



Extracellular laminin regulates hematopoietic potential of pluripotent stem cells through integrin β 1-ILK- β -catenin-JUN axis

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ABSTRACT

Recombinant matrices have enabled feeder cell-free maintenance cultures of human pluripotent stem cells (hPSCs), with laminin 511-E8 fragment (LM511-E8) being widely used. However, we herein report that hPSCs maintained on LM511-E8 resist differentiating to multipotent hematopoietic progenitor cells (HPCs), unlike hPSCs maintained on LM421-E8 or LM121-E8. The latter two LM-E8s bound weakly to hPSCs compared with LM511-E8 and activated the canonical Wnt/ β -catenin signaling pathway. Moreover, the extracellular LM-E8-dependent preferential hematopoiesis was associated with a higher expression of integrin β 1 (ITGB1) and downstream integrin-linked protein kinase (ILK), β -catenin and phosphorylated JUN. Accordingly, the lower coating concentration of LM511-E8 or addition of a Wnt/ β -catenin signaling activator, CHIR99021, facilitated higher HPC yield. In contrast, the inhibition of ILK, Wnt or JNK by inhibitors or mRNA knockdown suppressed the HPC yield. These findings suggest that extracellular laminin scaffolds modulate the hematopoietic differentiation potential of hPSCs by activating the ITGB1-ILK- β -catenin-JUN axis at the undifferentiated stage. Finally, the combination of low-concentrated LM511-E8 and a revised hPSC-sac method, which adds bFGF, SB431542 and heparin to the conventional method, enabled a higher yield of HPCs and higher rate for definitive hematopoiesis, suggesting a useful protocol for obtaining differentiated hematopoietic cells from hPSCs in general.

1. Introduction

Since the reporting of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) (Thomson et al., 1998; Takahashi et al., 2007), researchers have applied human pluripotent stem cells (hPSCs) as a stable cell source for regenerative medicine and the establishment of disease models to elucidate the pathogenesis and develop novel treatments. For the culture of hPSCs to maintain pluripotency, mouse embryonic fibroblasts (MEFs) are used as feeder cells, while Matrigel, collagen, fibronectin, proteoglycan, vitronectin, E-cadherin, laminin, and combinations of these molecules are used as feeder-free scaffolds (Nakashima and Omasa, 2016). Among the intact laminin

isoforms, laminin-511, -521, -332, and -111 support hPSC proliferation in the undifferentiated state (Nakashima and Omasa, 2016), and truncated LM511-E8, which is composed of the E8 component of the recombinant laminin α 5, β 1, and γ 1 chains, was proposed as an extracellular scaffold for hPSC maintenance, as it can be easily synthesized and shows higher integrin affinity than intact laminins (Miyazaki et al., 2012). Good manufacturing process (GMP)-grade LM511-E8 has been commercialized and widely used to maintain the undifferentiated state of hPSCs under conditions that meet GMP criteria with simple procedures in Japan (Miyazaki et al., 2017). LM511-E8 binds tightly to integrin α 6 β 1 on the hPSC membrane, and hPSCs maintain their pluripotency through PI3K/AKT and Fyn-RhoA-ROCK signaling (Miyazaki

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et al., 2012; Nakashima and Omasa, 2016). Meanwhile, several reports show that LM-E8s can support cell type-specific differentiation to, for example, ocular cells, endothelial progenitor cells and hepatoblast-like cells from hPSCs (Shibata et al., 2018; Yap et al., 2019), but how the application of different extracellular laminin isoforms to undifferentiated hPSCs induces intracellular pathways to promote different cellular differentiation fates has not been characterized.

For the hematopoietic differentiation of hPSCs, various monolayer two-dimensional (2D) cultures and three-dimensional (3D) embryoid body methods have been reported (Hansen et al., 2019; Sturgeon et al., 2014; Takayama et al., 2008). All of them basically differentiate hematopoietic cells through the primitive streak-like state and beyond the mesodermal state. However, it is still uncertain which method is most efficient for yielding functional hematopoietic cells (Hansen et al., 2019). We previously established the feeder-dependent “hPSC-sac method” by culturing hPSCs on mesenchymal type C3H10T1/2 mouse stromal cells with only one cytokine, vascular endothelial growth factor (VEGF), in a 2D culture system (Takayama et al., 2008). This simple method yields multipotent hematopoietic progenitor cells (HPCs) in a sac-like structure, which further differentiate into various types of blood cells. This was the first procedure to generate megakaryocytes and functional platelets from hPSCs and has also been widely used to differentiate erythroblasts, neutrophils, monocytes, and T lymphocytes (Chao et al., 2017; Nakamura et al., 2014; Nishimura et al., 2013; Takei et al., 2018). However, the number of HPCs obtained is small compared to the number of hPSCs at the start of the differentiation.

In this study, we addressed the impaired hematopoietic commitment of hPSCs maintained on various LM-E8 scaffolds. We compared different LM-E8s to find the mechanism and signaling pathway that affect the hematopoietic differentiation potential of hPSCs and sought various measures to overcome this impairment. We assessed the combination of factors that enhance the efficiency of the hPSC-sac method to establish the “revised hPSC-sac method”. Finally, we integrated these measures to enhance the yield of HPCs and assessed terminal hematopoietic differentiation with remarks on the definitive-type.

