TITLE: Biomimetic Aorta-Gonad-Mesonephros on-a-Chip to Study Human Developmental Hematopoiesis.

AUTHORS

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ABSTRACT

A fundamental limitation in the derivation of hematopoietic stem and progenitor cells is the imprecise understanding of human developmental hematopoiesis. Herein we established a multilayer microfluidic Aorta-Gonad-Mesonephros (AGM)-on-a-chip to emulate developmental hematopoiesis from human pluripotent stem cells. We show that the AGM-chip efficiently derives endothelial to hematopoietic transition (EHT) in the presence of both mesenchymal stroma and endothelial cells. The AGM-chip could dissect the cellular and molecular mechanisms of human developmental hematopoiesis.

INTRODUCTION

The hematopoietic system sustains life through an almost limitless production of blood cells and resistance to infection. The generation of all blood cells originates in the embryonic tissue called aorta-gonad-mesonephros (AGM)[1,2]. At around 12-15 days post coitum (dpc) of human embryos, a subset of endothelial cells called hemogenic endothelium (HE) in the AGM are specified to hematopoietic progenitor cells (HPCs) through endothelial to hematopoietic transition (EHT)[2]. Capturing the precise mechanisms of human hematopoietic development, especially EHT process, will benefit efforts to generate blood cells *in* vitro for cell therapy, drug screening and disease modeling.[3-5]. Herein we propose a novel method to model human hematopoietic development in an organ-on-a-chip system[6-11]. We developed a microfluidic AGM-on-a-chip which resembles key features of the cellular components of human AGM, including the HE, mesenchymal stroma (MS) and endothelial cells (ECs)[2]. We previously isolated a MS cell line from murine AGM that supports EHT *in vitro* (AGMS-3)[12], which we use in the current study to recapitulate mesenchymal stroma in the AGM. We also used human umbilical vein endothelial

cells (HUVECs) as a platform of ECs because they have the capacity to support hematopoiesis *in vitro*[13] and are easy to handle. We demonstrate that the AGM-chip efficiently induces EHT from human pluripotent stem cells (hPSCs) compared with regular suspension culture, providing a robust generation of human blood cells. Of note, the presence of both MS cells and ECs renders EHT with functional HPCs *in vitro*. We propose our AGM-chip as a novel platform to study the cellular and molecular mechanisms of human hematopoietic development.

RESULTS

The AGM-chip is biomimetic both in terms of structure and cellular interactions during hematopoietic development (fig. 1A-B). We employed hPSC-derived HE (hPSC-HE), HUVECs as a model of ECs, and AGMS-3 cells as a model of MS into the AGM-chip. The device consists of two polydimethylsiloxane (PDMS) microchannels separated by a 3-um porous membrane, which allows cellular interactions between each cell layer (fig. 1C-D). This system realizes to recover differentiated cells from the outlet for analyses by flowing fluid through the microchannel. We inputted hPSC-HE and AGMS-3 cells in the upper layer, and HUVECs in the lower layer (fig. 1B). We coated the device with fibronectin and inputted AGMS-3 cells and HUVECs in the device one day prior to the injection of hPSC-HE (fig. 1D). Hematopoietic specification of hPSC-HE was verified on the chip format (fig. 2). We then examined the hematopoietic specification of GFP-labeled hPSC-HE in the AGM-chip. We observed the formation of round-shaped GFP+ cells, indicating hematopoietic transition (fig. 2). We noted that both HUVECs and AGMS-3 cells interacted with the GFP+ cells through the porous membrane, consistent with the notion that ECs and MS cells coax hematopoietic specification of HE[14] (fig. 2). We examined EHT as the yields of hematopoietic progenitor cells (HPCs) from an entire AGM-chip (fig. 3A). We found that both the AGM-chip and suspension culture produced HPCs at slimier yields at both 1- and 2-weeks of culture. The presence of feeder cells (AGMS-3 cells and HUVECs) significantly rendered HPCs

