures was 4.3-fold in HPCs (Figure 5(c)) and 2.2-fold in HSCs (Figure 5(d)). These differences between cocultures and controls demonstrate the expansion advantages of coculturing HCs with stromal cells.

Figure 6 shows the relationship between culture densities and expansions of primitive HCs for which total cell densities on day 1 were chosen as indexes of culture density. It is clear that low-density cultures induced high expansion of these cells in the cocultures but high expansion was achieved in the high-density control cultures. In the OP9 or C3H10T1/2 cocultures, HSC expansion was comparable (Figure 6(b)) but HPC expansion was far better in C3H10T1/2 cocultures than OP9 cocultures (Figure 6(a)), suggesting that C3H10T1/2 cells are preferable for expanding primitive HCs in FLCs.

From these results, it was confirmed that coculturing FLCs with C3H10T1/2 stromal cells under low-density conditions is optimal for primitive HC expansion.

DISCUSSION

Ex vivo expansion of HSCs has largely been investigated by mimicking an *in vivo* hematopoietic microenvironment. This microenvironment is comprised of a 3D structure composed of many cell types, including HCs and stromal cells, that interact with each other to control HSC expansion and maintenance (26). With respect to stromal cells, diverse cell types derived from bone marrow, cord blood and Wharton jelly, as well as stromal cell lines, have been applied to these cocultures (9,21,27-29) and it has been revealed that these stromal cell types decisively influence HC expansion. This multi-pronged support, via secretion of soluble factors (stem cell factor [SCF], thrombopoietin [TPO], etc.) and direct contact between stromal cells and HSCs, is also important for HC expansion (7-10,30,31).

Under 3D culture conditions, cell-cell interactions are enhanced compared to conventional 2D cultures. Here, high density cultures induce augmentation of cell-cell interactions and cell contact inhibition simultaneously, creating opposing effects on cell growth. Thus, examining HC expansion under 3D coculture conditions with different HC densities was essential for optimizing HC expansion conditions to facilitate subsequent HSC transplantation. In this study, high HC expansion was obtained in FLC cocultures with stromal cells at low-seeding densities (Figures 5, 6) and cocultures with densities lower than 1 x 10^7 cells/cm^3 (D1) would be expected to induce a higher HC expansion than that obtained in this study. A similar effect of culture densities has also been reported in 3D cocultures of bone marrow-derived HCs with an ST2 stromal cell line (14). These results are thought to be caused by lowered contact inhibition in the low-density cultures (13).

Interestingly, an opposite effect of culture density on HC expansion was found in the control cultures (Figures 5, 6). Because FLCs were seeded as an HC source without further purification in this study, the FLCs were supposed to contain several types of cells, including

stromal cells. We speculate that scarce cell-cell interactions between HCs and the stromal cells present in the low-density control FLCs caused lowered HC expansion and this expansion was enhanced with more frequent cell-cell interaction by increasing culture density. In the cases of OP9 or C3H10T1/2 cocultures, the cell-cell interactions between HCs and stromal cell layers formed prior to the cocultures were considered to be sufficient and less contact inhibition in the low-density cocultures might have contributed to the high HC expansion we observed (Figures 5, 6) (13). These results strongly suggest the existence of stromal cells in FLCs as well as the importance of cell-cell interactions between HCs and stromal cells in HC expansion.

In previous studies, we applied three types of stromal cells (DAS 104-4 and DAS 104-8 stromal cell lines, in addition to bone marrow-derived stromal cells) to FLC cocultures (17,18). However, the performance of each stromal cell line (DAS 104-4 and DAS 104-8) in supporting HC expansion was unstable and gradually decreased with passaging. To examine the effects of culture density on HC expansion, usage of stromal cells with stable performances are indispensable (9). Thus, in this study, we used two stromal cell lines (OP9 and C3H10T1/2) that were expected to exhibit stable performance due to commercial availability and wide application as stromal cells (9,19,20).

Between these stromal cell lines, the ability to support HSC expansion was comparable but HPCs were far better in C3H10T1/2 than OP9 (Figures 5, 6). In the coculture experiments with C3H10T1/2 cells (Figure 4), rapid expansion of primitive HCs was detected during the first week, indicating that a coculturing period of only 1 week is sufficient for this stromal cell line. Furthermore, passaging cultures of C3H10T1/2 are easier than those of OP9 due to a higher growth rate and no decrease in cell performance with increasing passage

number was observed. From these facts, C3H10T1/2 is concluded to be superior over OP9 for HC expansions derived from FLCs, similar to a previous report in which these stromal cell lines were applied to induce HCs from human embryonic stem cells (19).