2. Materials and methods

2.1. Cells

The hESC lines KhES-3 and KthES11 (hES 1) were obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan), and H1 (hES 2) was obtained from the WiCell Research Institute (Madison, WI, USA) with approval from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The hiPSC lines 1383A8 (hiPSC 1), 692D2 (hiPSC 2), TkDN-Sev2 and MK04 were established in house (Ito et al., 2018; Okita et al., 2013). Use of the cells was approved by an ethics committee at Kyoto University, and all studies involving the use of human samples were conducted in accordance with the Declaration of Helsinki.

2.2. Reagents

The canonical Wnt/ β -catenin signaling pathway activator CHIR99021 (6 μ M) was purchased from Tocris (Bristol, UK), ILK inhibitor Cpd22 (1 μ M) from Calbiochem (Merck, Darmstadt, Germany), Wnt inhibitor Wnt-C59 (1 μ M) from Selleck Chemicals (Houston, TX, USA), and SP600125 (10 μ M) from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Maintenance of hPSCs

hPSCs were maintained on Mitomycin C-treated MEFs, as previously described (Hirata et al., 2013), or on laminin-E8 fragments (LM-E8s). LM-E8s (LM521-E8, LM511-E8, LM421-E8, LM411-E8, LM332-E8, LM321-E8, LM311-E8, LM121-E8, and LM111-E8) were provided by one of the authors (K.S.), and LM511-E8 was purchased from Nippi (Tokyo,

Japan). 6-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland. #92406) were precoated with LM-E8s at a concentration of 0.5 μ g/cm², except LM411-E8 (1.0 μ g/cm²), for 1 h at 37°C in CO₂ incubators. For LM511-E8, diluted precoating was done as shown in Fig. 3B. hPSCs were cultured over 5 days on each scaffold in AK02N medium (Ajinomoto, Tokyo, Japan) with 10 μ M Y27632 (Wako, Osaka, Japan). For passaging, the cells were detached by treatment with 0.5 $\times$ TrypLE Select (Gibco, Carlsbad, CA, USA) and 5 mM EDTA/DPBS for 4 min at 37°C in a CO₂ incubator and were scraped and then seeded at 0.1 $\times$ 10⁵ cells/well onto an LM-E8-coated well (Nakagawa et al., 2015).

2.4. Hematopoietic cell differentiation

2.4.1. Differentiation from hPSCs to HPCs by the hPSC-sac method

Small clumps of equivalent numbers (0.7 to 0.8 $\times$ 10⁵ cells) of hPSCs treated with 1 mM CaCl₂ (Sigma-Aldrich) and 20% KSR (Invitrogen, Carlsbad, CA, USA) or 181 PU/ml Disperse I (Wako) were transferred onto a 10 cm culture dish (#150466, Thermo Fisher Scientific, Waltham, MA, USA) coated with mitomycin C-treated C3H10T1/2 cells (Riken Bio-Resource Center, Tsukuba, Ibaraki, Japan) on gelatin and cocultured in hematopoietic cell differentiation medium consisting of IMDM (Sigma-Aldrich) with 15% fetal bovine serum (FBS), insulin/transferrin/selenite solution and penicillin streptomycin-L-glutamine solution (Gibco; Thermo Fisher Scientific), 50 μ g/ml ascorbic acid (Sigma-Aldrich), 450 μ M α -monothiolglycerol (Sigma-Aldrich), and 20 ng/ml recombinant human VEGF (Wako). This differentiation was based on the hPSC-sac method (Hirata et al., 2013; Takayama et al., 2008). We modified the method to place the cell culture dishes in a CO₂ incubator with hypoxia (5% O₂) on days 1–7. In the revised hPSC-sac method, 50 ng/ml basic FGF (Wako, Osaka, Japan) and 10 μ M SB431542 (Wako) were added on days 4–7, and 10 U/ml Heparin (AY pharmaceuticals, Tokyo, Japan) on days 4–10 (FSH + H in Fig. 5B). The media was replaced every 3–4 days. On day 9 or 14 of the culture, the cells were collected with a cell scraper and filtered with a cell strainer. The differentiated cells were counted using trypan blue exclusion staining. Then we analyzed and sorted the cells by FACSVerse and FACS Aria II (BD Biosciences, San Jose, CA, USA). We calculated the number of differentiated cells from the number of cells on dishes and percentage of target cells.

2.4.2. Erythrocyte, megakaryocyte and platelet differentiation

The sorted CD34⁺CD43⁺HPCs or human cord blood CD34⁺CD133⁺ cells (purchased from ZenBio, Research Triangle, NC, USA) were transferred onto mitomycin C-treated C3H10T1/2 cells in differentiation medium without VEGF. For erythroid-lineage differentiation, 50 ng/ml human SCF (R&D Systems, Minneapolis, MN, USA), 5 U/ml EPO (Kyowa Hakko Kirin, Tokyo, Japan), 10 ng/ml human TPO (R&D), 50 μ M Trolox (Cayman, Ann Arbor, MI, USA), and 50 μ M IBMX (Sigma-Aldrich) were added (modified from Hirata et al., 2013). For megakaryocyte/platelet-lineage differentiation, 50 ng/ml human SCF, 50 ng/ml human TPO, 25 units/ml Heparin, 750 nM SR1 (Selleck), 10 μ M Y27632 (Wako), and 15 μ M KP-457 (Kaken Pharmaceutical, Tokyo, Japan) were added (Ito et al., 2018). Nonadherent cells were counted using trypan blue exclusion staining and analyzed on days 21 (day + 21) and 10 (day + 10) after the differentiation of CD34⁺CD43⁺HPCs to erythrocyte and megakaryocyte lineages, respectively. We calculated the number of differentiated cells from the number of cells and percentage of target cells. To estimate the number of platelets, Trucount Tubes (BD biosciences) were used.