from the AGM-chip at 2 weeks (with and without feeder, p=0.013) (fig. 3B). Consistent with HPC numbers, colony-forming-unit (CFU) capacity depends on the presence of feeder cells at 2-weeks of culture (fig. 3C), indicating for the role of feeder cells in sustaining functional HPCs in culture. Moreover, CFU capacity of the AGM-chip was relatively higher than that of suspension culture at both 1-week (p=0.051) and 2-week (p=0.08) of culture in the presence of feeder cells (Fig. 3C). These data demonstrate the advantage of our AGM-chip in producing HPCs over suspension culture, indicating microenvironmental cues play an important role in the induction of EHT. To assess the EHT capacity of an intact AGM-chip, we harvested cells from the outflow of the device by perfusing culture medium using a ring pump at a flow rate of 20 uL min^{-1} (fig. 4A). We found that around 1.5% of the outlet cells were CD34+CD45+ HPCs and confirmed their colony-forming capacity (fig. 4B-C). These data demonstrate that the AGM-chip produces HPCs through EHT.

DISCUSSION

In this paper, we present an *in vitro* AGM-on-a-chip platform as biomimetic AGM, which is where blood cells emerge during development[15]. We demonstrated that perfusion culture in the AGM-chip yields more EHT than suspension cell culture indicated by the presence of HPCs. Finally, we established that feeder cells support EHT. We expect the AGM-chip could be a powerful experimental tool with which end users can assess the effects of target signaling, small molecules and genetic interventions in human developmental hematopoiesis.

Conflicts of interest

There are no conflicts to declare.

ACKNOWLEDGEMENTS

We would like to thank Dr. Misaki Ouchida for graphical assistance, Ms. Harumi Watanabe for administrative assistance, Ms. Yuka Ozaki and Kayo Yano for their technical assistance, and Dr. Peter Karagiannis for reading and editing the paper. This work was supported by the Core Center for iPS Cell Research of Research Center Network for Realization of Regenerative Medicine from the Japan Agency for Medical Research and Development (AMED) [M.K.S.], the Program for Intractable Diseases Research utilizing Disease-specific iPS cells (AMED: 17935423) [M.K.S.], and the Center for Innovation program of Japan Science and Technology Agency (JST) [R.O. and M.K.S.]. R.S. is a recipient of Early Career KAKENHI, iPS Academia Japan, and Sen-shin Medical Research Foundation (SMRF) fellowships. This work was also supported by AMED under Grant No. JP18gm5810008, JSPS KAKENHI Grant No. JP17H02082, and the Kyoto University Hakubi Project.

AUTHOR CONTRIBUTIONS

R.S., R.O. and Y-s.T. designed the study, conducted the experiments, interpreted the data and wrote the manuscript. A.L., T.M., C.M., E.S. and K.K. conducted the experiments. T.N. established AGMS-3 cell line. Y-s.T., A.N., and M.K.S. supervised the study. R.S., R.O., Y-s.T., A.N. and M.K.S. commented and wrote the paper.

DISCLOSURES

The authors declare they have no competing financial interests.

METHODS

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yu-suke Torisawa (torisawa.yusuke.6z@kyoto-u.ac.jp).

Cell lines

All the experiments of this study were performed with 409B2 iPSCs or CBA11 iPSCs (Ohta, 2019). HUVECs were either purchased from Angio-Proteomie (GFP-HUVEC, cAP-01001GFP) or Lonza (issue Acquisition Number: 29000). AGMS-3 was isolated and cultured as described previously (Xu 1998).

hPSC culture

The maintenance of hPSCs was done using iMatrix-511 (Matrixome) in mTeSR1 media (STEMCELL Technologies). Media were changed every other day, and the cells were passaged as single cells every 7 days using TrypLE Express (Life technologies).

Cell Culture

Green fluorescent protein-expressing and red fluorescent protein-expressing HUVECs (GFP-HUVECs and mCherry-HUVECs, respectively, Angio-Proteomie) were cultured in endothelial cell growth medium-2 (EGM-2, Lonza). All the experiments were conducted using HUVECs of

passage 7 or lower. AGMS-3 cells were cultured in alpha-MEM (Thermo Fischer Scientific) containing 15% FBS, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin.