In general, HC expansion is strongly influenced by culture conditions, especially in stimulating factors used, and HSC expansions > 100-fold have been reported in 2-week cocultures with stromal cells bathed in cytokine cocktails (including SCF and TPO) (27,28,32). Coculture conditions used in this study were quite different from those in previous studies, and the highest expansion of HSCs in cocultures with C3H10T1/2 cells was 8.3-fold without exogenous cytokines. This value is lower than both previous reports and our previous 3D cocultures with DAS 104-8 stromal cells in which expansion of HSCs > 30-fold was achieved under the conditions similar to those in this study (17). However, the expansion in this study is arguably sufficient for UCB transplantation if comparable expansion of primitive HCs in UCB is achieved.

With respect to freeze-thawed stromal-cell TECs, HC expansion was far better in the FLC cocultures with these stromal-cell TECs than with TECs without freezing (17). In these cocultures, the growth of stromal cells in the TECs was obviously suppressed after thawing (18), suggesting that growth inhibition of stromal cells by TEC freezing contributed to high expansion of HCs similar to general inhibition treatments by γ-irradiation of anticancer reagents. On the other hand, in our previous study using fibroblast TECs, roughly 80% of viable cells were recovered after the freeze-thaw process and this value was comparable to that of control cryopreservation using suspended cells (23). In addition, we observed no morphological changes of FLC TECs before and after the freezing process (33). Therefore, we hypothesize that freezing TECs did not induce considerable decreases in viability or

morphological changes of stromal cells in the TECs. However, the possibility that stromal cells used in this study proliferated during the cocultures after thawing cannot be denied. Thus, comparison of HC expansion in the cocultures of FLCs with untreated, freeze-thawed, and γ-irradiated stromal cells will be required in addition to confirming growth of C3H10T1/2 and OP9 stromal cell lines within the TECs after thawing.

Within mouse FLCs, we observed $CD34^+$ cells as HSCs in line with our previous studies (17,18,24). Percentages of $CD34^+$ cells on day 1 in the control 3D cultures (5.2%, Figure 1) were lower compared with whole FLCs (13.9 ± 1.3%) reported on the same embryonic day (34) and these percentages were comparable to those on day 1 obtained in our previous study (17). Thus, the percentages were found to be reduced during the early stage of cultures up to day 1, including cell seeding.

Expression of CD34 on mouse HSCs is altered by aging and HSCs in mouse FLCs (embryonic days 12-18) have been confirmed to express CD34 (35,36), whereas HSCs in mouse adults do not express CD34 (37). Here, there is a possibility that CD34 expression on the HSCs changes temporally over the culture period in a manner similar to aging. To evaluate such effects of our expansion method with high accuracy, other mouse HSC markers are required in addition to CD34.

Several markers of mouse HSCs have been reported, including lineage-negative (Lin^-) c-$kit^+CD34^+Sca\text{-}1^+$ in mouse fetuses or neonates, and Lin^-c-$kit^+CD34^-Sca\text{-}1^+$ in mouse adults (35,36). $CD150^+CD48^-CD41^-$ is also reported to be a common HSC marker profile in adult bone marrow and fetal liver (7,31,37,38), making this combination of markers worth investigating. Here, high purification using multiple markers enhances HSC purity, but also reduces cell numbers in the HSC fraction. This reduction complicates evaluation of HSC

expansion, especially in the 3D cultures using porous polymer scaffolds, because sufficient recovery of 3D-cultured cells that are dispersed into single cells for FACS analysis is difficult due to possible formation of cell aggregates within the scaffolds (17). Thus, combinations of HSC markers suited for 3D cultures of mouse FLCs should be determined from the viewpoints of HSC purity and practical FACS analyses of cultured cells (39).

In the present study, we clarified that coculturing FLCs with stromal cells in the low-density cultures is optimal for expanding primitive HCs (Figures 5, 6). For clinical application to UCB transplantation, therefore, cocultures of HCs derived from human UCB with stromal cells in the low-density cultures might be effective to expand primitive HCs. However, usage of human stromal cell lines are likely to be problematic due to immunological issue, and stromal cell isolation from UCB is also difficult (40). These facts might prevent the establishment of useful coculture systems composed of UCB-derived HCs and stromal cells. In this case, solitary UCB cell cultures under high-density conditions will be advantageous for HC expansion. Investigations of optimal conditions for expansion of primitive HCs derived from UCB as well as 3D cultures of UCB cells at different densities are currently progressing in our laboratory.

In conclusion, we investigated the effects of FLC density and stromal cell types (C3H10T1/2 and OP9) on the expansion of primitive HCs in 3D cocultures. Cocultures of FLCs with stromal cells were effective to expand primitive HCs while C3H10T1/2 was superior over OP9 for HC expansion. The FLC densities strongly affected primitive HC expansion and the effect differed between cocultures and solitary FLC cultures. In the cocultures, higher expansion of the primitive HCs was achieved when FLCs were seeded at low cell densities whereas the expansion increased under high-density conditions in the

solitary FLC cultures. These results strongly suggest the existence of stromal cells in the FLCs and demonstrate that culture density is a pivotal factor for effective HC expansion.