2.4.3. T Cell lineage differentiation

24-well plates coated with StemSpan lymphoid differentiation coating material (STEMCELL Technologies, Vancouver, BC, Canada) were incubated at room temperature as described in the manufacturer's protocol. The sorted CD34⁺CD43⁺HPCs or human cord blood CD34⁺CD133⁺ cells were seeded in wells and incubated in StemSpan

SFEMII medium (STEMCELL Technologies) with 10 ng/ml TPO, 10 ng/ml SCF, 5 ng/ml Flt3L (Peprotech, Rocky Hill, NJ, USA), and 20 ng/ml IL-7 (Miltenyi Biotec, Bergisch Gladbach, Germany) on days 0 to + 10. Then from days + 10 to + 21, they were incubated in StemSpan SFEMII medium with 100 ng/ml TPO, 50 ng/ml SCF, 50 ng/ml Flt3L, 50 ng/ml IL-7, 15 μ M SB203580 (Calbiochem), and 30 ng/ml SDF1 α (R&D Systems) (modified from Iriguchi et al., 2021; Minagawa et al., 2018). All cells in the well were scraped and collected. We counted the cells using trypan blue exclusion staining and stained for flow cytometry analysis and then calculated the number of differentiated cells from the number of cells and percentage of target cells.

2.4.4. Hematopoietic colony-forming cell assay

The sorted CD34⁺CD43⁺HPCs were cultured in semisolid media (MethoCult H4434 Classic; STEMCELL Technologies). At day + 21, colony forming units (CFUs) were counted under a microscope.

2.5. Statistical analysis

Data analysis and plots were generated using Prism (GraphPad). Data are represented as the mean + standard error of the mean (SEM) when $n = 3$ or more. Two-tailed student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparisons test was used to determine statistical significance. The level of significance was set at 0.05.

3. Results

3.1. Laminin 511-E8 scaffold restricts hematopoietic differentiation through downregulation of the canonical Wnt/ β -catenin signaling pathway

Feeder-free maintenance on LM511-E8 fragments has become standard to providing hiPSCs for regenerative medicine (Doi et al., 2014; Sasaki et al., 2015; Shibata et al., 2018; Takebe et al., 2017; Umekage et al., 2019). However, we noticed that unlike hPSCs maintained on MEFs, hPSCs maintained on LM511-E8 at a regular concentration of 0.5 μ g/cm² poorly formed sac-like structures and yielded a low number of CD34⁺CD43⁺HPCs from equivalent numbers of hPSCs (Supplemental figure S1A-C). We also differentiated the hPSCs maintained on different scaffolds of LM-E8 isoforms with the same hPSC-sac method on C3H10T1/2 cells (Fig. 1A). hPSCs maintained on 0.5 μ g/cm² LM421-E8 or LM121-E8 could successfully induce CD34⁺CD43⁺HPCs, but not if maintained on six other LM-E8 types (Fig. 1B). This observation held for a different hPSC line (Fig. 1C). These results suggest that the scaffolds used only during the maintenance culture of hPSCs modulate the hematopoietic differentiation of hPSCs afterward. To identify which genes were affected in the hPSCs maintained by the different LM-E8 scaffolds, we performed a transcriptome analysis using microarrays and averaged the gene expressions in hiPSCs on LM-E8s in two groups: hematopoietic LM-E8s (LM421 and LM121) and non-hematopoietic LM-E8s (Fig. 1D). Forty-three genes were upregulated more than two-fold and had an FDR < 0.05 in hiPSCs maintained on the hematopoietic LM-E8 group compared with the non-hematopoietic group (Supplemental figure S1D and Supplemental table 2). An upstream regulator analysis by Ingenuity Pathway Analysis (IPA, Qiagen Inc, Hilden, Germany) indicated that CTNNB1 (β -catenin) was the most relevant upstream molecule (Fig. 1D and Supplemental figure S1E). We confirmed the upregulation of CTNNB1 downstream genes in another hPSC line on hematopoietic LM-E8 group by qRT-PCR (Supplemental figure S1F). Accordingly, the addition of a GSK3 β inhibitor, CHIR99021, which is known as a canonical Wnt/ β -catenin signaling pathway activator (Kitajima et al., 2016), in the maintenance culture of hiPSCs on LM511-E8 increased the sac-like structure formation and CD34⁺CD43⁺HPC yield on day 14 (Fig. 1E-G). These results suggest that poor activation of the canonical Wnt/ β -catenin signaling pathway in maintained hPSCs explains the impaired hematopoietic commitment potential.

