International Journal of Hematology

A portable platform for stepwise hematopoiesis from human pluripotent stem cells within PET-reinforced collagen sponges

--Manuscript Draft--

Manuscript Number:	IJHM-D-15-00940R2
Full Title:	A portable platform for stepwise hematopoiesis from human pluripotent stem cells within PET-reinforced collagen sponges
Article Type:	Original Article
Section/Category:	Japan
Corresponding Author:	Megumu K. Saito Kyoto University JAPAN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Kyoto University
Corresponding Author's Secondary Institution:	
First Author:	Yoshinori Sugimine
First Author Secondary Information:	
Order of Authors:	Yoshinori Sugimine
	Akira Niwa
	Hiroyuki Matsubara
	Kazuki Kobayashi
	Yasuhiko Tabata
	Toshio Heike
	Tatsutoshi Nakahata
	Megumu K. Saito
Order of Authors Secondary Information:	

Funding Information:		
	Japan Agency for Medical Research and Development	Dr. Tatsutoshi Nakahata Dr. Megumu K. Saito
	Japan Society for the Promotion of Science	Dr. Tatsutoshi Nakahata Dr. Megumu K. Saito
	Japan Society for the Promotion of Science (JP25860856)	Dr. Akira Niwa

Abstract: Various systems for differentiating hematopoietic cells from human pluripotent stem cells (PSCs) have been developed, although none have been fully optimized. In this report, we describe the development of a novel three-dimensional system for differentiating hematopoietic cells from PSCs using collagen sponges (CSs) reinforced with poly (ethylene terephthalate) (PET) fibers as a scaffold. PSCs seeded onto CSs were differentiated in a stepwise manner with appropriate cytokines under serum-free and feeder-free conditions. This process yielded several lineages of floating hematopoietic cells repeatedly for more than one month. On immunohistochemical staining, we detected CD34+ cells and CD45+ cells in the surface and cavities of the CS. Taking advantage of the portability of this system, we were able to culture multiple CSs together floating in medium, making it possible to harvest large numbers of hematopoietic cells repeatedly. Given these findings, we suggest that this novel three-dimensional culture system may be useful in the large-scale culture of PSC-derived

	hematopoietic cells.
Response to Reviewers:	N/A
Additional Information:	
Question	**Response**
Is your submission applicable to any of cases below? (Please refer to the latest *IJH* Information for Authors) 1) “Duplicate Publication” which has been published previously or currently under consideration for publication elsewhere. 2) “Secondary Publication” which is based on material published in other journals or online.	No If Yes, please provide an explanation below. ------

Original article

Title

A portable platform for stepwise hematopoiesis from human pluripotent stem cells within PET-reinforced collagen sponges

Short title (45 letters including space)

PSC-derived hematopoiesis in collagen sponges

Authors

Yoshinori Sugimine[1,2,4], Akira Niwa[1,4], Hiroyuki Matsubara[1], Kazuki Kobayashi[1], Yasuhiko Tabata[3], Toshio Heike[2], Tatsutoshi Nakahata[1] and Megumu K. Saito[1,*]

Affiliations

[1]Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, 6068507, Japan

[2]Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, 6068507, Japan

[3]Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, 6068507, Japan

[4]These authors contributed equally to this work.

***Correspondence**

Megumu K. Saito

Address: 53 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.

E-mail: msaito@cira.kyoto-u.ac.jp

Phone: +81 75-366-7089 FAX: +81 75-366-7088

Abstract

Various systems for differentiating hematopoietic cells from human pluripotent stem cells (PSCs) have been developed, although none have been fully optimized. In this report, we describe the development of a novel three-dimensional system for differentiating hematopoietic cells from PSCs using collagen sponges (CSs) reinforced with poly (ethylene terephthalate) (PET) fibers as a scaffold. PSCs seeded onto CSs were differentiated in a stepwise manner with appropriate cytokines under serum-free and feeder-free conditions. This process yielded several lineages of floating hematopoietic cells repeatedly for more than one month. On immunohistochemical staining, we detected CD34+ cells and CD45+ cells in the surface and cavities of the CS. Taking advantage of the portability of this system, we were able to culture multiple CSs together floating in medium, making it possible to harvest large numbers of hematopoietic cells repeatedly. Given these findings, we suggest that this novel three-dimensional culture system may be useful in the large-scale culture of PSC-derived hematopoietic cells.

Keywords

Human embryonic stem cells; human induced pluripotent stem cells; collagen sponge; hematopoietic differentiation; hematopoietic progenitor cells

MAIN TEXT (4,501 words, not including references and figure legends)

Introduction

Recent advances in the field of stem cell biology have opened up possibilities of applying human pluripotent stem cells (PSCs) for regenerative medicine and disease analysis. Induced pluripotent stem cells (iPSCs) are PSCs that can be established from somatic cells from individuals, and therefore are useful for personalized medicine, such as cell transplantation and disease-specific iPSC studies [1-3]. For these purposes, it is necessary to establish differentiation systems which can obtain a large number of highly purified, terminally differentiated cells, and such strategies are required for various cell types.

Various systems for differentiating hematopoietic cells from PSCs have been developed, such as three-dimensional (3D) cultures formed as embryoid body[4-7], two-dimensional (2D) cultures with feeder cells[8-15], 2D feeder-free systems[16-18] and so on. Since each system has its own advantages and disadvantages, no single optimal culture system has been obtained to date. An important fact regarding any culture system is that non-hematopoietic components composed by extracellular matrices and supporting cells are required to maintain the hematopoietic progenitors in the differentiation system [19, 20]. It is known that various supporting cells, such as osteoblasts[21, 22], vascular

endothelial cells [23, 24], stromal cells[25, 26], megakaryocytes[27-29] and neural cells[30, 31], have a key role in generating the hematopoietic cells from the aorta-gonad-mesonephros region[32-34] and bone marrow *in vivo*. The hematopoietic niche plays a major role in the maintaining hematopoietic stem/progenitor cells (HSPCs) and retaining their differentiation capacity by producing cytokines and extracellular matrix molecules, as well as by facilitating signal communication through cell adhesion[20, 35, 36].

Systems for the hematopoietic cell differentiation from PSCs are considered to give rise to both hematopoietic cells and the cells constituting the *in vitro* niche in the same dish [37, 38]. Naturally, most PSC-derived hematopoietic inductions without existing stromal cells contain simultaneous hematopoietic and niche-composing cell development in the same dish [37, 38]. Amongst them, our recently established 2D feeder-free culture has brought about robust and orderly hematopoietic cell derivation from PSCs without any serum or animal-derived stromal cells [16, 17]. Only by stepwise tuning of cytokine cocktails, PSCs automatically differentiate into both functional blood and other lineage cells as autologous planar microenvironment for hematopoietic progenitor cells (HPCs), resulting in sustainable HPC and mature blood cell production [16, 17]. Therefore, we considered that an *in vitro* 3D hematopoietic niche comprising scaffolds and supporting cells could be constructed by applying our 2D-hematopoietic

differentiation system.

In this report, we developed a novel hematopoietic differentiation system from PSCs using collagen sponges (CSs) reinforced by the incorporation of poly(ethylene terephthalate) (PET) fibers as a 3D-scaffold. CSs enabled the production of an *in vitro* hematopoietic microenvironment comprising hematopoietic progenitors, supporting cells and extracellular structures. Since those 3D-scaffolds are portable and facilitate the recovery of hematopoietic cells, they can be useful for the large-scale production of PSC-derived hematopoietic cells applicable for transplantation or a disease analysis.

Material and Methods

Study ethics

The methods were carried out in accordance with the declaration of Helsinki and the Japanese guidelines on the use of human embryonic stem cells. The study plan using human embryonic stem cells (ESCs) was approved by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Preparation of collagen sponges

Collagen sponges mechanically reinforced by the incorporation of poly(ethylene terephthalate) (PET) fibers (PETCol-24w, hereafter referred to as CSs) were purchased from MedGEL (Tokyo, Japan)[39]. In order to remove air bubbles, six CSs were put in a 50 mL conical tube (Becton-Dickinson, Franklin Lakes, USA) containing 10 mL of maintenance medium for PSCs, and were centrifuged at 10000 rpm for five minutes. After centrifugation, the CSs were picked up with a spatula, and placed onto 24-well plates (**Fig. 1**).

Cytokines used for culture

Recombinant human basic fibroblast growth factor (bFGF) was purchased from Wako

Pure Chemical Industries, Ltd. (Osaka, Japan). The following cytokines were purchased from R&D Systems (Minneapolis, USA): BMP-4, VEGF, SCF, IL-3, Thrombopoietin (TPO), Flt-3 Ligand (FL3), GM-CSF and M-CSF. Recombinant human erythropoietin (EPO) was purchased from EMD Biosciences (San Diego, USA).

Maintenance of human PSCs

This study used human ESCs (cell line: KhES1) and iPSCs (cell lines: 201B7, 409B2 and CB-A11). The human ES cell line, KhES1, was kindly provided by Dr. Norio Nakatsuji. Human iPS cell lines 201B7 [2] and 409B2 [1] were kindly provided by Dr. Shinya Yamanaka. CB-A11 is an iPSC clone established from cord-blood hematopoietic cells [17]. These PSCs were maintained on mitotically-inactivated SNL feeder cells with Primate ES Cell Medium (ReproCELL, Kanagawa, Japan) supplemented with 5 ng/mL bFGF, or tissue culture dishes coated with growth factor-reduced Matrigel (Becton-Dickinson, Franklin Lakes, USA) in mTeSR1 serum-free medium (STEMCELL Technologies, Vancouver, Canada). The medium was replaced every day.