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Figure Legends

Figure 1. Flow cytometry analysis of three-dimensional (3D) cultured fetal liver cells (FLCs) on day 1 (Control). Analyses stained by (A) control labeled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), (B) anti-Ter119 labeled with PE, (C) anti-B220 labeled with FITC, (D) anti-c-kit labeled with PE, and (E) anti-CD34 labeled with FITC antibodies.

Figure 2. Changes over time in total cell and hematopoietic cell (HC) numbers in 3D culture experiments of FLCs (Control). Mean ± standard deviation (SD) (n = 5-6). Open triangles represent 3D cultures of FLCs seeded at 1 x 10^7 cells/cm^3 (D1); closed squares represent 3D cultures of FLCs seeded at 2 x 10^7 cells/cm^3 (D2); closed circles represent 3D cultures of FLCs seeded at 1 x 10^8 cells/cm^3 (D10). Numbers of (A) total cells, (B) $Ter119^+$ erythriod cells, (C) $B220^+$ B cells, (D) $c\text{-}kit^+$ hematopoietic progenitor cells (HPCs) and (E) $CD34^+$ hematopoietic stem cells (HSCs). *$p < 0.05$ vs. day 1; $^{\dagger}p < 0.05$ vs. D1; $^{\ddagger}p < 0.05$ vs. D2.

Figure 3. Changes over time in total cell and HC numbers in 3D coculture experiments of FLCs with OP9 stromal cells. Mean ± SD (n = 3). Open triangles represent 3D cocultures of FLCs seeded at 1 x 10^7 cells/cm^3 (D1); closed squares represent 3D cocultures of FLCs seeded at 2 x 10^7 cells/cm^3 (D2); closed circles represent 3D cultures of FLCs seeded at 1 x 10^8 cells/cm^3 (D10). Numbers of (A) total cells, (B) $Ter119^+$ erythriod cells, (C) $B220^+$ B cells, (D) $c\text{-}kit^+$ HPCs and (E) $CD34^+$ HSCs. *$p < 0.05$ vs. day 1; $^{\dagger}p < 0.05$ vs. D1; $^{\ddagger}p < 0.05$ vs. D2; $^{\#}p < 0.05$ vs. control cultures.

Figure 4. Changes over time in total cell and HC numbers in 3D coculture experiments of FLCs with C3H10T1/2 stromal cells. Mean ± SD (n = 5). Open triangles represent 3D

cocultures of FLCs seeded at 1 x 10^7 cells/cm^3 (D1); closed squares represent 3D cocultures of FLCs seeded at 2 x 10^7 cells/cm^3 (D2); closed circles represent 3D cultures of FLCs seeded at 1 x 10^8 cells/cm^3 (D10). Numbers of (A) total cells, (B) $Ter119^+$ erythriod cells, (C) $B220^+$ B cells, (D) c-kit^+ HPCs and (E) $CD34^+$ HSCs. *$p < 0.05$ vs. day 1; $^{\dagger}p < 0.05$ vs. D1; $^{\ddagger}p < 0.05$ vs. D2; $^{\#}p < 0.05$ vs. control cultures; $^{\S}p < 0.05$ vs. cocultures with OP9 cells.

Figure 5. Expansion of HCs in 3D cocultures of FLCs with stromal cells. Mean ± SD. Hatched bars represent 3D cocultures and control cultures of FLCs seeded at 1 x 10^7 cells/cm^3 (D1); gray bars represent 3D cocultures and control cultures of FLCs seeded at 2 x 10^7 cells/cm^3 (D2); closed bars represent 3D cocultures and control cultures of FLCs seeded at 1 x 10^8 cells/cm^3 (D10). Expansions of (A) $Ter119^+$ erythroid cells, (B) $B220^+$ B cells, (C) c-kit^+ HPCs and (D) $CD34^+$ HSCs. The number of runs under each condition are the same as in Figures 1-3. $^{\dagger}p < 0.05$ vs. D1; $^{\ddagger}p < 0.05$ vs. D2; $^{\#}p < 0.05$ vs. control cultures; $^{\S}p < 0.05$ vs. cocultures with OP9 cells.

Figure 6. Relationship between culture density and expansion of primitive HCs. Mean ± SD. Open triangles represent 3D cultures of FLCs (control) (n = 5-6); closed squares represent 3D cocultures of FLCs with OP9 stromal cells (n = 3); closed circles represent 3D cocultures of FLCs with C3H10T1/2 cells (n = 5). Relationships between total cell densities on Day 1 and expansion of (A) c-kit^+ HPCs or (B) $CD34^+$ HSCs.

