TITLE:

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

AUTHOR(S):

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CITATION:

Sugimine, Yoshinori. A portable platform for stepwise hematopoiesis from human pluripotent stem cells within PET-reinforced collagen sponges. 京都大学, 2022, 博士(医学)

ISSUE DATE:

2022-01-24

URL:

<https://doi.org/10.14989/doctor.r13464>

RIGHT:

電子ジャーナルのサイトへのリンク The original publication is
available at www.springerlink.com doi: 10.1007/s12185-016-2088-x

International Journal of Hematology

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--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:	<table><tr><td>Japan Agency for Medical Research and Development</td><td>Dr. Tatsutoshi Nakahata Dr. Megumu K. Saito</td></tr><tr><td>Japan Society for the Promotion of Science</td><td>Dr. Tatsutoshi Nakahata Dr. Megumu K. Saito</td></tr><tr><td>Japan Society for the Promotion of Science (JP25860856)</td><td>Dr. Akira Niwa</td></tr></table>		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
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	hematopoietic cells.
Response to Reviewers:	N/A
Additional Information:	
Question	Response
<p>Is your submission applicable to any of cases below? (Please refer to the latest IJH Information for Authors)</p> <p>1) "Duplicate Publication" which has been published previously or currently under consideration for publication elsewhere.</p> <p>2) "Secondary Publication" which is based on material published in other journals or online.</p>	<p>No If Yes, please provide an explanation below. -----</p>

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

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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.

1
2
3 1 **Keywords**
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6 2 Human embryonic stem cells; human induced pluripotent stem cells; collagen sponge;
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9 3 hematopoietic differentiation; hematopoietic progenitor cells
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1 **MAIN TEXT (4,501 words, not including references and figure legends)**

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3 **Introduction**

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

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

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

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

1 differentiation system.

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

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3 **1 Material and Methods**
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6 **2 Study ethics**
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10 3 The methods were carried out in accordance with the declaration of Helsinki and the
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12 4 Japanese guidelines on the use of human embryonic stem cells. The study plan using
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14 5 human embryonic stem cells (ESCs) was approved by the Ministry of Education, Culture,
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17 6 Sports, Science and Technology (MEXT) of Japan.
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25 **8 Preparation of collagen sponges**
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28 9 Collagen sponges mechanically reinforced by the incorporation of poly(ethylene
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30 10 terephthalate) (PET) fibers (PETCol-24w, hereafter referred to as CSs) were purchased
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33 11 from MedGEL (Tokyo, Japan)[39]. In order to remove air bubbles, six CSs were put in a
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36 12 50 mL conical tube (Becton-Dickinson, Franklin Lakes, USA) containing 10 mL of
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39 13 maintenance medium for PSCs, and were centrifuged at 10000 rpm for five minutes. After
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42 14 centrifugation, the CSs were picked up with a spatula, and placed onto 24-well plates (**Fig.**
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54 **17 Cytokines used for culture**
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57 18 Recombinant human basic fibroblast growth factor (bFGF) was purchased from Wako
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1 Pure Chemical Industries, Ltd. (Osaka, Japan). The following cytokines were purchased
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6 from R&D Systems (Minneapolis, USA): BMP-4, VEGF, SCF, IL-3, Thrombopoietin
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9 (TPO), Flt-3 Ligand (FL3), GM-CSF and M-CSF. Recombinant human erythropoietin
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12 (EPO) was purchased from EMD Biosciences (San Diego, USA).
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19 **Maintenance of human PSCs**

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22 This study used human ESCs (cell line: KhES1) and iPSCs (cell lines: 201B7, 409B2 and
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25 CB-A11). The human ES cell line, KhES1, was kindly provided by Dr. Norio Nakatsuji.
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27
28 Human iPS cell lines 201B7 [2] and 409B2 [1] were kindly provided by Dr. Shinya
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31 Yamanaka. CB-A11 is an iPSC clone established from cord-blood hematopoietic cells
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34 [17]. These PSCs were maintained on mitotically-inactivated SNL feeder cells with
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37 Primate ES Cell Medium (ReproCELL, Kanagawa, Japan) supplemented with 5 ng/mL
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40 bFGF, or tissue culture dishes coated with growth factor-reduced Matrigel (Becton-
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43 Dickinson, Franklin Lakes, USA) in mTeSR1 serum-free medium (STEMCELL
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46 Technologies, Vancouver, Canada). The medium was replaced every day.
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54 **Seeding of clumped PSCs onto CSs**