Seeding of clumped PSCs onto CSs

PSCs were treated with CTK solution consisting of 0.1 mg/mL collagenase IV (Invitrogen,

Tokyo, Japan), 0.25% trypsin (Invitrogen, Tokyo, Japan), 0.1 mM CaCl2 (Nacalai tesque, Kyoto, Japan) and 20% KSR for one minute at room temperature, washed twice with phosphate-buffered saline (PBS) and detached with a scraper after adding 1.5 mL of mTeSR1 medium. They were then transferred into a 15 mL conical tube (Becton-Dickinson, Franklin Lakes, USA) and dissociated by pipetting. The tube was left standing for one minute to precipitate the clumped PSCs. Thereafter, the supernatant and small cell-clumps were removed and the clumps were recovered. Clumps in 300-500 μm diameter are seeded onto CSs in 24-well plates (**Fig. 1**). By matching the size of CS and culture well, clumps of PSCs could be surely distributed on CS.

Seeding of single-cell suspensions PSCs onto CSs

PSCs were treated with CTK solution for one minute at room temperature, washed twice with PBS, treated with 1 mL of Accumax (Innovative Cell Technologies, Inc., San Diego, USA) for 10 minutes at 37°C, dissociated with a 1000 μL pipette tip and transferred into a 15 mL conical tube containing 9 mL of PBS. Cells were washed twice in order to remove the collagenase contained within Accumax, then were centrifuged at 1500 rpm for five minutes at room temperature. The cellular pellet was suspended with 500-1000 μL of mTeSR1 containing 10 μM of Y27632 (Abcam, Tokyo, Japan). A 50 μL aliquot of the

cell suspension containing the indicated numbers of PSCs was gently dripped on the center of a CS. One milliliter of mTeSR1 medium was added the next day, and cells were cultured one extra day before starting the differentiation.

Hematopoietic cell differentiation

At the start of differentiation (day 0), CSs were transferred into 12-well plates. Thereafter, CSs were transferred into another 12-well plates with appropriate cytokine-containing medium on days depicted (**Fig. 1**). The CSs were cultured in the same wells until floating hematopoietic cells emerged. The media and cytokines were used as described previously [16, 17]. In brief, BMP4 (80 ng/mL) was added to the mTeSR1 medium from day 0 through day 4 in order to induce primitive streak cell. Then, the mTeSR1 medium was replaced by StemPro-34 serum-free medium (Gibco, Tokyo, Japan) containing 2 mM GlutaMAX (Invitrogen, Tokyo, Japan) on day 4, which was supplemented with a cytokine cocktail composed of VEGF (80 ng/mL), basic FGF (25 ng/mL) and SCF (100 ng/mL). On day 6, the medium were replaced with StemPro-34 serum-free medium containing different cytokines to induce specific hematopoietic lineages. For myeloid cell induction, the culture medium contained with SCF (50 ng/mL), IL-3 (50 ng/mL), TPO (5 ng/mL) and FL3 (50 ng/mL) was changed every four days. G-CSF (10 ng/mL) was added from

day 14 to 25 when phagocytosis assay was performed. For monocytic cell induction, the culture medium contained with SCF (50 ng/mL), IL-3 (50 ng/mL), TPO (Thrombopoietin) (5 ng/mL), M-CSF (50 ng/mL) and Flt-3 ligand (50 ng/mL) was changed on day 10. On day 14, the medium was replaced with a cytokine cocktail containing Flt-3 ligand (50 ng/mL), GM-CSF (25 ng/mL) and M-CSF (50 ng/mL) and changed every four days. For erythroid cell induction, the culture medium contained with EPO (5 IU/mL), IL-3 (50 ng/mL) and SCF (50 ng/mL), was changed every two days.

Recovery of hematopoietic cells from CSs

To recover adherent cells from the CSs on day 6, the CSs were placed along the lateral wall of a 50 mL conical tube, then centrifuged at 7000 rpm for 10 seconds in order to drain off the medium from the CS. Then, 1 mL of Accumax was added to the CSs. After incubation for 10 minutes at 37°C, 9 mL of PBS was added, and the samples were mixed well. The CSs were removed from the tubes after being squeezed with a spatula, and the tubes were centrifuged at 1500 rpm for five minutes to obtain a cellular pellet. To recover floating cells, CSs were transferred into another well containing fresh culture medium. The leftover media were recovered and centrifuged at 1500 rpm for five minutes to obtain a cellular pellet. Floating hematopoietic cells could usually be obtained every five days.

Phagocytosis assay

Phagocytosis was detected using chemiluminescent microspheres (luminol-binding carboxyl hydrophilic microspheres; TORAY, Tokyo, Japan), as previously described [16]. Briefly, 2×10^4 floating cells on day 25 were suspended in 50 ml of the reaction buffer (HBSS containing 20 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid [HEPES]) per tube. To activate the system, 5 ml of chemiluminescent microspheres was added, and light emission was recorded continuously. During the measurement, the samples were kept at 37 °C. To inhibit phagocytosis, 1.75 mg of cytochalasin B (Sigma-Aldrich) was added to the sample. Chemiluminescence from the microspheres was detected using an EnVision plate reader (PerkinElmer).

Scanning electron microscopy (SEM)

CSs were fixed with 4% paraformaldehyde and 2% glutaraldehyde overnight at 4°C. After post-fixation with 1% OsO4 for three hours, the CSs were dehydrated, dried and coated with a thin layer of platinum palladium. The specimens were examined with a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan).

Culture of multiple CSs in a flask

Twelve CSs were transferred together into a 50 mL tissue culture flask (Becton-Dickinson, Franklin Lakes, USA) containing 25 mL of culture medium on differentiation day 18. To recover the floating cells, the flask was gently shaken, and the cultured medium containing floating cells was recovered. Subsequently, the media was centrifuged, and the cellular pellet was suspended in fresh medium.

Flow cytometric analysis

The data from the flow cytometric analysis were collected using the MACS Quant™ Analyzer (Miltenyi Biotec, Bergisch-Gladbach, Germany) and analyzed utilizing the FlowJo software package (Tree Star Inc., Ashland, USA). The following antibodies were purchased from BD Biosciences (San Jose, USA): CD34-PE (clone: 581), CD43-APC (clone: 1G10), CD45-PE (clone: HI30), CD71-APC (clone: L01.1), CD235a-PE (clone: GA-R2 (HIR2), and CD271-FITC (clone:C40-1457). CD14-APC (clone: RMO52) antibody was purchased from Beckman Coulter (Brea, USA). KDR (CD309)-Alexa fluor 647 (clone: HKDR-1) and VE-Cadherin (CD144)-PE (clone:BV9) were purchased from Biolegend (San Diego, USA).

May-Giemsa staining

Cells were seeded onto glass slides using CYTOSPIN 4 (Thermo Scientific, Waltham, USA) and were stained with May-Grünwald and Giemsa staining solution (MERCK, Darmstadt, Germany) following the manufacturer's instructions.

RNA preparation and real-time quantitative PCR

RNA samples were prepared using silica gel membrane-based spin-columns (RNeasy Mini-Kit; Qiagen, Valencia, CA) and subjected to reverse transcription using random hexamer primers (Life Technologies, Tokyo, Japan) with a Sensiscript-RT Kit (Qiagen, Valencia, CA). All procedures were performed according to the manufacturer's instructions. For real-time quantitative PCR, TaqMan Gene Expression Assays system (Applied Biosystems, Carlsbad, CA) was used: ZFP42 (Hs01938187_s1), Nanog (Hs04260366_g1), T (Hs00610080_m1), APLNR (Hs02597618_s1), MIXL1 (Hs00430824_g1), CDH5 (Hs00901463_m1), RUNX1 (Hs02558380_s1) and GAPDH (Hs00266705_g1) as internal control. The instrument used was StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Carlsbad, CA).

Histological analysis of cells in CSs

Cultured CSs were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan.) after media were absorbed in paper towel. After freezing in −80°C overnight, sections were obtained with cryostat microtmes. Samples were examined with hematoxylin and eosin and Masson's trichrome stains. Anti-human CD34 (clone: QBEnd/10, Novocastra Laboratories, Newcastle, U.K.) and anti-human CD45 (clone: 2B11+PD7/26, DAKO, Glostrup, Denmark) murine antibodies were used for detecting cells in CSs**.** DAB staining was performed with VECTASTAIN ABC kit. (VECTOR, Burlingame, CA) according to the manufacturer's protocol.

Colony formation assay

At the indicated days of culture, cells were recovered from CSs as described above (see **Recovery of hematopoietic cells from CSs** section). After recovery, the cells were resuspended with 10 mL medium and incubated in another tissue-culture dish (#3003, Becton–Dickinson) for 10 min to eliminate adherent non-hematopoietic cells [40]. Floating cells were collected and dispersed by 40-micrometer strainers. After dead cells were eliminated by labeling with Dead-Cert Nanoparticles (#DC-001, ImmunoSolv, Edinburgh, UK), live hematopoietic cells were cultured at a concentration of 1×10^3 (for counting CFU-G) or 1×10^4 (for counting CFU-Mix, BFU-E, and CFU-GM) cells/ml in

35-mm petri dishes (#1008; Becton-Dickinson) using 1 ml/dish of MethoCult GF+ semisolid medium (#4435; STEM- CELL Technologies) as previously described. Colonies were counted after 14–21 days of incubation, and colony types were determined according to the criteria described previously [41-43] by in situ observation using an inverted microscope. The abbreviations used for the clonogenic progenitor cells are as follows: CFU-Mix, mixed colony-forming units; BFU-E, erythroid burst- forming units; CFU-GM, granulocyte–macrophage colony-forming units; and CFU-G, granulocyte colony-forming units.