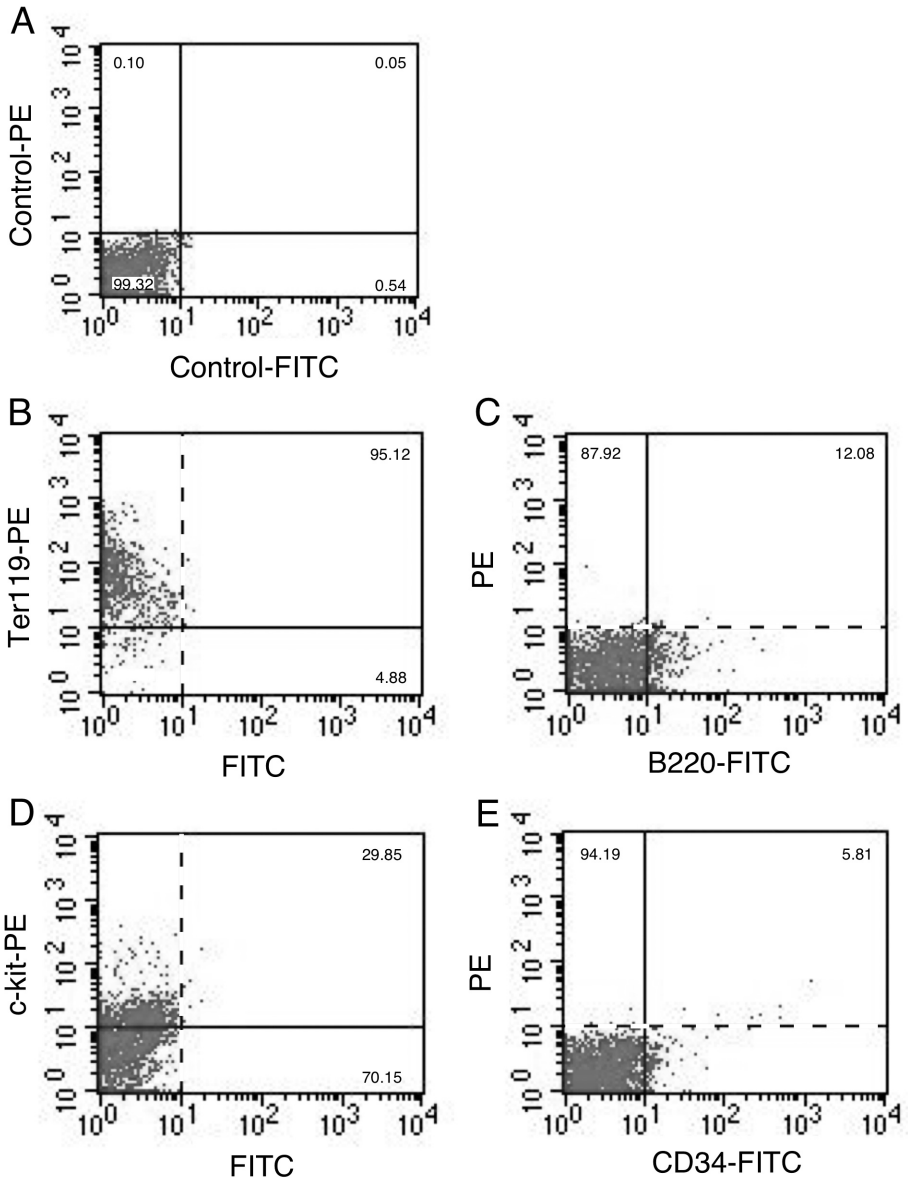
A
0.10
0.05
99.32
0.54
Control-PE
Control-FITC
B
95.12
4.88
Ter119-PE
FITC
C
87.92
12.08
PE
B220-FITC
D
29.85
70.15
c-kit-PE
FITC
E
94.19
5.81
PE
CD34-FITC