Hemogenic endothelial differentiation

hPSC spheroids were formed as described previously (JoVE, 2019), suspended in mTeSR1 (StemCell Technologies) containing 1.25 µg/mL iMatrix-511 (Matrixome), and plated onto non-coated culture plates. After three days, the medium was replaced with Essential 8 (Life technologies) containing 4 µM CHIR99021 (WAKO), 80 ng/mL BMP4 (R&D systems), and 80 ng/mL VEGF (R&D systems). After two more days, the medium was replaced with Day 2 differentiation medium: Essential 6 with 1 µM SB431542, 80 ng/mL VEGF, and 100 ng/mL Stem cell factor (SCF). Finally, two days later, the cells were harvested with TrypLE express, and CD34+ cells were isolated by MACS.

Colony forming unit assay

After 1 or 2 weeks of culture, CD34+ cells were sorted by MACS and cultured at a concentration of 1×10^3 cells/mL in one well of a 6-well plate (#1008; Becton-Dickinson) with 1 mL/well MethoCult GF+ semisolid medium (#4435; STEMCELL Technologies). Colonies were counted after 14 days of incubation using an inverted microscope by three individuals.

Flow cytometry

Cells grown in culture or harvested from animal tissues were stained with 4:200-1:200 dilution of each antibody for at least 30 min on ice in the dark with the antibodies CD34-APC and CD45-PE. Unless specifically indicated, all the antibodies used are against human cells. Acquisitions were

done on a BD FACSAria II cell sorter or BD LSRFortessa cytometer. Sorting was performed on the BDFACS Aria II cell sorter. Flow cytometry data were analyzed using FlowJo V.10.

Fabrication of Microfluidic Device

The microfluidic device consists of two layers of microchannels separated by a semipermeable membrane. The microchannel layers were fabricated from PDMS using a soft lithographic method (Duffy, 1998). PDMS prepolymer (Sylgard 184, Dow Corning) at a ratio of 10:1 base to curing agent was cast against a mold composed of SU-8 2150 (MicroChem) patterns formed on a silicon wafer. The cross-sectional size of the microchannels was 1 mm in width and 300 um in height. To introduce solutions into the channels, access holes were punched through the PDMS using a 6-mm biopsy punch. Two PDMS layers were bonded to a semipermeable PET membrane containing 3.0 um pores (#353091 Falcon) using a thin layer of liquid PDMS prepolymer as a mortar (Wu, 2005). PDMS prepolymer was spin-coated (4000 rpm for 60 s) onto a glass slide. Subsequently, both the top and bottom channel layers were placed on the glass slide to transfer the thin layer of PDMS prepolymer onto the embossed PDMS surfaces. The membrane was then placed onto the bottom layer and sandwiched with the top layer. The combined layers were left at room temperature for 1 day to remove air bubbles and then put into an oven at 60 ^{o}C overnight to cure the PDMS glue.

Microfluidic Cell Culture

The microfluidic device was sterilized in UV light for 2 hours. Following the sterilization, the semipermeable membrane of the device was coated with fibronectin (#33016015, Gibco) by incubating the microchannels with a fibronectin solution (100 ug mL^{-1} in PBS) at room temperature

for 2 hours. The channels were then rinsed with culture medium prior to cell seeding. A suspension of HUVECs (5 x 10^4 cells per 10 uL) was introduced into the bottom channel, and then the device was inverted and incubated at 37 ^oC for 1 hour to allow the cells to adhere onto the bottom side of the membrane. Once cellular attachment was confirmed, the device was flipped back, and the microchannels were filled with culture medium (EGM-2). After 1 day in culture, a suspension of AGMS-3 cells (2 x 10^4 cells per 10 uL) was introduced into the top channel. After incubation at 37 ^oC for 1 hour, culture medium (alpha-MEM) was introduced into the top channel and incubated at 37 ^oC for 1 day to form layers of endothelium and mesenchymal stroma. Subsequently, a suspension of HE cells (5 x 10^4 cells per 10 uL) was introduced into the top channel and incubated at 37 ^oC for 2 hours to enable cell attachment. The reservoirs were filled with EHT medium (150 uL in each reservoir), and the device was put in an incubator (37 ^oC, 5% CO_2). The device was cultured for up to 14 days with replacement of the culture medium every 2 or 3 days.