Results

Induction of mesodermal progenitor cells (MPCs) on CSs from clumps of undifferentiated PSCs

We previously reported robust hematopoietic differentiation protocols from human PSCs using 2D feeder-free culture system on Matrigel-coated dishes [16, 17]. Therefore, we first evaluated whether the principle of these 2D feeder-free methods could be applied to hematopoietic differentiation on CSs. We seeded 20 undifferentiated PSC clumps of 300-500 μm in diameter, containing 2.5 x 10^4 ± 0.39 x 10^4 cells in total, onto each CS and incubated overnight in maintenance medium (**Figs. 1a and 1b**) before starting differentiation. Scanning electron microscopy (SEM) imaging confirmed that clumps of PSCs (cell line: KhES1) adhered onto collagen and PET fibers (**Fig. 2a**). For initial differentiation, BMP4 for first 4 days and VEGF, SCF and bFGF for subsequent 2 days were added to induce KDR+CD34+ MPCs (**Fig. 2b**). We obtained this population from all tested PSC clones (cell lines: KhES1, 201B7, 402B2, and CB-A11) at day 6 (**Fig. 2c**) with comparable efficacy to the previously reported monolayer protocol [16, 17], though the frequency varied among clones. These results demonstrated that CSs can let PSCs follow the directed differentiation into MPCs in 3D condition.

We next evaluated the expression of genes specifically expressed PSCs (cell

lines: KhES1 and CB-A11) or MPCs. While PSC-specific genes such as ZFP42[44] and NANOG[44] were almost undetectable, genes specifically expressed in mesodermal progenitors (T[16] and MIXL1[16]), hematopoietic progenitors (RUNX1[45]) and endothelial progenitors (APLNR[46] and CDH5[47]) were upregulated in day 6 progenitors (**Figs. 3a and 3b**). These data confirmed that, in the present system, cells were driven into hemoangiogenic mesodermal progenitors and indicated that the remaining undifferentiated cells were minimal.

Production and evaluation of hematopoietic cells in CSs

Our previous study has shown that KDR+CD34+ fraction includes cells harboring differentiation potential into hematopoietic cells[16]. Therefore, we next tried inducing hematopoietic lineages by switching cytokines to hematopoietic cocktail (**Fig. 4a**). With the myeloid differentiation setting, nearly 100% of floating living cells showed hematopoietic-cell marker CD43+CD45+ (**Fig. 4b**) and some of them exhibited lobular neutrophil-like or foamy macrophage-like morphology (**Fig. 4c**). The floating hematopoietic cells spontaneously emerged from CSs out into medium approximately from day 20 to 50 (**Fig. 4d**). During the culture period, we were able to repeatedly recover hematopoietic cells every five days, simply by collecting the media containing floating

cells (**Fig. 4d**). Since approximately half a million hematopoietic cells could be obtained during each recovery, our CS method repeatedly yielded 20 hematopoietic cells for each initial PSC. Modifying this setting with additional G-CSF stimulation induced the production of functional myeloid and neutrophic cells that successfully showed the phagocytic activity (**Fig. 4e**). In contrast, with the monocytic differentiation setting, recovered living cells are mostly $CD14^{+}CD45^{+}$, and showed foamy monocyte/ macrophage-like appearance (**Figs. 4f and 4g**). Finally, with the erythroid differentiation setting, $CD71^{+}CD235a^{+}$ erythroid cells were obtained (**Fig. 4h**). These cells had basophilic cytoplasm with high N/C ratio, compatible to erythroid progenitor cells (**Fig. 4i**). We dissociated cells adhered onto CSs and found that $CD34^{+}$ progenitor and $CD45^{+}$ hematopoietic cell populations were indeed cultivated (**Fig. 4j**). $CD34^{+}$ progenitors sustainably remained inside the CS for longer than 4 weeks (**Fig. 4k**), and, more intriguingly, non-hematopoietic cells positive for the endothelial marker VE-cadherin or the mesenchymal stem/stromal cell marker CD271 continued to be observed throughout the culture period (**Figs.4l and 4m**).

To evaluate the hematopoietic outcome in this system, we next performed a sequential colony forming assay, and found that the CS system harbors colony forming cells until day 30 (**Fig. 4n**). The clonogenicity of the produced cells was skewed to

myeloid clones. We consider this is consistent with the finding that our differentiation condition predominantly produces myeloid cells without erythropoietin. To exclude the reactivation of transgenes integrated in iPSCs, we tracked the expression pattern of reprograming factors during hematopoietic differentiation and confirmed that there was no aberrant expression of transgenes (OCT4, SOX2, KLF4, MYCN) (**Fig. 5**).

All data described above showed that CS can support stepwise differentiation of clumped PSCs into hematopoietic lineages. More remarkably, because all medium changes are achieved just by transferring CSs into another well contained with fresh medium every 2-5 day, hematopoietic cells in this method can be very easily and repetitively recovered by harvesting cultured medium.

Hematopoietic differentiation on CSs from single-cell suspensions of PSCs on CSs.

Since the average porous size of a CS is approximately 200 μm [39], clumps of PSCs are considered not to infiltrate into the CS. This implies that the clumps adhered onto the CSs. The cells then began to differentiate on the surface of the CSs, and some cells subsequently infiltrated into the CSs and formed three-dimensional structures (see **Fig. 7**). To take advantage of the CS's three-dimensional structure, we next tried to start initial differentiation inside of CS. For, this, we evaluated whether the CSs can support the

hematopoietic differentiation of PSCs dissociated into single cells (see **Fig. 1a**). We enzymatically dissociated PSCs and prepared single cell suspensions. We confirmed the attachment of individual cells onto collagen sheets when the suspension was dripped onto CSs in 24-well plates (**Fig. 6a**). After overnight incubation with maintenance medium for undifferentiated PSCs, the CSs were transferred into 12-well plates containing differentiation medium. Thereafter, the differentiation proceeded in a similar fashion as was observed for the way to clumped PSCs. As a result, KDR+CD34+ progenitors emerged on day 6 (**Fig. 6b**), as same as clumped PSCs. To determine the optimal cell number of undifferentiated PSCs per one CS, we tested several starting cell numbers. As shown in **Figs. 6c-6e**, 1×10^5 of KhES1 cells per one CS yielded the largest number of KDR+CD34+ fraction, but the optimal seeding number of PSCs varied in each PSC clone (data not shown). Similarly to the clumped PSC method, floating hematopoietic cells emerged from the CSs in culture medium from approximately day 20. In the presence of a myeloid cytokine cocktail, these floating living cells showed myeloid-like surface markers and morphology (**Figs. 6f and 6g**).

Topographical characterization of hematopoietic cells in CSs

In order to specify the identity of the cellular components in the CS, we next performed

immunohistochemical staining of the CS (**Fig. 7a**). We found that the CD34+ cells and collagen fibers form a sac-like structure, and the CD45+ hematopoietic cells are found into the cavity of the CS (**Figs. 7b and 7c**). We also evaluated the cell-cell interactions and topographical characterization of the hematopoietic cells and microenvironment-comprising cells on the scaffold using SEM. As expected, collagen fibers were degraded during differentiation due to the digestion by cellular collagenase, while the 3-dimensional structures were maintained by PET fibers (**Figs. 8a and 8b**). When clumped PSCs were differentiated, globular hematopoietic-like cells were attached on the sheet-like cells bridging PET fibers (**Fig. 8c**). Overall, the immunohistology and SEM imaging characterized the *in vitro* hematopoietic microenvironment on CSs, which were composed of PET fibers, hematopoietic cells and supporting non-hematopoietic cells, with a close positional relationship. Interestingly, these components were corresponds to previously described hematopoietic microenvironment developed *in vitro* [48].

Hematopoietic differentiation from multiple CSs in a single flask

Major advantages of current CS method are its portability, scalability and simplicity. Conventional 2D-methods generally require numbers of dishes for large-scale culture because of their own narrow ranges in optimal cell density. On the other hands, embryonic

body culture is easily portable and expandable through a floating condition. However, a hemangioblastic progenitor, an origin of HSPCs, has been reported to emerge only in very limited vascular endothelial spaces inside self-organized structures. As a result, harvesting hematopoietic cells usually requires dissociation or reseeding of embryonic bodies [5, 7]. Our method enables directed specification into hematopoietic lineage via mesoderm and blood harvest only by collecting medium. To validate the possibility of our CS method for productive large-scale culture, we next collected 12 CSs together after initial differentiation from clumped PSCs and cultured in a 50 mL flask containing the medium with myeloid cytokine cocktail (**Fig. 9a**). Thereafter, CSs were cultured in floating condition, and hematopoietic cells were recovered each time of changing medium. As shown in **Fig. 9b**, approximately one to three million cells were harvested at time repetitively over 2 weeks from 12 CSs. Since each of CS initially contained $2.5x10^4$ PSCs, the yield of hematopoietic cells was about three to 10 cells per one PSC at each recovery. Recovered cells were positive for CD43 and CD45 and included morphologically immature cells (**Figs. 9c and 9d**). Although further optimization of the culture conditions with multiple CSs is necessary, our portable CS niche harboring hematopoietic cells provides a promising platform for large-scale derivation of hematopoietic cells from PSCs in the future.