Figure 1

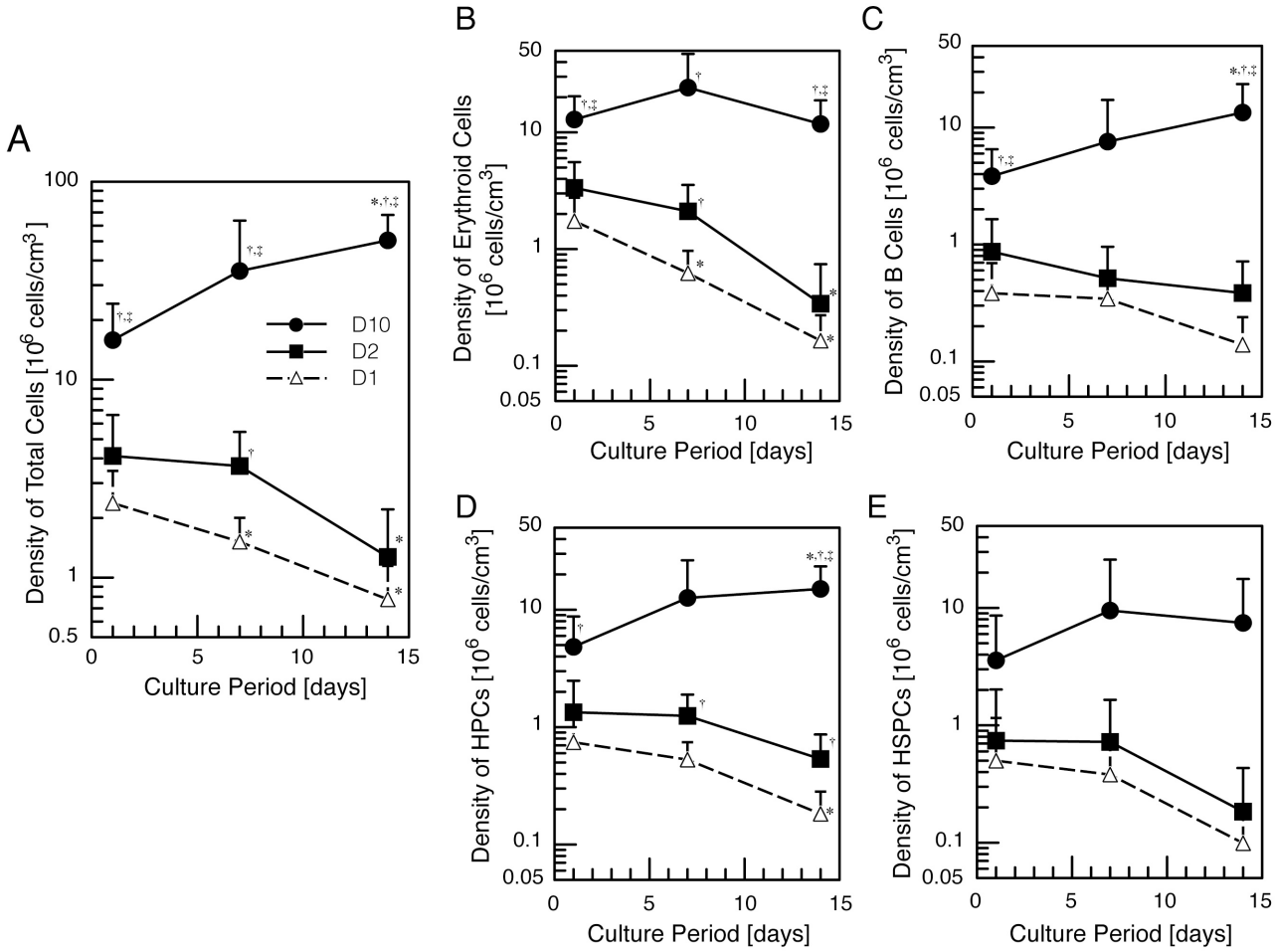
A
Density of Total Cells [10^6 cells/cm^3]
Culture Period [days]
D10
D2
D1
B
Density of Erythroid Cells [10^6 cells/cm^3]
Culture Period [days]
C
Density of B Cells [10^6 cells/cm^3]
Culture Period [days]
D
Density of HPCs [10^6 cells/cm^3]
Culture Period [days]
E
Density of HSPCs [10^6 cells/cm^3]
Culture Period [days]