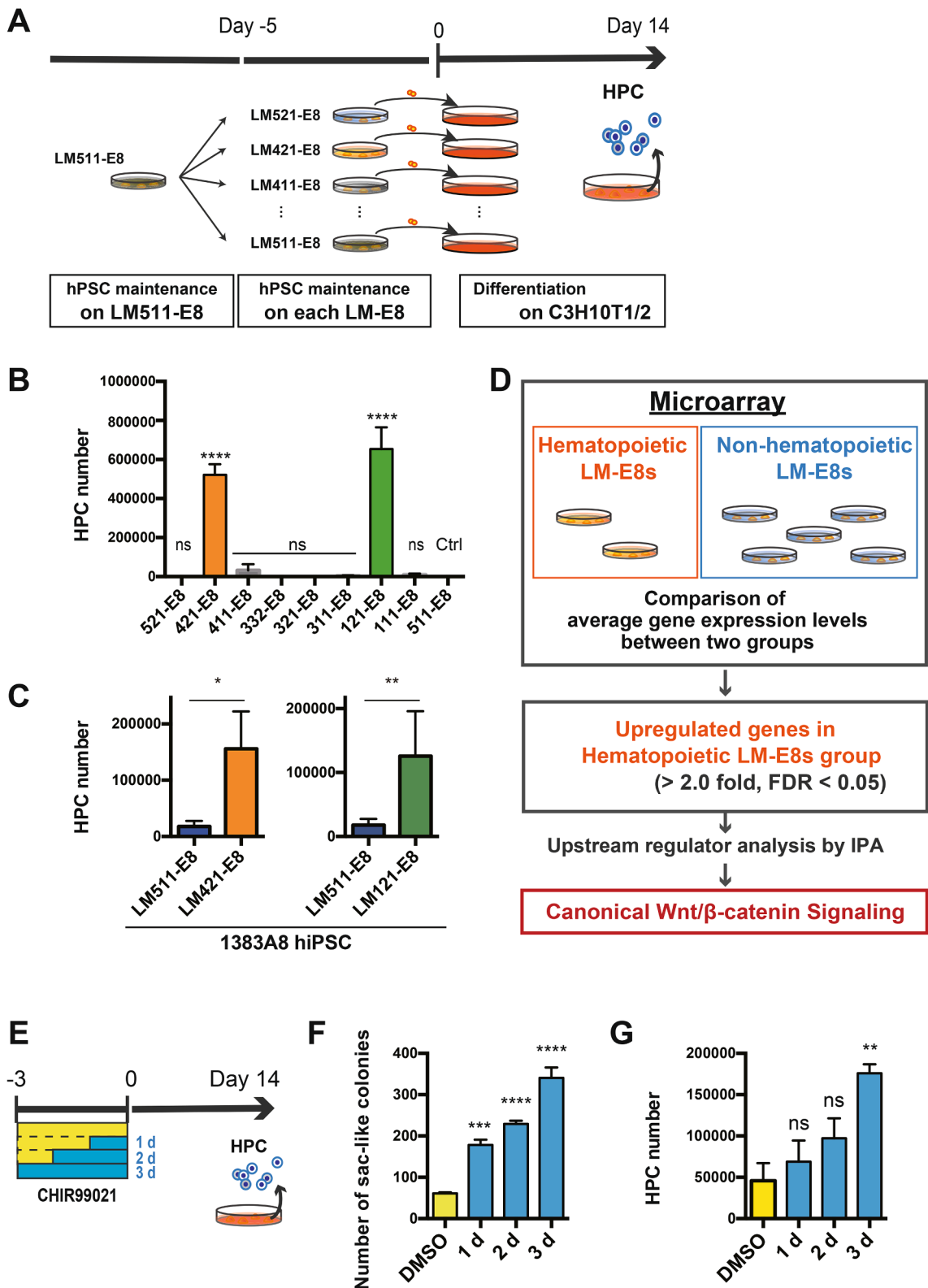
3.2. hPSCs on hematopoietic LM-E8 present lower adhesion but express higher levels of ITGB1 and ILK

Laminins are molecules that bind to an integrin complex for cell adhesion. Integrin α 6 β 1 is the most dominant integrin complex expressed on hPSCs (Nishiuchi et al., 2006). Moreover, the cytoplasmic domain of ITGB1, which is the β subunit of integrin α 6 β 1, activates the canonical Wnt/ β -catenin signaling pathway via integrin-linked protein kinase (ILK) in many cells (HEK293 and MDA231 human cell lines, ovarian carcinoma and melanoma cells, etc.) (Burkhalter et al., 2011; Oloumi et al., 2006; Piva et al., 2017). To verify how LM-E8 scaffolds affect the canonical Wnt/ β -catenin signaling pathway through ITGB1 on undifferentiated hPSCs, we first checked the protein expression of ITGB1 and found that it was relatively higher in hPSCs maintained on hematopoietic LM421-E8 and LM121-E8 compared with non-hematopoietic LM511-E8 or LM521-E8 by immunofluorescence microscopy and flowcytometry analysis (Fig. 2A, 2B and Supplemental figure S2A). So too were the expressions of ILK, β -catenin and phosphorylated JUN (pJUN), which is a downstream molecule of the Wnt/ β -catenin signaling pathway (Qu  lo et al., 2004; Troussard et al., 1999), and included in the 43 genes upregulated in LM421-E8- or LM121-E8-maintained hPSCs (Fig. 2C, Supplemental figure S1D and S2B). Notably, although the hPSC colonies did not show typical round morphology with defined edges, the pluripotent phenotype of the hPSCs was consistent across all laminins examined (Supplemental figure S2C). Unexpectedly, the cell adhesion assay, which reflects the mechanical interaction strength between laminins and ITGB1, revealed that the strength of the adhesion was weaker with hPSCs on hematopoietic LM421-E8 and LM121-E8 than on non-hematopoietic LM511-E8 or LM521-E8 (Fig. 2D). These results suggest the weaker adhesion of hPSCs cultured on LM421-E8 or LM121-E8 results in the upregulation of ITGB1 and downstream ILK to activate the canonical Wnt/ β -catenin signaling pathway.