Discussion

Since collagen is one of the main components of the extracellular matrix, which facilitates cell adhesion and proliferation, three-dimensional collagen-based scaffolds are widely used for *in vitro* cell culture. Type I collagen, a major component of bone marrow niche, has become used more to incubate various blood cells such as cord blood-derived mononuclear cells and leukemic cells [49, 50]. However, long-term *in vitro* culture with collagen-based scaffolds results in their degradation, mainly due to enzymatic digestion by the incorporated cells [39]. In this study, we used PET-reinforced CSs as the scaffold for hematopoietic differentiation, since PET fibers are insensitive to biological degradation, while still possessing biocompatibility. Indeed, PET incorporation suppressed the shrinkage of CSs and maintained the porous structure of the scaffold during long-term cell culture, making it possible to maintain the culture system for more than one month [39]. In our system, it seems that the niche-like structure was maintained by the 3D cellular rearrangement in the PET-fiber framework even after the original collagen scaffolds were degraded.

In the CS method, the yield per clump at each recovery was comparable to or slightly lower than that of the 2D method. However, since the CS method requires a smaller volume of medium (2 ml vs. 10 ml) and is sustainable for more than 50 days, the

relative costs of obtaining hematopoietic cells are reduced compared to the 2D method. Further optimization of the culture conditions, such as the cytokines and media, is needed to improve the output of the system. Additionally, the substrate elasticity might also affect the output of the hematopoietic differentiation system, as was previously shown in the expansion of HSPCs [51].

Despite the recent advances in technology, it remains challenging to develop *bona fide* human HSPCs with bone marrow reconstitution activity in immunodeficient mice at the single-cell level. [52] In this situation, there are concerns whether the hematopoietic cells grown in an artificial *in vitro* niche can appropriately migrate and adapt to the *in vivo* niche in the host mice. Since our system enables the transplantation of hematopoietic progenitors with their *in vitro* microenvironment, we transplanted CSs with differentiated PSCs directly into immunodeficient mice to evaluate whether transplantable putative HSPCs might be harbored in this niche. However, preliminary experiments involving the surgical transplantation of CSs into subcutaneous tissues failed to detect any circulating hematopoietic cells in the host mice (data not shown). Since there was no residual sponge *in vivo*, we concluded that the collagen was digested before the hematopoietic cells could migrate into the *in vivo* niche of the host mice. We therefore consider that the procedures used for transplantation, such as the site and timing of

transplantation, need to be further optimized.

Contrary to conventional methods that require clumped or aggregated human PSCs to start differentiation, our 3D-CS system can incorporate human PSCs dissociated into single cell suspension, allowing them to differentiate into hematopoietic lineages. However, the single-cell method is relatively unstable, presumably because a substantial number of the initially seeded PSCs are detached and lost. In conventional 2D cultures, collagens are not the optimal extracellular matrix protein for the maintenance and propagation of human PSCs. Recently, recombinant fragments of laminin E8 were reported to efficiently support the adhesion and expansion of human PSCs [53]. Similar to the maintenance of PSCs, laminin-containing mixtures of extracellular matrix, such as Matrigel, lead to robust cell adhesion and propagation in hematopoietic differentiation systems [16, 17]. Also, the different matrix gives a lineage specific differentiation, such as neuron, muscle, mesoderm, etc [54-56]. Therefore, coating or incorporation of substrate materials with other matrices, such as laminins, may improve the hematopoietic output of the current differentiation system.

In this report, as a proof-of-concept experiment for the application of the 3D-CS system, we showed that floating culture with multiple CSs could allow for the easy recovery of a large number of hematopoietic cells for more than a month. The large-scale

culture with this kind of "portable niche" would be a potentially useful concept for future applications of human PSC-derived hematopoietic cells, for example drug screening or cell therapy. Additionally, our system was constructed using defined conditions without serum and exogenous feeder/stromal cells, which is preferable for future applications for regenerative medicine. Further optimization of the system might provide an opportunity to accelerate the clinical application of PSC-derived hematopoietic cells.

Acknowledgements

We thank Y. Sasaki and M. Yamane for their technical assistance, H. Watanabe for the administrative assistance and M. Osawa, M. Yanagimachi, T. Tanaka, M. Matsui and S. Tajima for critical discussions. We are grateful to Center for Anatomical, Pathological and Forensic Medical Researches, Graduate School of Medicine, Kyoto University for Immunohistochemistry (IHC) analysis. Funding was provided by grants from the Ministry of Health, Labour and Welfare to T.N. and M.K.S., grants from the Leading Project of MEXT to T.N., a grant from the Funding Program for World-Leading Innovative Research and Development on Science and Technology (FIRST Program) of the Japan Society for the Promotion of Science (JSPS) to T.N., JSPS KAKENHI Grant Number JP25860856 to A.N., Japan Science and Technology Agency CREST to T.N., the

Program for Intractable Diseases Research utilizing Disease specific iPS cells from Japan Agency for Medical Research and Development (AMED) to T.N., the grant for Core Center for iPS Cell Research of Research Center Network for Realization of Regenerative Medicine from AMED to T.N. and M.K.S, and grants from the JSPS to T.N. and M.K.S. All of the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicting interests

The authors declare that they have no conflict of interest.

REFERENCES

1. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S et al. **A more efficient method to generate integration-free human iPS cells**. *Nature methods.* 2011;**8**:409-12.
2. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K et al. **Induction of pluripotent stem cells from adult human fibroblasts by defined factors**. *Cell.* 2007;**131**:861-72.
3. Takahashi K, Yamanaka S. **Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors**. *Cell.* 2006;**126**:663-76.
4. Kennedy M, Firpo M, Choi K, Wall C, Robertson S, Kabrun N et al. **A common precursor for primitive erythropoiesis and definitive haematopoiesis**. *Nature.* 1997;**386**:488-93.
5. Wang L, Menendez P, Shojaei F, Li L, Mazurier F, Dick JE et al. **Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression**. *J Exp Med.* 2005;**201**:1603-14.
6. Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S et al. **Development of definitive endoderm from embryonic stem cells in culture**. *Development (Cambridge, England).* 2004;**131**:1651-62.
7. Oshima M, Endoh M, Endo TA, Toyoda T, Nakajima-Takagi Y, Sugiyama F et al. **Genome-wide analysis of target genes regulated by HoxB4 in hematopoietic stem and progenitor cells developing from embryonic stem cells**. *Blood.* 2011;**117**:e142-50.
8. Takayama N, Nishimura S, Nakamura S, Shimizu T, Ohnishi R, Endo H et al. **Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells**. *J. Exp. Med.* 2010;**207**:2817-30.
9. Takayama N, Nishikii H, Usui J, Tsukui H, Sawaguchi A, Hiroyama T et al. **Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors**. *Blood.* 2008;**111**:5298-306.
10. Vodyanik MA, Bork JA, Thomson JA, Slukvin, Ii. **Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential**. *Blood.* 2005;**105**:617-26.
11. Nakano T, Kodama H, Honjo T. **In vitro development of primitive and definitive erythrocytes from different precursors**. *Science (New York, N.Y.* 1996;**272**:722-4.
12. Nakano T, Kodama H, Honjo T. **Generation of lymphohematopoietic cells from embryonic stem cells in culture**. *Science (New York, N.Y.* 1994;**265**:1098-101.

13. Choi KD, Vodyanik MA, Togarrati PP, Suknuntha K, Kumar A, Samarjeet F et al. **Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures**. *Cell Rep.* 2012;**2**:553-67.
14. Choi KD, Vodyanik MA, Slukvin, Ii. **Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors**. *The Journal of clinical investigation.* 2009;**119**:2818-29.
15. Choi KD, Vodyanik M, Slukvin, Ii. **Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells**. *Nat. Protoc.* 2011;**6**:296-313.
16. Niwa A, Heike T, Umeda K, Oshima K, Kato I, Sakai H et al. **A novel serum-free monolayer culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors**. *PLoS One.* 2011;**6**:e22261.
17. Yanagimachi MD, Niwa A, Tanaka T, Honda-Ozaki F, Nishimoto S, Murata Y et al. **Robust and highly-efficient differentiation of functional monocytic cells from human pluripotent stem cells under serum- and feeder cell-free conditions**. *PLoS One.* 2013;**8**:e59243.
18. Salvagiotto G, Burton S, Daigh CA, Rajesh D, Slukvin, Ii, Seay NJ. **A defined, feeder-free, serum-free system to generate in vitro hematopoietic progenitors and differentiated blood cells from hESCs and hiPSCs**. *PLoS One.* 2011;**6**:e17829.
19. Morrison SJ, Shah NM, Anderson DJ. **Regulatory mechanisms in stem cell biology**. *Cell.* 1997;**88**:287-98.
20. Adams GB, Scadden DT. **The hematopoietic stem cell in its place**. *Nat. Immunol.* 2006;**7**:333-7.
21. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC et al. **Osteoblastic cells regulate the haematopoietic stem cell niche**. *Nature.* 2003;**425**:841-6.
22. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG et al. **Identification of the haematopoietic stem cell niche and control of the niche size**. *Nature.* 2003;**425**:836-41.
23. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. **SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells**. *Cell.* 2005;**121**:1109-21.
24. Raynaud CM, Butler JM, Halabi NM, Ahmad FS, Ahmed B, Rafii S et al. **Endothelial cells provide a niche for placental hematopoietic stem/progenitor cell expansion through broad transcriptomic modification**. *Stem Cell Res.* 2013;**11**:1074-90.
25. Corselli M, Chin CJ, Parekh C, Sahaghian A, Wang W, Ge S et al. **Perivascular**

support of human hematopoietic stem/progenitor cells. *Blood.* 2013;**121**:2891-901.

26. Pinho S, Lacombe J, Hanoun M, Mizoguchi T, Bruns I, Kunisaki Y et al. **PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion**. *J. Exp. Med.* 2013;**210**:1351-67.