Figure 2

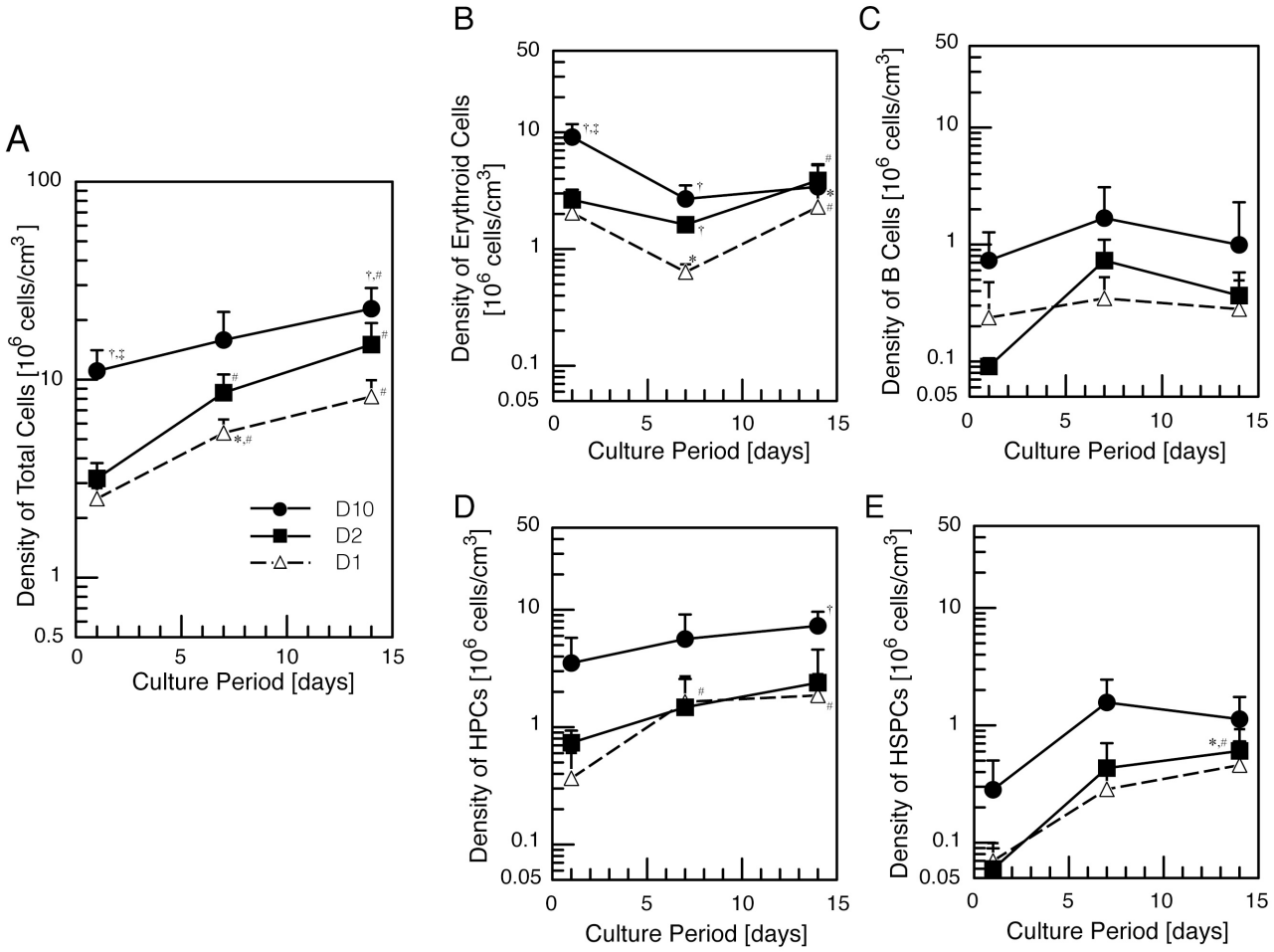
A
Density of Total Cells [10^6 cells/cm^3]
100
10
1
0.5
0
5
10
15
Culture Period [days]
D10
D2
D1
B
Density of Erythroid Cells [10^6 cells/cm^3]
50
10
1
0.1
0.05
Culture Period [days]
C
Density of B Cells [10^6 cells/cm^3]
Culture Period [days]
D
Density of HPCs [10^6 cells/cm^3]
Culture Period [days]
E
Density of HSPCs [10^6 cells/cm^3]
Culture Period [days]

Figure 3

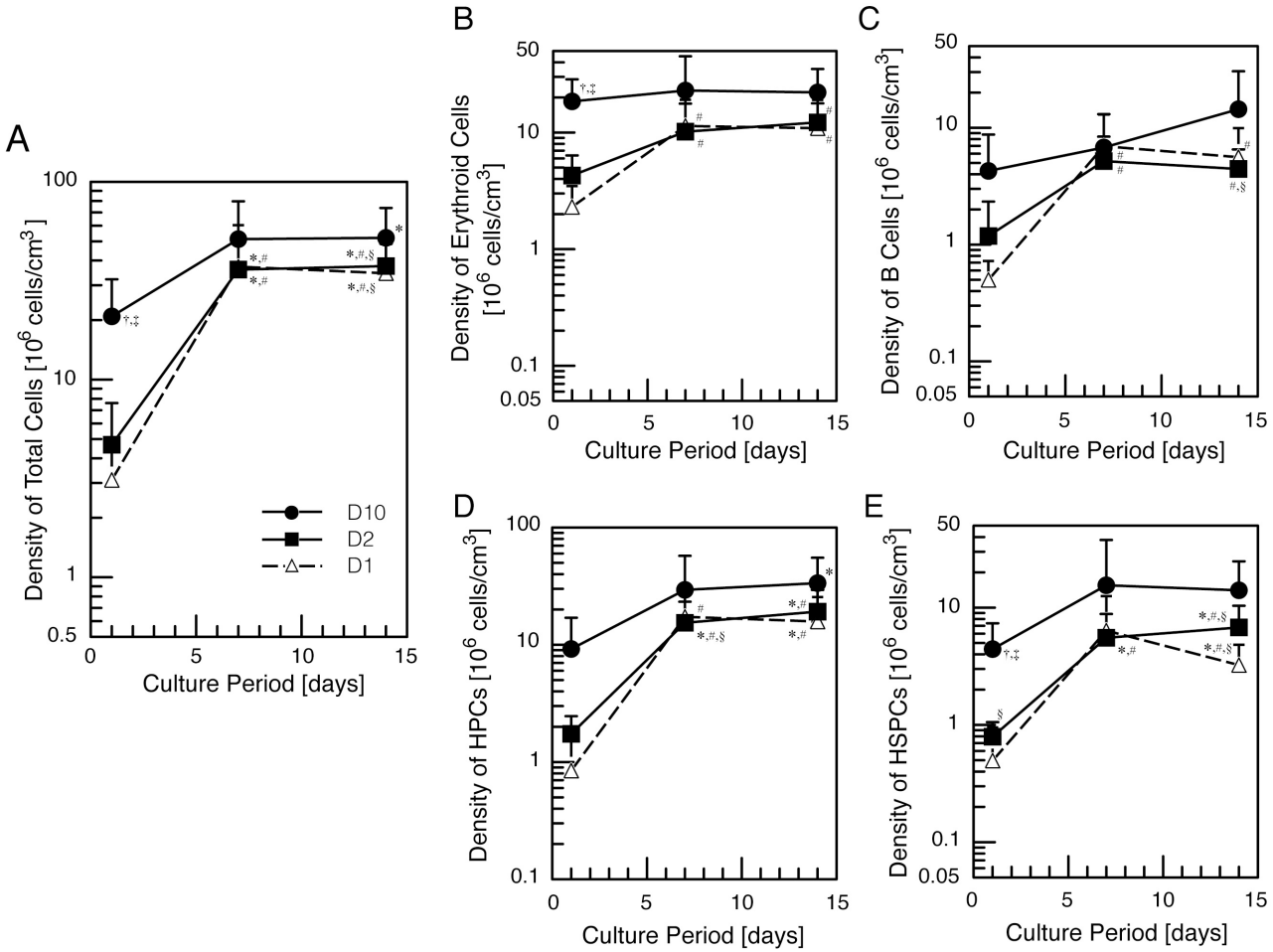
A
Density of Total Cells [10^6 cells/cm^3]
Culture Period [days]
D10
D2
D1
B
Density of Erythroid Cells [10^6 cells/cm^3]
Culture Period [days]
C
Density of B Cells [10^6 cells/cm^3]
Culture Period [days]
D
Density of HPCs [10^6 cells/cm^3]
Culture Period [days]
E
Density of HSPCs [10^6 cells/cm^3]
Culture Period [days]