3.3. A lower coating concentration of non-hematopoietic LM511-E8 provides a higher HPC yield and enhances ITGB1 expression on hPSCs

We accordingly questioned whether decreasing the binding avidity by simply lowering the concentration of LM511-E8 for the culture plate coating could induce hematopoietic differentiation. Serial dilution of LM511-E8 decreased the binding avidity of hPSCs (Fig. 3A) and increased the expression levels of ITGB1 and ILK (Fig. 3B, 3C, Supplemental figure S3A and B) in undifferentiated hPSCs and also the differentiated CD34⁺CD43⁺HPC yield from hPSCs (Fig. 3D). The dilution did not affect the pluripotent phenotype of the hPSCs (Supplemental figure S3C). We also observed that a dilution of $\times 1/32$ or greater (dilution factor ≥ 32) could not keep hPSCs attached to the dish for maintenance culture (data not shown).

The expression level of ITGB1 on hPSCs cultured on LM511-E8 $\times 1/8$ (dilution factor = 8, concentration = 0.0625 μ g/cm²) was comparable to that on hPSCs cultured on LM121-E8 or LM421-E8 (Fig. 3E). The expression of β -catenin and pJUN were also upregulated by the dilution of LM511-E8 (Fig. 3F and Supplemental figure S3D). Additionally, the partial positive correlation of the ITGB1 expression and CD34⁺CD43⁺HPC yield was observed in four hPSC cell lines assessed at different LM511-E8 dilutions (Supplemental figure S4A). The dilution effect of LM511-E8 was conserved between several hPSC lines (Supplemental figure S4B-G). Moreover, dilution of another non-hematopoietic LM-E8 isoform, LM521-E8 (concentration = 0.03125 μ g/cm²), also upregulated ITGB1, its downstream signals, and the differentiated CD34⁺CD43⁺HPC yield (Supplemental figure S4H-J). These results suggested that the higher weak adhesion-associated ITGB1 levels might represent a crucial mechanism for enhanced hematopoiesis through the canonical Wnt/ β -catenin signaling pathway even if hPSCs are maintained on "non-hematopoietic type" isoforms such as LM511-E8 or LM521-E8.



(caption on next page)

Fig. 1. Extracellular laminin scaffolds determine the hematopoietic potential of hPSCs by controlling the canonical Wnt/ β -catenin signaling pathway. (A) Schema of the experiments for comparing the effect of LM-E8s on the maintenance of hPSCs for HPC differentiation (basic conditions = $0.5 \mu\text{g}/\text{cm}^2$). MK04 hiPSCs maintained on LM511-E8 with feeder-free culture were transferred to different LM-E8-coated plates. Five days later, each hiPSC line was differentiated to HPCs with the same hPSC-sac method on C3H10T1/2 feeder cells (the hPSC-sac method is shown in Supplemental figure S1A). (B) The number of $\text{CD}34^+\text{CD}43^+$ HPCs on day 14 differentiated from 0.7 to 0.8×10^5 MK04 hiPSCs. MK04 hiPSCs were maintained on various LM-E8s until day 0. $n \geq 3$, mean $\pm$ SEM. **** $P < 0.0001$ vs. control LM511-E8 (Ctrl) by Dunnett's test. n.s., not significant. (C) The number of $\text{CD}34^+\text{CD}43^+$ HPCs on day 14 differentiated from 0.7 to 0.8×10^5 1383A8 hiPSCs. 1383A8 hiPSCs were maintained on LM-E8s until day 0. $n \geq 3$, mean $\pm$ SEM. * $P = 0.0103$, ** $P = 0.0087$ by t test. (D) A flowchart to identify the specific signaling in hPSCs on hematopoietic LM-E8s from microarray analyses. Ingenuity Pathway Analysis (IPA) indicated the canonical Wnt/ β -catenin signaling pathway as the upstream regulator. (See also Supplemental figure S1D and S1E.) (E) 1383A8 hiPSCs maintained on LM511-E8 were pre-activated for the canonical Wnt/ β -catenin signaling pathway by CHIR99021 on days 0 to 3 and then differentiated by the conventional hPSC-sac method for 14 days. Yellow and blue columns represent the addition of DMSO and CHIR99021, respectively. (F) The number of sac-like structure colonies per equivalent number of hPSCs in (E). $n \geq 3$, mean $\pm$ SEM. **** $P < 0.001$ and **** $P < 0.0001$ vs. DMSO by Dunnett's test. (G) The number of $\text{CD}34^+\text{CD}43^+$ HPCs on day 14 differentiated from 0.7 to 0.8×10^5 hPSCs in (E). $n = 3$, mean $\pm$ SEM. ** $P < 0.01$ vs. DMSO by Dunnett's test. n.s., not significant.

3.4. ILK activates the canonical Wnt/ β -catenin signaling pathway to induce hematopoietic differentiation

We next pre-treated hPSCs on low-concentrated LM511-E8 x1/8 with several inhibitors prior to hPSC-sac differentiation (Fig. 4A). Treatment with any of an ILK inhibitor (Cpd22), WNT inhibitor (Wnt-C59), or JNK inhibitor (SP600125) lowered the $\text{CD}34^+\text{CD}43^+$ HPC yield similarly (Fig. 4B). Accordingly, the knockdown of ILK by doxycycline-inducible shRNA (Fig. 4C and Supplemental figure S5A and S5B) reduced the expression of β -catenin and pJUN (Fig. 4D and Supplemental figure S5C) and the $\text{CD}34^+\text{CD}43^+$ HPC yield (Fig. 4E). Given that the inhibitors were applied for only 2 days before differentiation (Fig. 4A) and that the knockdown of ILK did not affect the number of hPSCs (Supplemental figure S5D), we concluded that the downregulation of ILK signaling had minimal effect on cell survival or proliferation during differentiation. Together, these results suggest that ILK, a downstream molecule of ITGB1, is activated through the canonical Wnt/ β -catenin signaling pathway to induce the hematopoietic differentiation of hPSCs.