27. Heazlewood SY, Neaves RJ, Williams B, Haylock DN, Adams TE, Nilsson SK. **Megakaryocytes co-localise with hemopoietic stem cells and release cytokines that up-regulate stem cell proliferation**. *Stem Cell Res.* 2013;**11**:782-92.

28. Olson TS, Caselli A, Otsuru S, Hofmann TJ, Williams R, Paolucci P et al. **Megakaryocytes promote murine osteoblastic HSC niche expansion and stem cell engraftment after radioablative conditioning**. *Blood.* 2013;**121**:5238-49.

29. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA et al. **Mesenchymal and haematopoietic stem cells form a unique bone marrow niche**. *Nature.* 2010;**466**:829-34.

30. Yamazaki S, Nakauchi H. **Bone marrow Schwann cells induce hematopoietic stem cell hibernation**. *Int J Hematol.* 2014;**99**:695-8.

31. Yamazaki S, Ema H, Karlsson G, Yamaguchi T, Miyoshi H, Shioda S et al. **Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche**. *Cell.* 2011;**147**:1146-58.

32. Medvinsky A, Dzierzak E. **Definitive hematopoiesis is autonomously initiated by the AGM region**. *Cell.* 1996;**86**:897-906.

33. Muller AM, Medvinsky A, Strouboulis J, Grosveld F, Dzierzak E. **Development of hematopoietic stem cell activity in the mouse embryo**. *Immunity.* 1994;**1**:291-301.

34. Medvinsky AL, Samoylina NL, Muller AM, Dzierzak EA. **An early pre-liver intraembryonic source of CFU-S in the developing mouse**. *Nature.* 1993;**364**:64-7.

35. Sugimura R, He XC, Venkatraman A, Arai F, Box A, Semerad C et al. **Noncanonical Wnt signaling maintains hematopoietic stem cells in the niche**. *Cell.* 2012;**150**:351-65.

36. Asada N, Katayama Y. **Regulation of hematopoiesis in endosteal microenvironments**. *Int J Hematol.* 2014;**99**:679-84.

37. Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A et al. **Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells**. *Blood.* 2003;**102**:906-15.

38. Wang L, Li L, Shojaei F, Levac K, Cerdan C, Menendez P et al. **Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties**. *Immunity.* 2004;**21**:31-41.

39. Takamoto T, Hiraoka Y, Tabata Y. **Enhanced proliferation and osteogenic**

differentiation of rat mesenchymal stem cells in collagen sponge reinforced with different poly(ethylene terephthalate) fibers. *J. Biomater. Sci. Polym. Ed.* 2007;**18**:865-81.

40. Suwabe N, Takahashi S, Nakano T, Yamamoto M. **GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation**. *Blood.* 1998;**92**:4108-18.

41. Nakahata T, Ogawa M. **Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies**. *Proceedings of the National Academy of Sciences of the United States of America.* 1982;**79**:3843-7.

42. Nakahata T, Ogawa M. **Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors**. *J. Clin. Invest.* 1982;**70**:1324-8.

43. Nakahata T, Spicer SS, Ogawa M. **Clonal origin of human erythro-eosinophilic colonies in culture**. *Blood.* 1982;**59**:857-64.

44. Son MY, Choi H, Han YM, Cho YS. **Unveiling the critical role of REX1 in the regulation of human stem cell pluripotency**. *Stem cells.* 2013;**31**:2374-87.

45. Ferrell PI, Xi J, Ma C, Adlakha M, Kaufman DS. **The RUNX1 +24 Enhancer and P1 Promoter Identify a Unique Subpopulation of Hematopoietic Progenitor Cells Derived from Human Pluripotent Stem Cells**. *Stem cells.* 2014

46. Vodyanik MA, Yu J, Zhang X, Tian S, Stewart R, Thomson JA et al. **A mesoderm-derived precursor for mesenchymal stem and endothelial cells**. *Cell stem cell.* 2010;**7**:718-29.

47. Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. **The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage**. *Nature.* 2009;**457**:892-5.

48. Leisten I, Kramann R, Ventura Ferreira MS, Bovi M, Neuss S, Ziegler P et al. **3D co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche**. *Biomaterials.* 2012;**33**:1736-47.

49. Mortera-Blanco T, Mantalaris A, Bismarck A, Aqel N, Panoskaltsis N. **Long-term cytokine-free expansion of cord blood mononuclear cells in three-dimensional scaffolds**. *Biomaterials.* 2011;**32**:9263-70.

50. Blanco TM, Mantalaris A, Bismarck A, Panoskaltsis N. **The development of a three-dimensional scaffold for ex vivo biomimicry of human acute myeloid leukaemia**. *Biomaterials.* 2010;**31**:2243-51.

51. Kumar SS, Hsiao JH, Ling QD, Dulinska-Molak I, Chen G, Chang Y et al. **The combined influence of substrate elasticity and surface-grafted molecules on the ex vivo expansion of hematopoietic stem and progenitor cells**. *Biomaterials.* 2013;**34**:7632-44.

52. Slukvin, Ii. **Hematopoietic specification from human pluripotent stem cells: current advances and challenges toward de novo generation of hematopoietic stem cells**. *Blood.* 2013

53. Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M et al. **Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells**. *Nature communications.* 2012;**3**:1236.

54. Brafman DA, Phung C, Kumar N, Willert K. **Regulation of endodermal differentiation of human embryonic stem cells through integrin-ECM interactions**. *Cell Death Differ.* 2013;**20**:369-81.

55. Zhang L, Stauffer WR, Jane EP, Sammak PJ, Cui XT. **Enhanced differentiation of embryonic and neural stem cells to neuronal fates on laminin peptides doped polypyrrole**. *Macromol. Biosci.* 2010;**10**:1456-64.

56. Liu Y, Wang X, Kaufman DS, Shen W. **A synthetic substrate to support early mesodermal differentiation of human embryonic stem cells**. *Biomaterials.* 2011;**32**:8058-66.

Figure Legends

Fig. 1. Hematopoietic differentiation from clumps of undifferentiated PSCs. (a) A schematic diagram of the culture system, cytokines and medium conditions for induction of common hemoanigogenic progenitor cells. (b) A representative photograph of CSs in a 24-well plate.

Fig. 2. Differentiation towards MPCs. (a) A SEM image of a PSC-clump on a CS. (b)

A representative flow cytogram of differentiated cells on CS derived from KhES1 (day 6). Dead cells and debris were excluded using DAPI staining. (c) The frequency of KDR+CD34+ fraction detected on day 6. The error bars indicate the S.D. All data are representative of at least three independent experiments.

Fig. 3. Expression of selected genes during differentiation. (a, b) Relative expression of PSC-specific (a) and MPC-specific (b) genes on day 0 (D0) and day 6 (D6). The error bars display the calculated maximum (RQMax) and minimum (RQMin) expression levels that represent standard error of the mean expression level (RQ value). Gene *GAPDH* was applied as endogenous control, and the samples of day 0 are set as standard. Samples were harvested from three independent experiments.

Fig. 4. Differentiation towards specific hematopoietic lineages. (a) Cytokine conditions for hematopoietic differentiation. **(b, c)** The appearance of myeloid cells (day 14). Flow cytograms (b) and May-Giemsa staining (c) are shown. Scale bars = 20 μm. (d) The number of hematopoietic cells recovered through prolonged culture. (e) Assay for phagocytosis-induced respiratory burst activity using chemiluminescent microspheres (luminol-binding microspheres). Abbreviation: RLU, relative light units. **(f-i)** The

appearance of monocytic (f, g) and erythroid (h, i) cells on day 14. Scale bars = 20 μm. **(j-m)** A flow cytogram of the cells inside the CSs: Blood cells on day 6 (j), 9, and 35 (k), and adhered non-hematopoietic cells on day 9 (l) and 35 (m). (n) Sequential clonogeneic assay of hematopoietic cells on CSs. Kh-ES1 cells were used in all experiments. Data are representative of at least three independent experiments. The error bars indicate the S.D.

Fig. 5. Re-activation of integrated transgenes monitored by real-time PCR in 409B2-iPSCs. The data from three independent experimental batches (#1-3) are shown. From 1.8×10^5 to 3.3×10^6 sample cells were applied for each assay. The error bars display the maximum (RQMax) and minimum (RQMin) expression levels that represent the standard error of the mean expression level (RQ value), standardized with the value of undifferentiated 201B7-iPSCs.

Fig. 6. Hematopoietic differentiation from single PSCs. (a) A SEM image of cells on a CS at the start of differentiation. Note that there is no obvious cellular aggregation. (b) A representative flow cytogram of cells derived from KhES1 on day 6. Dead cells and debris were excluded using DAPI staining. (c-e) The total cell number (c), frequency of CD34+KDR+ fraction (d) and estimated number of CD34+KDR+ fraction (e) obtained

from KhES1 on day 6. The error bars indicate the S.D. (f, g) The appearance of myeloid cells at day 28. Flow cytograms (f) and May-Giemsa staining (g) are shown. Scale bars = 20 μm. Kh-ES1 cells were used in all experiments. All data are representative of at least three independent experiments.

Fig. 7. Histological analysis of CSs. (a) A schema of the vertical section of a CS. Red rectangular areas are magnified. (b) Magnification of the middle to lower layer of the CS (area B). (c) Magnification of the upper surface of the CS (area C). Scale bars = 300 μm. Kh-ES1 cells were used in all experiments.

Fig. 8. Images obtained by scanning electron microscopy (SEM). (a) An image of a CS before cell seeding. (b) An image of a CS after differentiation with clumped PSCs. (c) A magnified images of cells differentiated from clumped PSCs on day 41. Kh-ES1 cells were used in all experiments.