Statistics and source data

Statistical analyses were done with t-test. We used Microsoft Excel for calculations and expressed the results as the means ± s.d. The source data for each graph is available in the supplementary tables.

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FIGURE LEGENDS

Figure 1. Construction of Aorta-Gonad-Mesonephros **(**AGM)-on-a-chip. (A) Scheme of the AGM region in the human embryo. Hemogenic endothelium (HE), mesenchymal stroma (MS) and

endothelial cells (EC) compose the AGM. (B) Scheme of the AGM-chip. HE and AGMS-3 cells are placed in the upper layer and HUVECs in the lower layer. A porous membrane in the middle allows the cells to interact through the layers. (C) Structure of the AGM-chip. The upper and lower layers are made of PDMS and sandwich the porous membrane. A ditch runs through the center of the chip and is where the cells are cultured. Medium is infused and circulates. (D) Photograph of the AGM-chip. Scale bar = 1 cm.

Figure 2. Cellular components of AGM-chip. Left; A bright-field microscopy image of HE seeded on the chip format without feeder cells. The formation of round-shaped hematopoietic cells is shown. Right; A confocal microscopy image of the AGM-chip with feeder cells. GFP-labeled HE cells (green), mCD13-stained AGMS-3 cells (magenta), and RFP-labelled HUVECs (cyan) are seen. Cross-sectional images are shown on the right and bottom. Scale bar = 100 um.

Figure 3. EHTin AGM-chip. (A) Experimental scheme. HE was seeded with or without feeder cells (AGMS-3 and HUVEC). At either 1 or 2 weeks, whole cells from the chip were collected and analyzed with HPC markers (CD34 and CD45) and with methylcellulose hematopoetic CFU assay. The bar graphs show the number of HPCs (B) (1 w; n=2, 2 w; n=3-5) and CFU (C) (1 w; n=3, 2 w; n=3-5). Each dot represents the result from one biologically independent experiment.

Figure 4. Recovery of HPCs from the outflow of AGM-chip. (A) Experimental scheme. GFP-labeled HE, AGMS-3 and HUVEC are seeded. After 1-week culture, cells from the outflow were analyzed with the HPC markers CD34 and CD45 and with CFU. (B) FACS plot of the outflow cells with CD34 and CD45. (C) A GFP+ colony under fluorescence microscopy. Scale bar = 200 um.