3.5. The combination of bFGF, TGF- β inhibitor and heparin increases hematopoietic differentiation efficiency in the hPSC-sac method

The hPSC-sac differentiation method, in which hPSCs are cultured on C3H10T1/2 or OP-9 feeder cells with VEGF, can generate HPCs inside sac-like structures (Takayama et al., 2008) and is widely used to derive various types of blood cells. This method has since been modified to add a hypoxia condition based on a report that showed hypoxia enhances mesoderm lineage differentiation (Ramírez-Bergeron et al., 2004 and Sano et al., Patent) (Supplemental figure S1A). We further sought to improve the hPSC-sac method by adding three soluble factors: basic fibroblast growth factor (bFGF), a transforming growth factor- β (TGF- β) inhibitor (SB431542), and heparin, on differentiation day 4 (Fig. 5A). bFGF is important for mesoderm differentiation (Flamme and Risau, 1992; Ng et al., 2016; Zhang et al., 2019); SB431542 enhances hematopoiesis from hPSCs by suppressing the TGF- β signal (Ng et al., 2016; Wang et al., 2012; Zhang et al., 2019); and heparin enhances bFGF action and inhibits CXCL4 to promote hematopoietic differentiation (Eslin et al., 2004; Spivak-Kroizman et al., 1994). The revised version of adding the combination of these three factors with prolonged heparin addition rather than adding just one or two dramatically increased the yield of $\text{CD}34^+\text{CD}43^+$ HPCs on day 14 more than 50-fold (average $\pm$ SEM; $19.11 \pm 7.245 \times 10^5$ vs. $0.36 \pm 0.095 \times 10^5$ HPCs/equivalent number of hPSCs; $P < 0.0001$) compared with the conventional hPSC-sac method (Fig. 5B).

3.6. Hemogenic endothelium-like cell yield increased with the revised hPSC-sac method

We next assessed the derivation of hemogenic endothelium (HE), which differentiates to HPCs (Boisset et al., 2010), during the conventional and revised hPSC-sac process. The *in vitro* yield of HE-like cells,

phenotyped as the $\text{CD}31^+\text{CD}117^+\text{CD}309^+\text{CD}73^+\text{CD}34^+\text{CD}144^+\text{CD}43^-\text{CD}45^-\text{CD}41^-$ population (Sturgeon et al., 2013) (Fig. 6A), was calculated by the cell number, which was significantly higher in the revised hPSC-sac method on day 9 from the equivalent number of hPSCs (Fig. 6B). In accordance with the higher number of HE-like cells, the expression of hematopoiesis-related genes, such as SPI1 (Nerlov and Graf, 1998), ERG (Loughran et al., 2008), TAL1, RUNX1c (Ditadi et al., 2015), and GFI1b (Khandanpour et al., 2010), was greatly enhanced (Fig. 6C). Notably, the expression of RUNX1c has been considered representative of definitive hematopoiesis (Ditadi et al., 2015). These results suggest that the revised hPSC-sac method increased the HPC yield by enhancing the HE-like cell population.

3.7. hPSCs on low-concentrated non-hematopoietic LM511-E8 with the revised hPSC-sac method deliver definitive hematopoiesis

Lastly, we compared the revised hPSC-sac method with the conventional method for hPSCs maintained on low-concentrated LM511-E8 x1/8 for terminal hematopoietic differentiation (Fig. 7A). The ratio of each differentiated blood cell lineage detected by the colony assay morphology was comparable from the same cell number of $\text{CD}34^+\text{CD}43^+$ HPCs (Fig. 7B and Supplemental figure S6A). As for erythroid differentiation by the revised method, the calculated count of $\text{CD}235^+$ erythroid cells expressing adult type beta-globin increased (Vinjamur et al., 2018) (Fig. 7C, Supplemental figure S6B and C). Next, regarding megakaryocyte-platelet differentiation, the revised hPSC-sac method tended to produce about a two-fold higher number of $\text{CD}41^+\text{CD}42b^+$ megakaryocytes and platelets (Fig. 7D, Supplemental figure S6D and E) from the same number of $\text{CD}34^+\text{CD}43^+$ HPCs. The efficiency for T cell lineage, which is considered to be derived from definitive hematopoiesis (Sturgeon et al., 2014), was also higher (Fig. 7E, Supplemental figure S6F and G). These results suggest that the revised sac method increases the rate of definitive hematopoiesis from hPSCs maintained on low-concentrated LM511-E8.

4. Discussion

We noticed hPSCs maintained on LM511-E8 ($0.5 \mu\text{g}/\text{cm}^2$), a GMP-grade scaffold, show poor *in vitro* hematopoietic differentiation following an authorized protocol for the GMP-grade generation of hiPSC stock lines at CiRA, Kyoto University, and the maintenance of these lines in any facilities for regenerative medicine in Japan (Doi et al., 2014; Sasaki et al., 2015; Shibata et al., 2018; Takebe et al., 2017; Umekage et al., 2019). Then we found that the several types of LM-E8 used as the scaffold for the maintenance culture altered the hematopoietic differentiation potential of a given hPSC line. Compared with hPSCs maintained on LM511-E8 at a determined concentration, hPSCs maintained on LM421-E8 or LM121-E8 showed weaker adhesion but higher ITGB1 expression levels along with the up-regulation of the downstream ILK-canonical Wnt/ β -catenin-pJUN signaling cascade. This effect in turn enhanced the hematopoietic differentiation potential. By targeting this