Fig. 9. The bulk culture of multiple CSs in a flask. (a) The gross appearance of 12 CSs suspended in a 50 mL flask. (b) The number of recovered hematopoietic cells from the bulk culture. (c, d) The appearance of myeloid cells obtained from the culture on day 34.

Flow cytograms (c) and May-Giemsa staining (d) are shown. Scale bars = 20 μm. Kh-ES1 cells were used in all experiments.

Figure 1

Click here to download Figure Figure 1.JPG

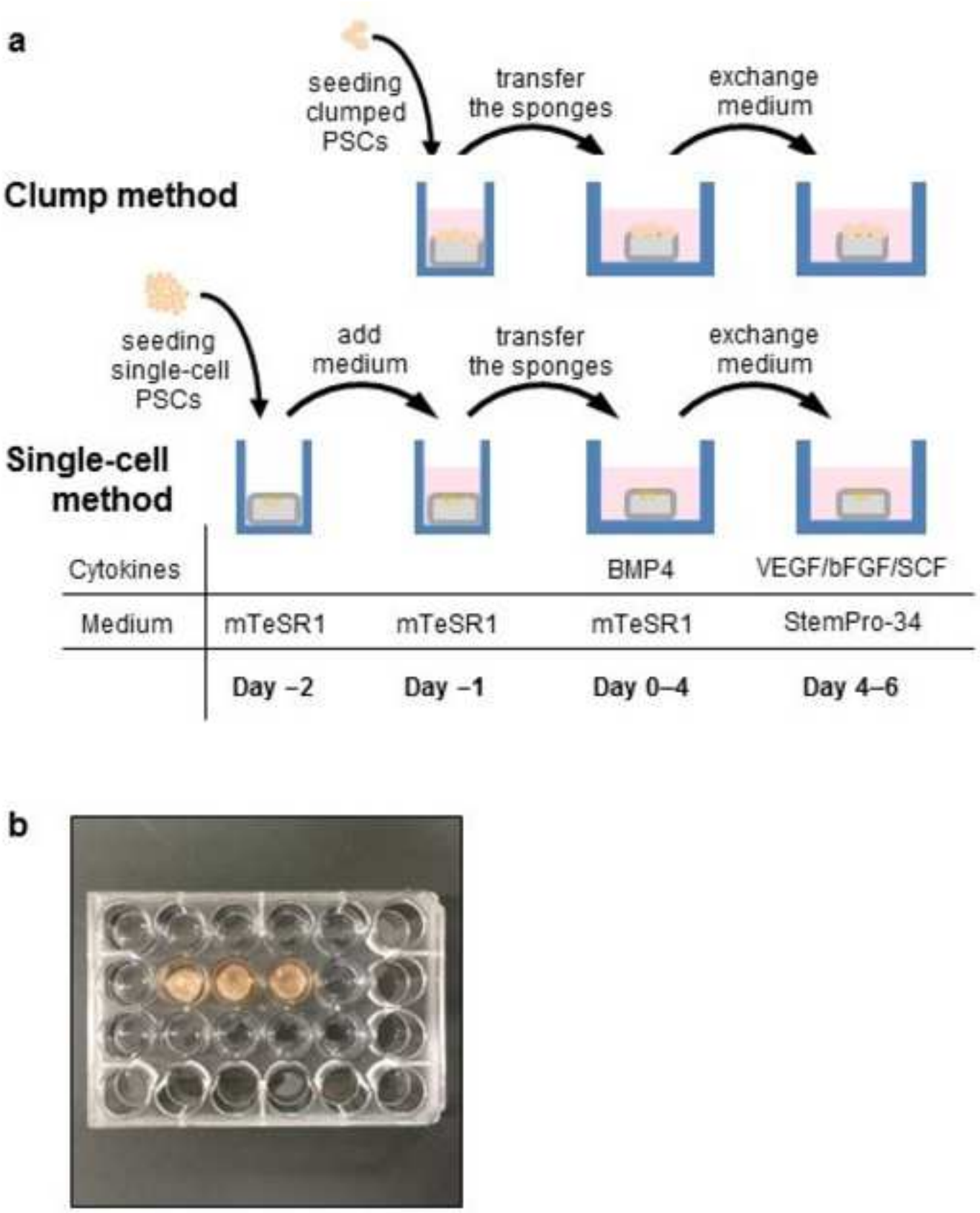

Figure 2

Click here to download Figure Figure 2.JPG

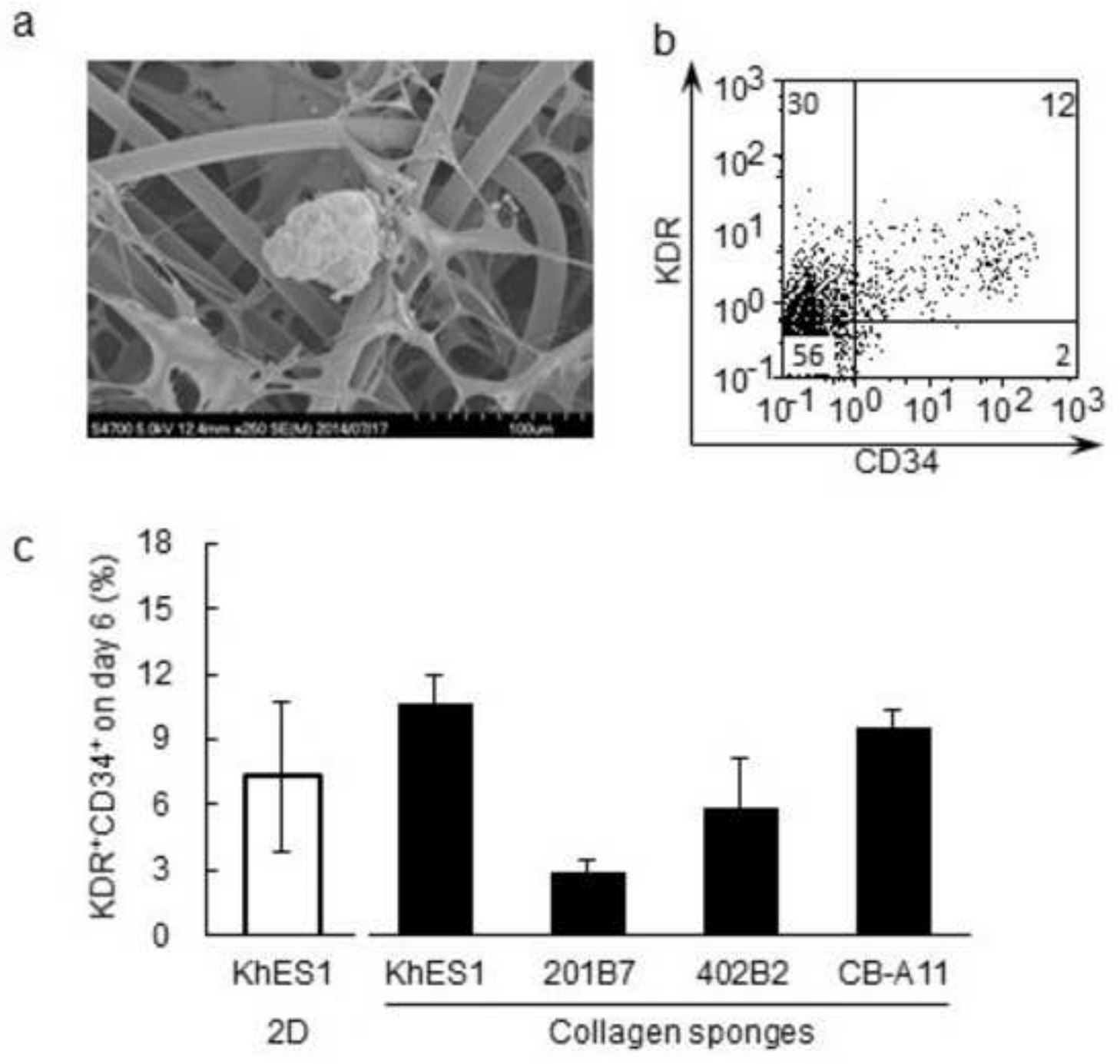

Figure 3

Click here to download Figure Figure 3.JPG