Figure 4

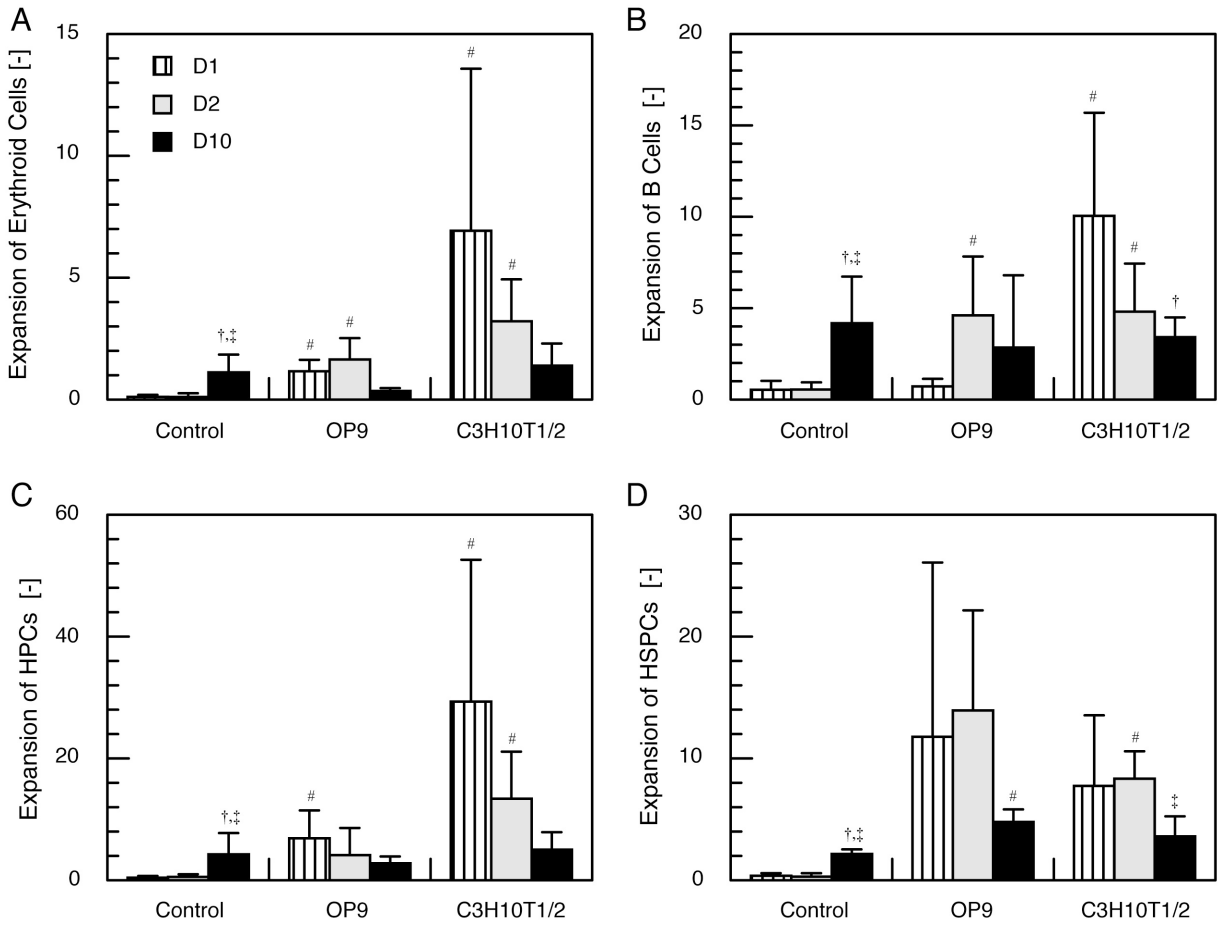
A
Expansion of Erythroid Cells [-]
15
10
5
0
D1
D2
D10
†,‡
#
#
#
#
Control
OP9
C3H10T1/2
B
Expansion of B Cells [-]
20
15
10
5
0
†,‡
#
#
#
†
Control
OP9
C3H10T1/2
C
Expansion of HPCs [-]
60
40
20
0
†,‡
#
#
#
Control
OP9
C3H10T1/2
D
Expansion of HSPCs [-]
30
20
10
0
†,‡
#
#
#
‡
Control
OP9
C3H10T1/2