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

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

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14 15 16 **Hematopoietic cell differentiation**

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19 6 At the start of differentiation (day 0), CSs were transferred into 12-well plates. Thereafter,
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22 7 CSs were transferred into another 12-well plates with appropriate cytokine-containing
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25 8 medium on days depicted (**Fig. 1**). The CSs were cultured in the same wells until floating
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28 9 hematopoietic cells emerged. The media and cytokines were used as described previously
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32 10 [16, 17]. In brief, BMP4 (80 ng/mL) was added to the mTeSR1 medium from day 0
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35 11 through day 4 in order to induce primitive streak cell. Then, the mTeSR1 medium was
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38 12 replaced by StemPro-34 serum-free medium (Gibco, Tokyo, Japan) containing 2 mM
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41 13 GlutaMAX (Invitrogen, Tokyo, Japan) on day 4, which was supplemented with a cytokine
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44 14 cocktail composed of VEGF (80 ng/mL), basic FGF (25 ng/mL) and SCF (100 ng/mL).
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47 15 On day 6, the medium were replaced with StemPro-34 serum-free medium containing
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51 16 different cytokines to induce specific hematopoietic lineages. For myeloid cell induction,
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54 17 the culture medium contained with SCF (50 ng/mL), IL-3 (50 ng/mL), TPO (5 ng/mL)
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57 18 and FL3 (50 ng/mL) was changed every four days. G-CSF (10 ng/mL) was added from
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1 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.

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2 **Phagocytosis assay**

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

12

13 **Scanning electron microscopy (SEM)**

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

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3 **1 Culture of multiple CSs in a flask**
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6 2 Twelve CSs were transferred together into a 50 mL tissue culture flask (Becton-Dickinson,
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9 3 Franklin Lakes, USA) containing 25 mL of culture medium on differentiation day 18. To
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12 4 recover the floating cells, the flask was gently shaken, and the cultured medium
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16 5 containing floating cells was recovered. Subsequently, the media was centrifuged, and the
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19 6 cellular pellet was suspended in fresh medium.
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25 **8 Flow cytometric analysis**
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28 9 The data from the flow cytometric analysis were collected using the MACS Quant™
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31 10 Analyzer (Miltenyi Biotec, Bergisch-Gladbach, Germany) and analyzed utilizing the
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35 11 FlowJo software package (Tree Star Inc., Ashland, USA). The following antibodies were
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38 12 purchased from BD Biosciences (San Jose, USA): CD34-PE (clone: 581), CD43-APC
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41 13 (clone: 1G10), CD45-PE (clone: HI30), CD71-APC (clone: L01.1), CD235a-PE (clone:
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44 14 GA-R2 (HIR2), and CD271-FITC (clone:C40-1457). CD14-APC (clone: RMO52)
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47 15 antibody was purchased from Beckman Coulter (Brea, USA). KDR (CD309)-Alexa fluor
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51 16 647 (clone: HKDR-1) and VE-Cadherin (CD144)-PE (clone:BV9) were purchased from
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54 17 Biolegend (San Diego, USA).
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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 microtomes. 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

1 35-mm petri dishes (#1008; Becton-Dickinson) using 1 ml/dish of MethoCult GF+
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6 semisolid medium (#4435; STEM- CELL Technologies) as previously described.
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10 Colonies were counted after 14–21 days of incubation, and colony types were determined
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13 according to the criteria described previously [41-43] by in situ observation using an
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16 inverted microscope. The abbreviations used for the clonogenic progenitor cells are as
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19 follows: CFU-Mix, mixed colony-forming units; BFU-E, erythroid burst- forming units;
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22 CFU-GM, granulocyte–macrophage colony-forming units; and CFU-G, granulocyte
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25 colony-forming units.
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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 $2.5 \times 10^4 \pm 0.39 \times 10^4$ 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

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

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

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

12 Since the average porous size of a CS is approximately 200 μm [39], clumps of PSCs are
13 considered not to infiltrate into the CS. This implies that the clumps adhered onto the CSs.
14 The cells then began to differentiate on the surface of the CSs, and some cells
15 subsequently infiltrated into the CSs and formed three-dimensional structures (see **Fig.**
16 **7**). To take advantage of the CS's three-dimensional structure, we next tried to start initial
17 differentiation inside of CS. For, this, we evaluated whether the CSs can support the
18

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 reseedling 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 2.5×10^4 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