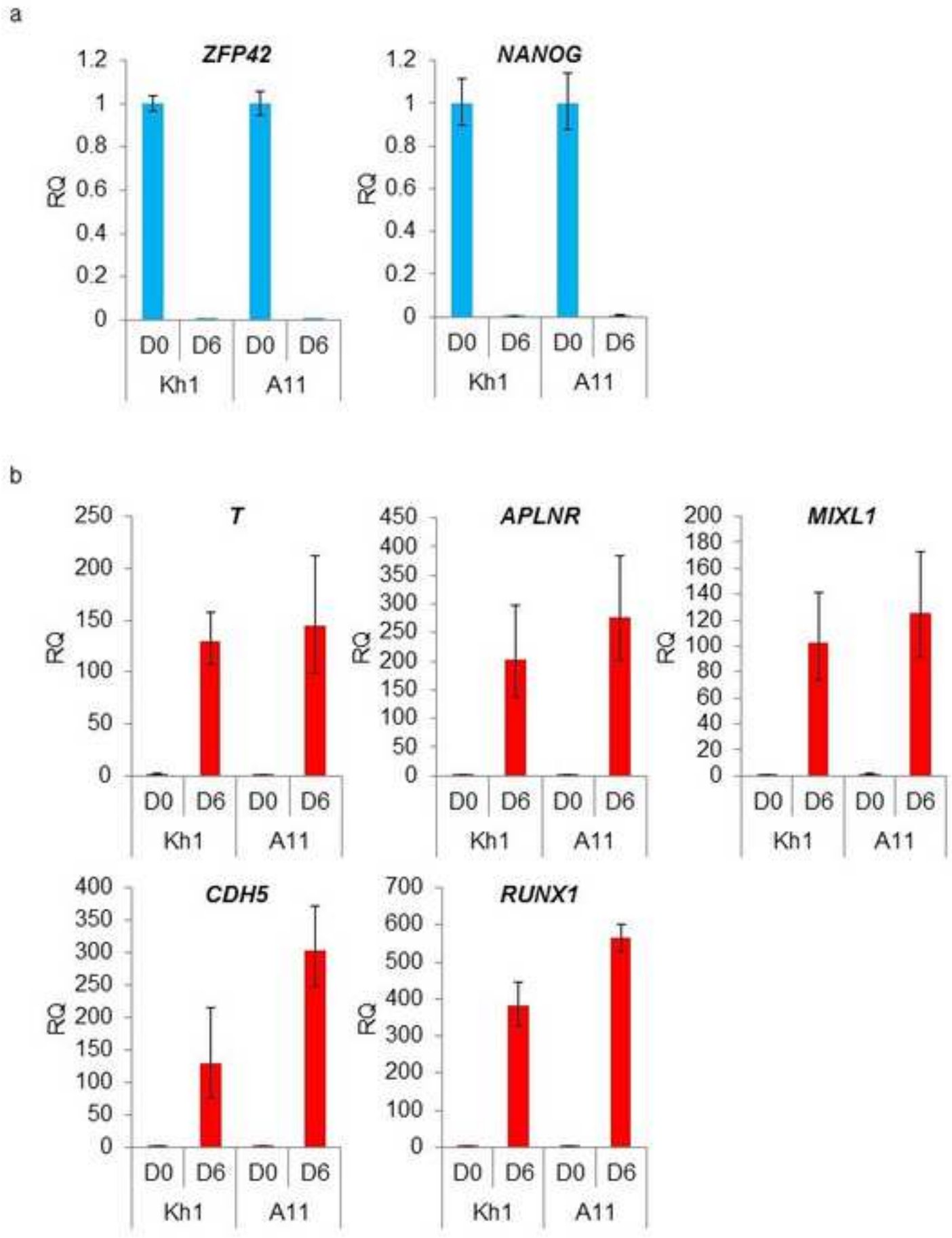

Figure 4

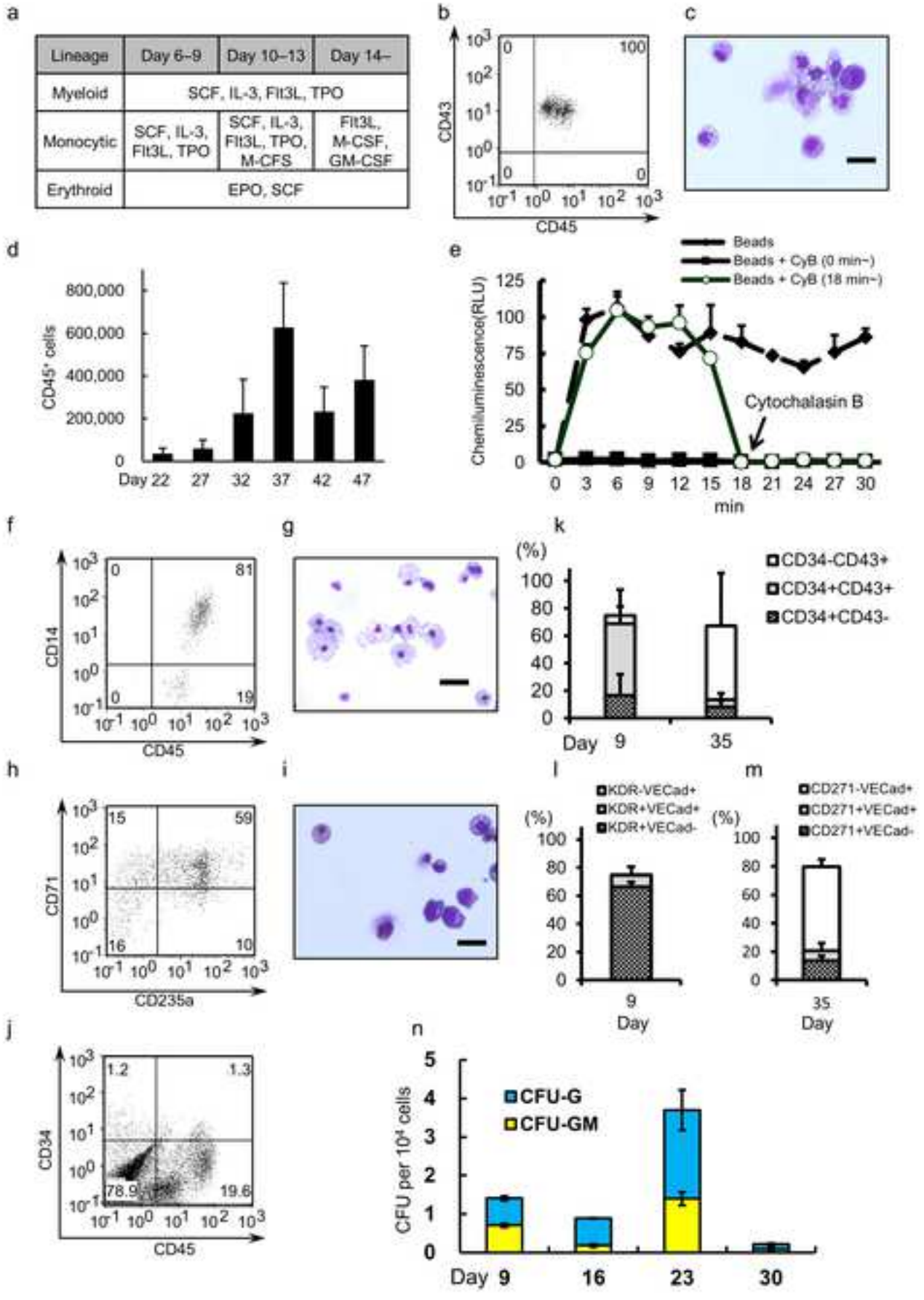
a
Lineage
Day 6–9
Day 10–13
Day 14–
Myeloid
SCF, IL-3, Flt3L, TPO
Monocytic
SCF, IL-3, Flt3L, TPO
SCF, IL-3, Flt3L, TPO, M-CFS
Flt3L, M-CSF, GM-CSF
Erythroid
EPO, SCF
b
CD43
CD45
c
d
CD45+ cells
Day 22 27 32 37 42 47
e
Beads
Beads + CyB (0 min–)
Beads + CyB (18 min–)
Chemiluminescence(RLU)
Cytochalasin B
min
f
CD14
CD45
g
k
(%)
CD34-CD43+
CD34+CD43+
CD34+CD43-
Day 9 35
h
CD71
CD235a
i
l
KDR-VECad+
KDR+VECad+
KDR+VECad-
(%)
9 Day
m
CD271-VECad+
CD271+VECad+
CD271+VECad-
(%)
35 Day
j
CD34
CD45
n
CFU per 10⁴ cells
CFU-G
CFU-GM
Day 9 16 23 30