Figure 5

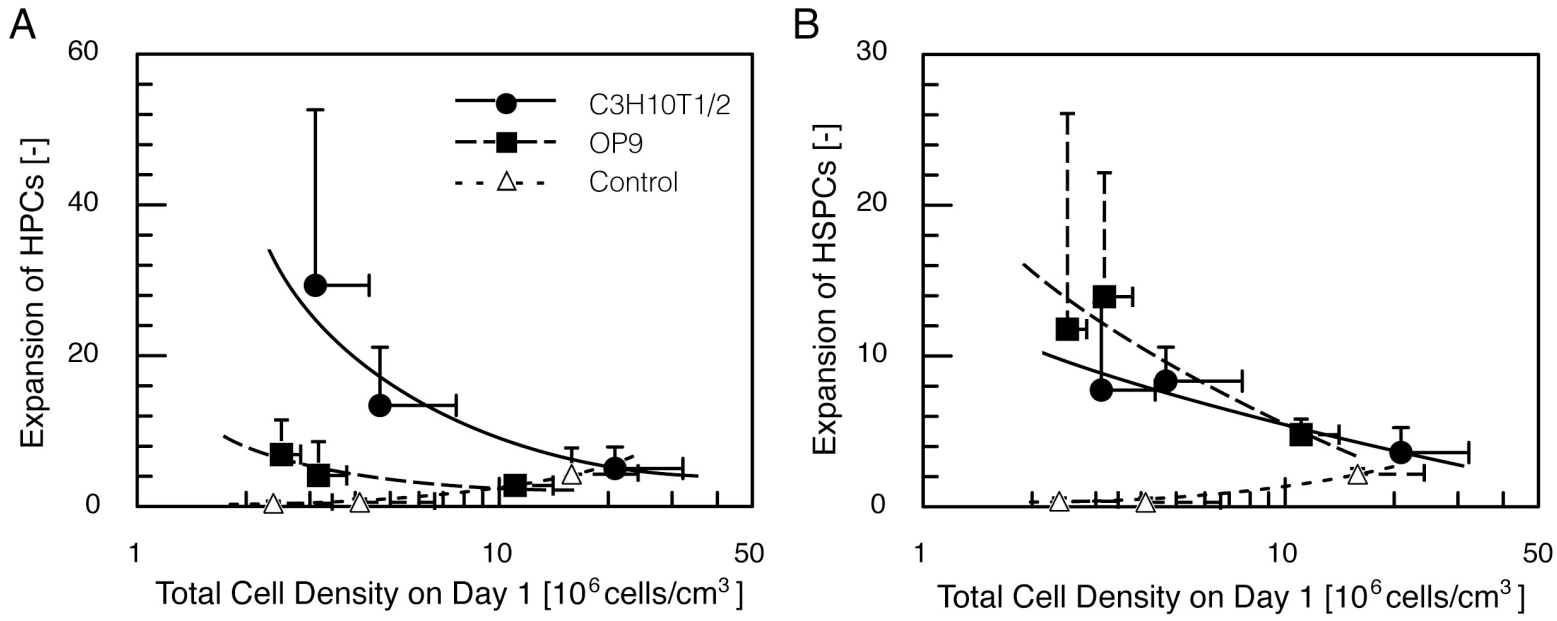
A
B
C3H10T1/2
OP9
Control
Expansion of HPCs [-]
Expansion of HSPCs [-]
Total Cell Density on Day 1 [10^6 cells/cm^3]
Total Cell Density on Day 1 [10^6 cells/cm^3]
0
20
40
60
10
30
1
10
50

Figure 6