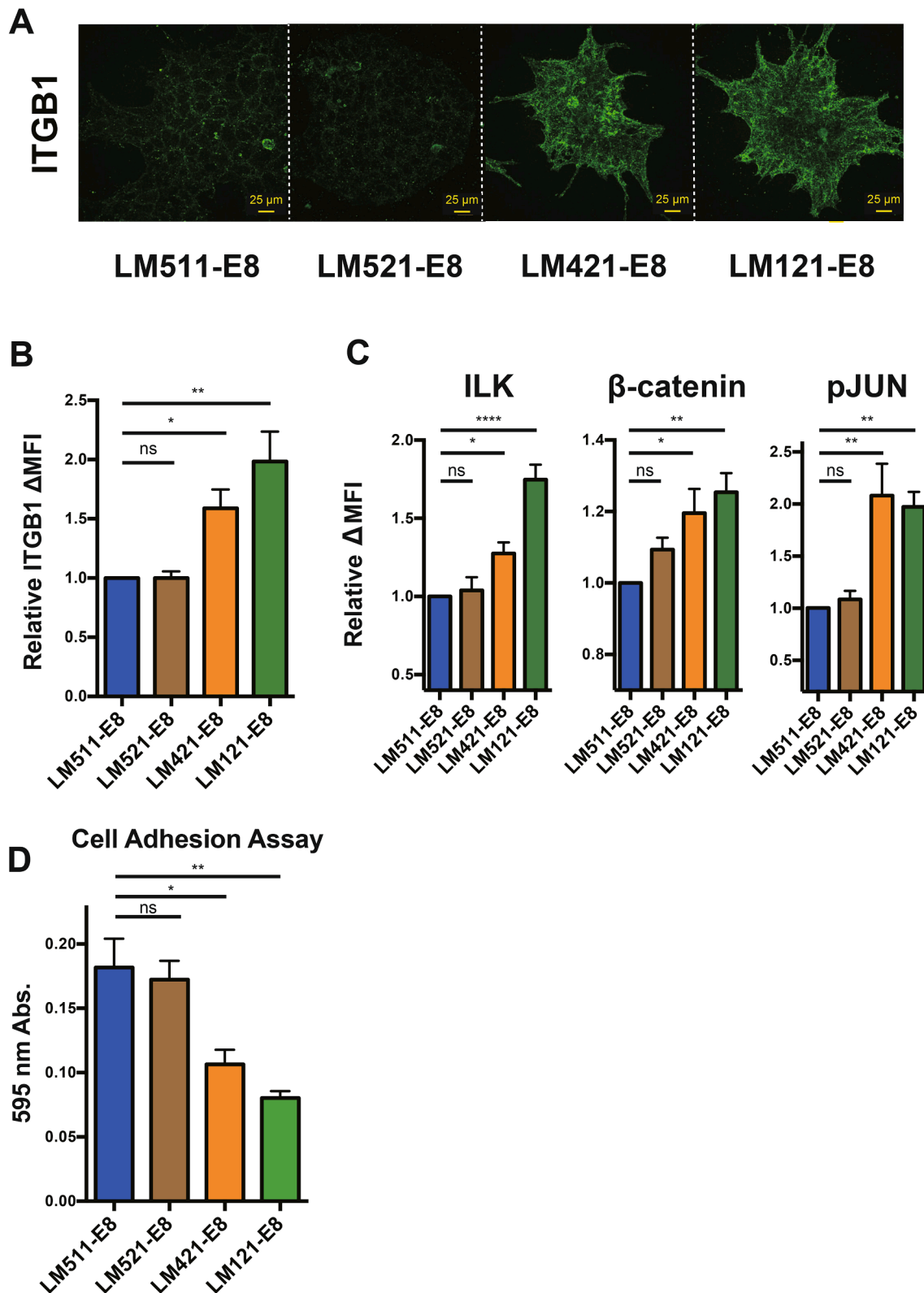


Fig. 2. Hematopoietic laminins have weaker binding avidity for hPSCs, resulting in higher ITGB1 expression level. (A) Fluorescence microscopy images of 1383A8 hiPSCs maintained on LM-E8s. hiPSCs on LM511-E8 and LM521-E8 express lower levels of ITGB1. (B) 1383A8 hiPSCs maintained on different LM-E8s were compared for ITGB1 expression measured by flowcytometry (values normalized to LM511-E8). $n \geq 3$, mean $\pm$ SEM; * $P < 0.05$ and ** $P < 0.01$ vs. LM511-E8 by Dunnett's test. n.s., not significant. (C) Flow cytometry analysis of the intracellular expression of integrin-linked kinase (ILK), β -catenin, and phosphorylated JUN (pJUN) in 1383A8 hiPSCs on each LM-E8 (values normalized to LM511-E8). Δ MFI represents mean fluorescence intensity (MFI) levels subtracted by the MFI of secondary conjugated antibody staining only. Δ MFI of LM511-E8 was set to 1. hiPSCs on LM511-E8 express lower levels of ITGB1, ILK, and β -catenin, similar to LM521-E8. $n \geq 3$, mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ vs. LM511-E8 by Dunnett's test. n.s., not significant. (D) The binding avidity for 1383A8 hiPSCs maintained on different LM-E8s by the cell adhesion assay. $n = 3$, mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. LM511-E8 by Dunnett's test. n.s., not significant.