Figure 5

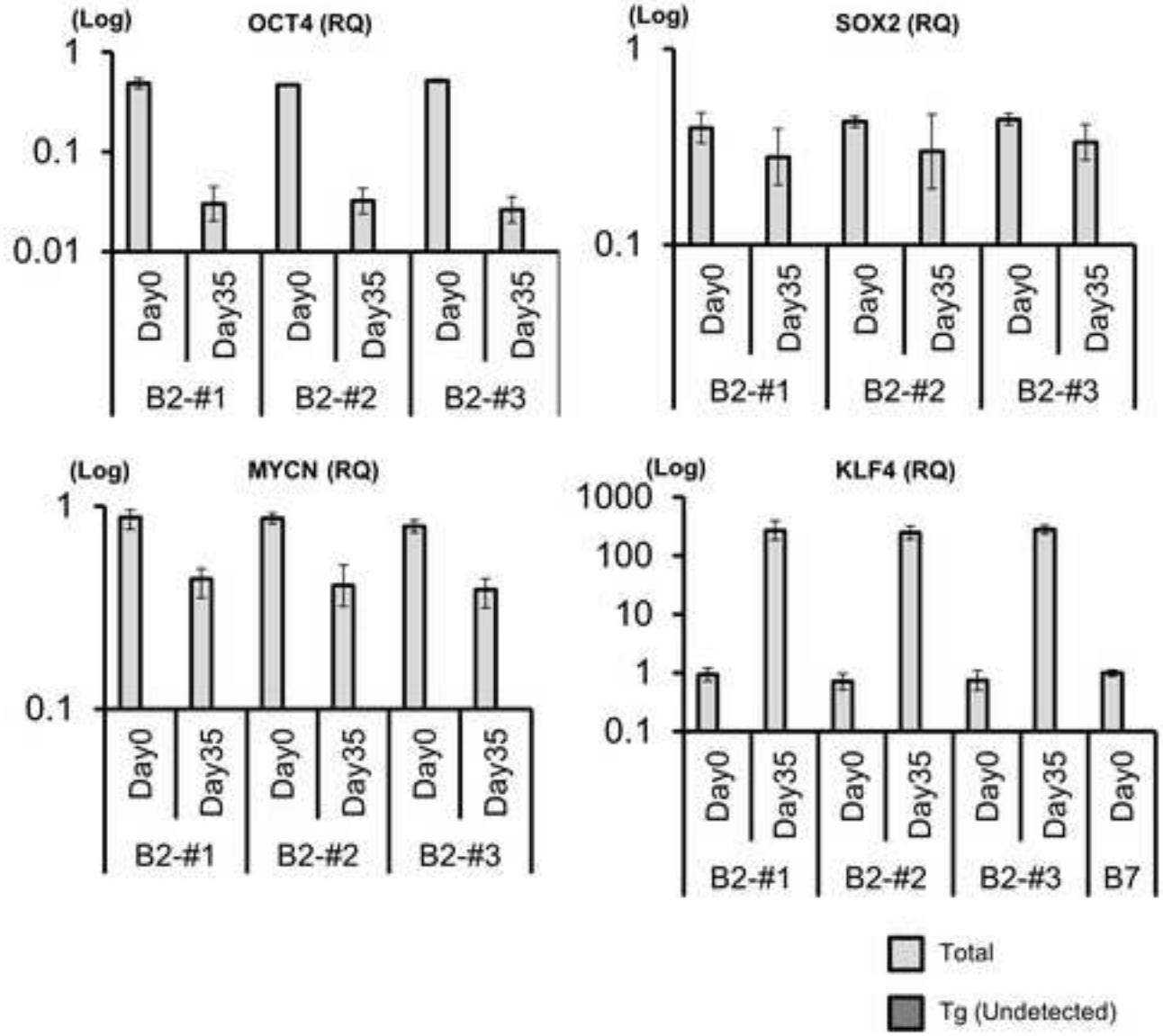

Figure 6

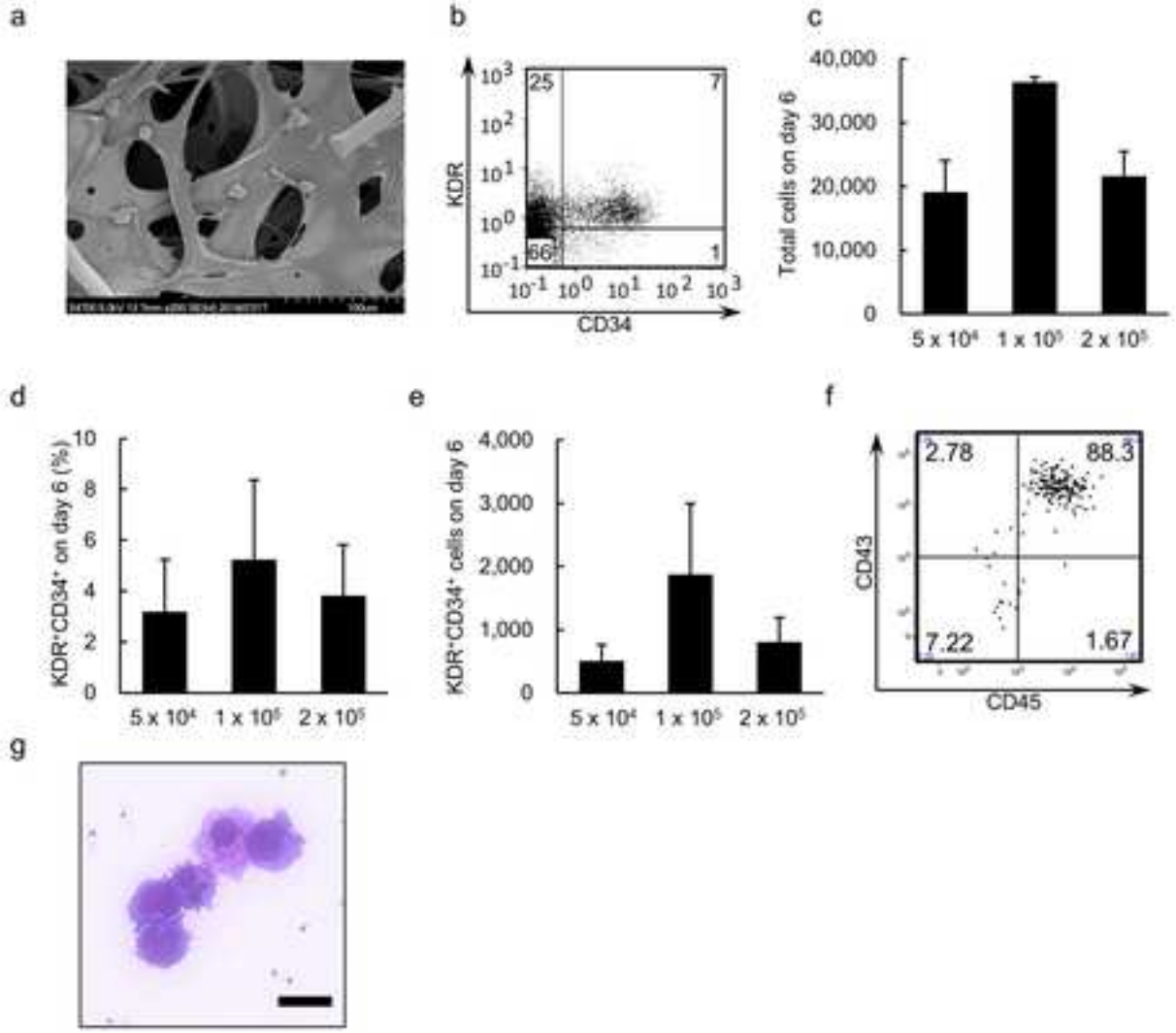

Figure 7

Figure 7

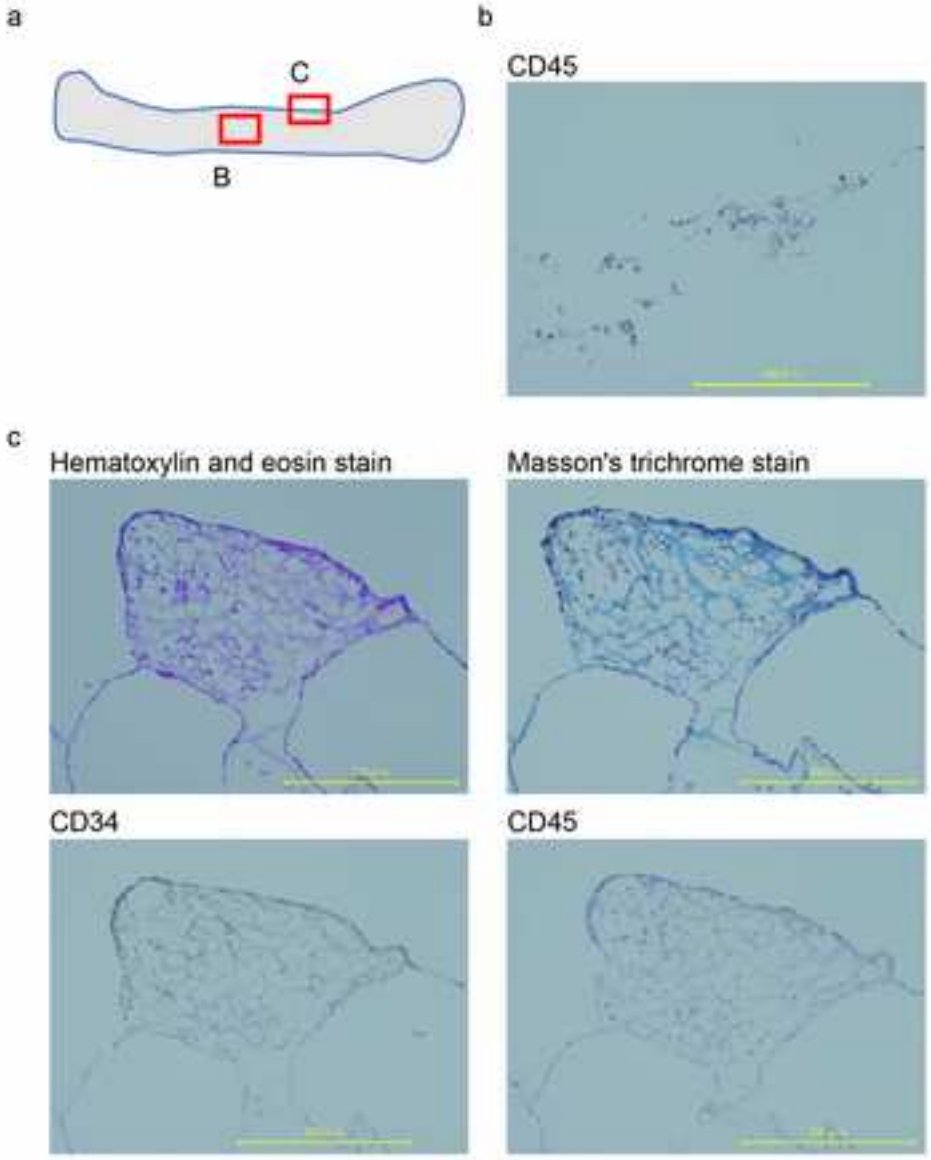

Figure 8

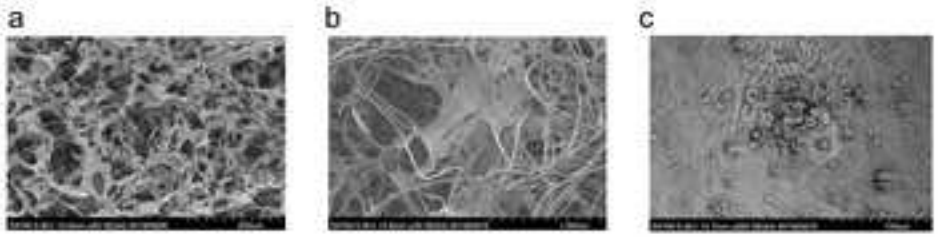

Figure 9