Figure 1

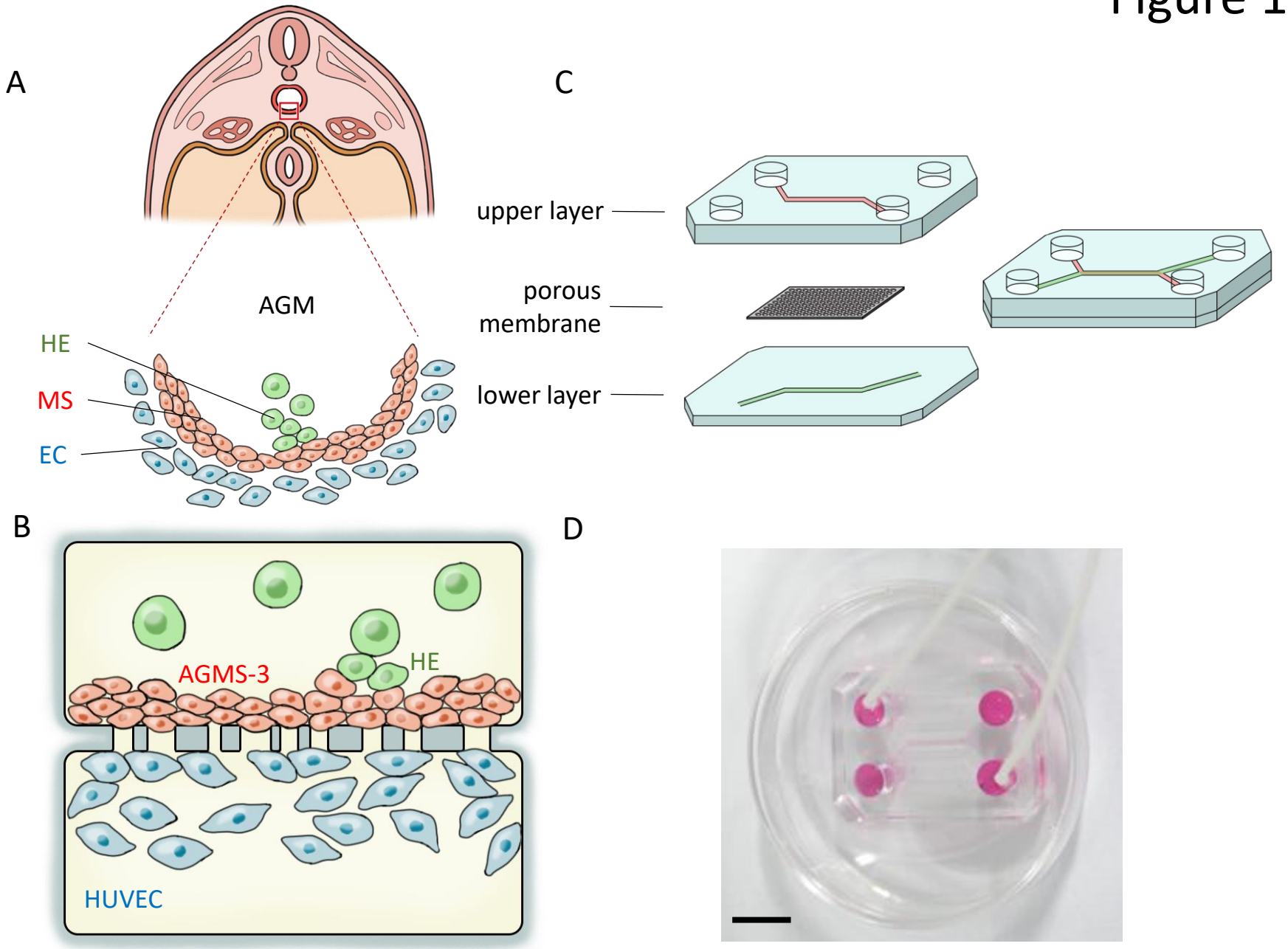

A
AGM
HE
MS
EC
C
upper layer
porous membrane
lower layer
B
AGMS-3
HE
HUVEC
D

Figure 2

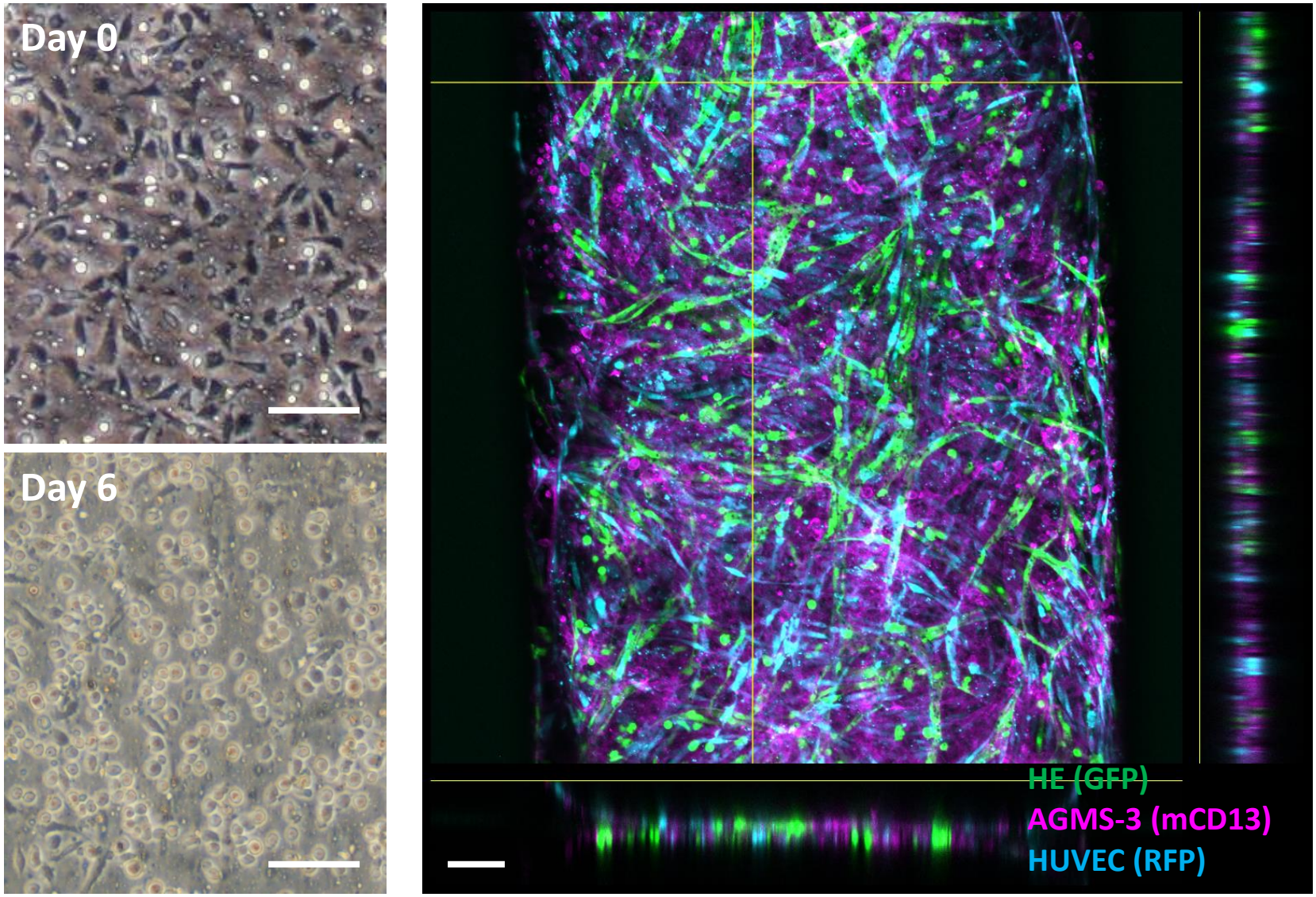


Figure 3

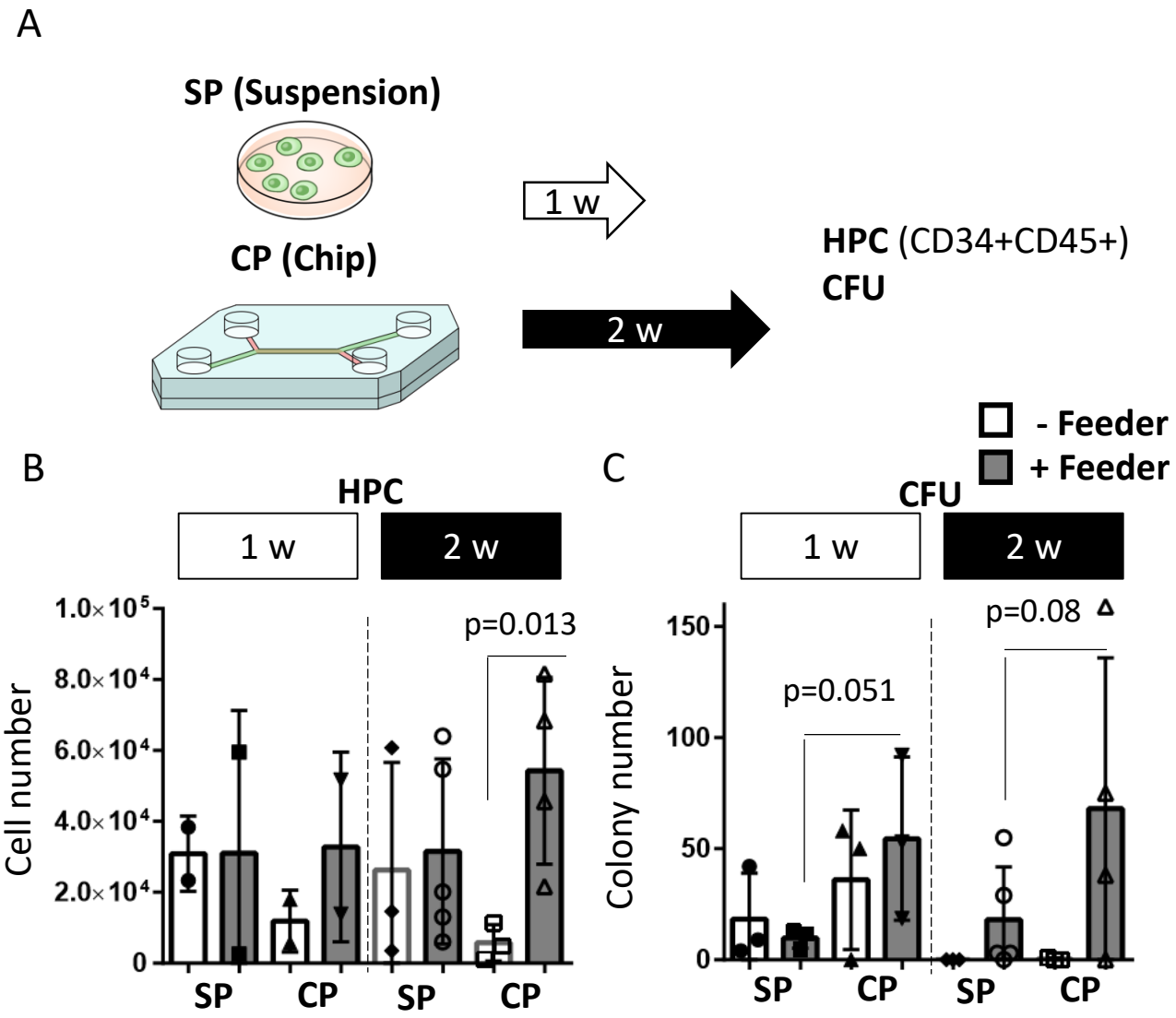
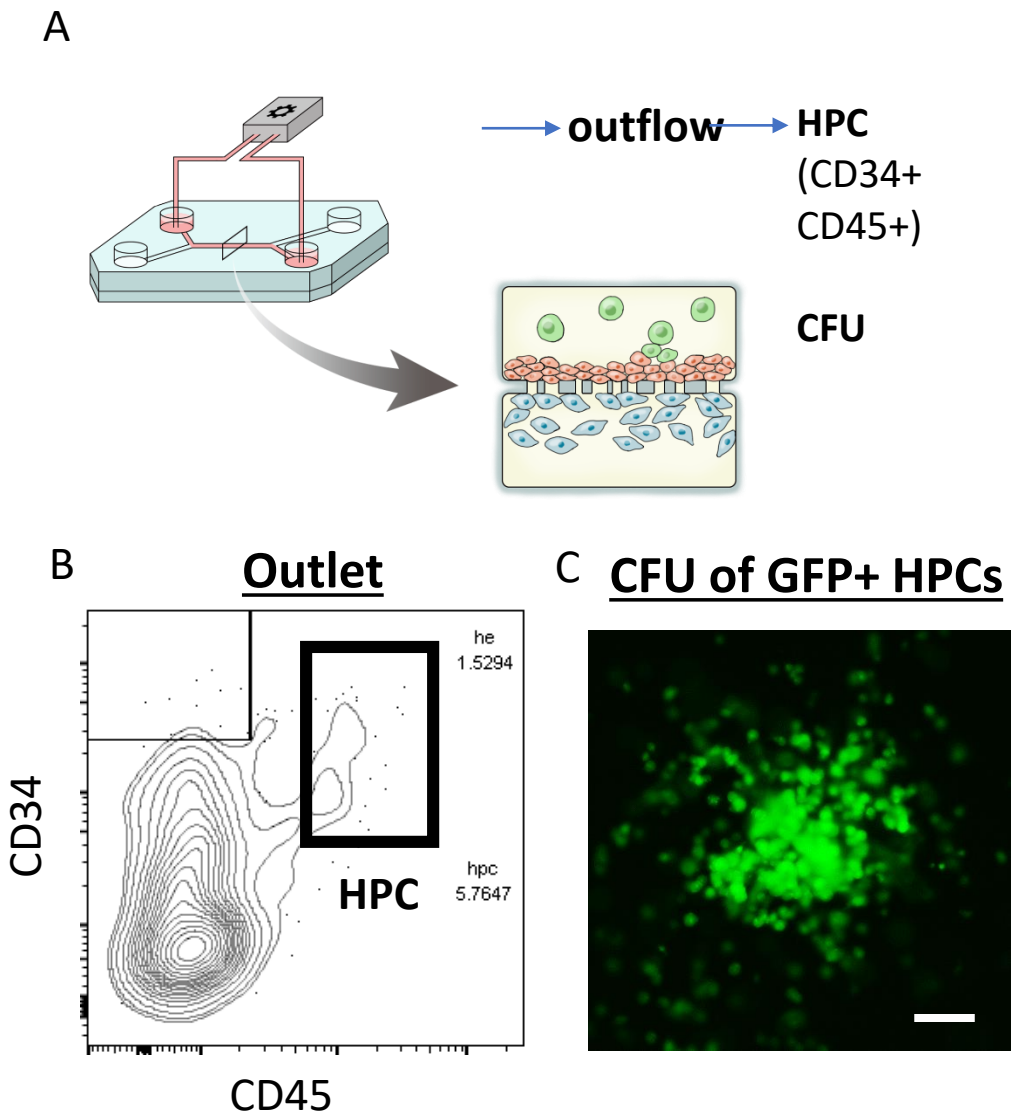

A
SP (Suspension)
CP (Chip)
1 w
2 w
HPC (CD34+CD45+)
CFU
B
HPC
1 w
2 w
Cell number
1.0×10⁵
8.0×10⁴
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
p=0.013
SP
CP
SP
CP
C
- Feeder
+ Feeder
CFU
1 w
2 w
Colony number
150
100
50
0
p=0.051
p=0.08
SP
CP
SP
CP

Figure 4


A
outflow
HPC
(CD34+
CD45+)
CFU
B
Outlet
he
1.5294
HPC
hpc
5.7647
CD34
CD45
C
CFU of GFP+ HPCs

1 week

SP-feeder	SP+feeder	CP-feeder	CP+feder
23400	2600	5712	13903
38400	59500	18076	51708

2 weeks

SP-feeder	SP+feeder	CP-feeder	CP+feder
60750	54720	11310	68340
3500	64000	1156	45600
14620	6000	4866	81600
	13000		21660
	20098		

1 week

SP-feeder	SP+feeder	CP-feeder	CP+feder
9	11.6	58	53
4	4.6	0	18.6
42	13	50	92

2 weeks

SP-feeder	SP+feeder	CP-feeder	CP+feder
0	55	1	75
0	29	0	38
0	3	0	159
	3		0
	0		