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

6 Despite the recent advances in technology, it remains challenging to develop
7 *bona fide* human HSPCs with bone marrow reconstitution activity in immunodeficient
8 mice at the single-cell level. [52] In this situation, there are concerns whether the
9 hematopoietic cells grown in an artificial *in vitro* niche can appropriately migrate and
10 adapt to the *in vivo* niche in the host mice. Since our system enables the transplantation
11 of hematopoietic progenitors with their *in vitro* microenvironment, we transplanted CSs
12 with differentiated PSCs directly into immunodeficient mice to evaluate whether
13 transplantable putative HSPCs might be harbored in this niche. However, preliminary
14 experiments involving the surgical transplantation of CSs into subcutaneous tissues failed
15 to detect any circulating hematopoietic cells in the host mice (data not shown). Since there
16 was no residual sponge *in vivo*, we concluded that the collagen was digested before the
17 hematopoietic cells could migrate into the *in vivo* niche of the host mice. We therefore
18 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

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

7 8 **Acknowledgements**

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

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1 Program for Intractable Diseases Research utilizing Disease specific iPS cells from Japan
2 Agency for Medical Research and Development (AMED) to T.N., the grant for Core
3 Center for iPS Cell Research of Research Center Network for Realization of Regenerative
4 Medicine from AMED to T.N. and M.K.S, and grants from the JSPS to T.N. and M.K.S.
5 All of the funders had no role in study design, data collection and analysis, decision to
6 publish, or preparation of the manuscript.
7

8 **Conflicting interests**

9 The authors declare that they have no conflict of interest.

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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)

1 A representative flow cytogram of differentiated cells on CS derived from KhES1 (day
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7 2 6). Dead cells and debris were excluded using DAPI staining. (c) The frequency of
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10 3 KDR+CD34+ fraction detected on day 6. The error bars indicate the S.D. All data are
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19 **Fig. 3. Expression of selected genes during differentiation.** (a, b) Relative expression
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22 7 of PSC-specific (a) and MPC-specific (b) genes on day 0 (D0) and day 6 (D6). The error
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25 8 bars display the calculated maximum (RQMax) and minimum (RQMin) expression levels
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29 9 that represent standard error of the mean expression level (RQ value). Gene *GAPDH* was
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32 10 applied as endogenous control, and the samples of day 0 are set as standard. Samples were
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41 **Fig. 4. Differentiation towards specific hematopoietic lineages.** (a) Cytokine
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44 14 conditions for hematopoietic differentiation. (b, c) The appearance of myeloid cells (day
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48 15 14). Flow cytograms (b) and May-Giemsa staining (c) are shown. Scale bars = 20 μ m. (d)
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51 16 The number of hematopoietic cells recovered through prolonged culture. (e) Assay for
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54 17 phagocytosis-induced respiratory burst activity using chemiluminescent microspheres
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57 18 (luminol-binding microspheres). Abbreviation: RLU, relative light units. (f-i) The
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1 appearance of monocytic (f, g) and erythroid (h, i) cells on day 14. Scale bars = 20 μ m.
2 (j-m) A flow cytogram of the cells inside the CSs: Blood cells on day 6 (j), 9, and 35 (k),
3 and adhered non-hematopoietic cells on day 9 (l) and 35 (m). (n) Sequential clonogenic
4 assay of hematopoietic cells on CSs. Kh-ES1 cells were used in all experiments. Data are
5 representative of at least three independent experiments. The error bars indicate the S.D.

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7 **Fig. 5. Re-activation of integrated transgenes monitored by real-time PCR in 409B2-**
8 **iPSCs.** The data from three independent experimental batches (#1-3) are shown. From
9 1.8×10^5 to 3.3×10^6 sample cells were applied for each assay. The error bars display the
10 maximum (RQMax) and minimum (RQMin) expression levels that represent the standard
11 error of the mean expression level (RQ value), standardized with the value of
12 undifferentiated 201B7-iPSCs.

13
14 **Fig. 6. Hematopoietic differentiation from single PSCs.** (a) A SEM image of cells on a
15 CS at the start of differentiation. Note that there is no obvious cellular aggregation. (b) A
16 representative flow cytogram of cells derived from KhES1 on day 6. Dead cells and debris
17 were excluded using DAPI staining. (c-e) The total cell number (c), frequency of
18 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 μ m. 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 μ m. 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.

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a **b**

Figure 3

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Figure 4

Figure 5

Figure 6

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