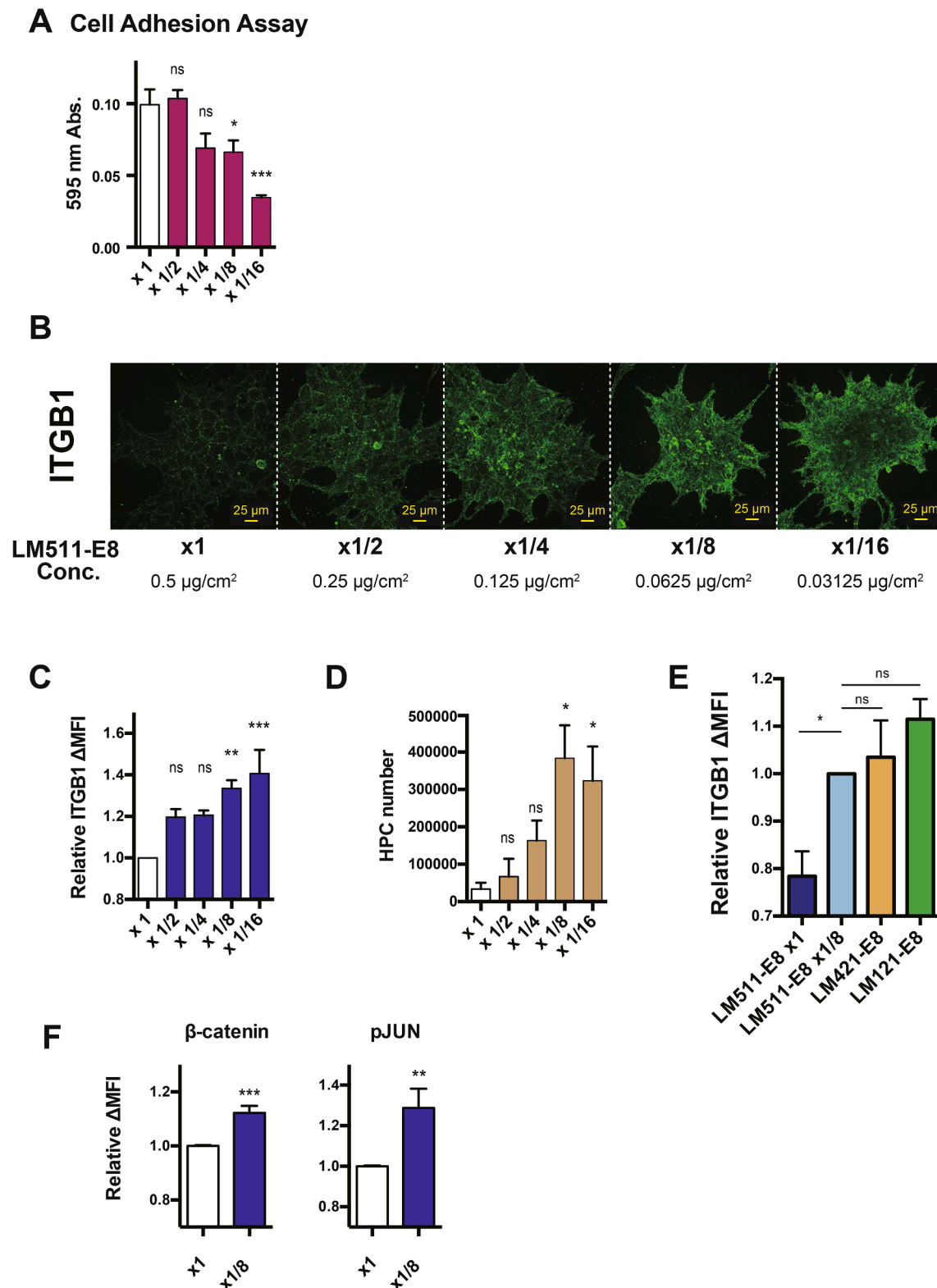


Fig. 3. Low concentration of non-hematopoietic LM511-E8 leads to increased ITGB1 expression and the hematopoietic differentiation of hPSCs. (A-D) 1383A8 hiPSCs maintained on serially diluted LM511-E8 were analyzed for their binding avidity (A), ITGB1 expression by fluorescence microscopy (B), normalized ITGB1 expression by flow cytometry (C), the number of CD34⁺CD43⁺HPCs on day 14 differentiated from 0.7 to 0.8×10^5 hPSCs (D), $n = 3$, mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. x1 concentration (no dilution) by Dunnett's test for (B-D). n.s., not significant. (E) 1383A8 hiPSCs maintained on different LM-E8s were compared for ITGB1 expression measured by flow cytometry (values normalized to LM511-E8 x1/8). Low-concentrated LM511-E8 x1/8 is comparable to that of LM121-E8 and LM421-E8. $n \geq 3$, mean $\pm$ SEM; * $P < 0.05$ vs. LM511-E8x1/8 by Dunnett's test. n.s., not significant. (F) Flow cytometry analysis of the intracellular expression of β -catenin and phosphorylated JUN (pJUN) in 1383A8 hiPSCs maintained on eight times-diluted LM511-E8 (x1/8; concentration of $0.0625 \mu\text{g}/\text{cm}^2$) relative to non-diluted LM511-E8 (x1). ΔMFI represents mean fluorescence intensity (MFI) levels subtracted by the MFI of secondary conjugated antibody staining only. ΔMFI of the non-diluted condition was set to 1. $n \geq 3$, mean $\pm$ SEM. ** $P = 0.0093$ and *** $P = 0.0004$ by t test.

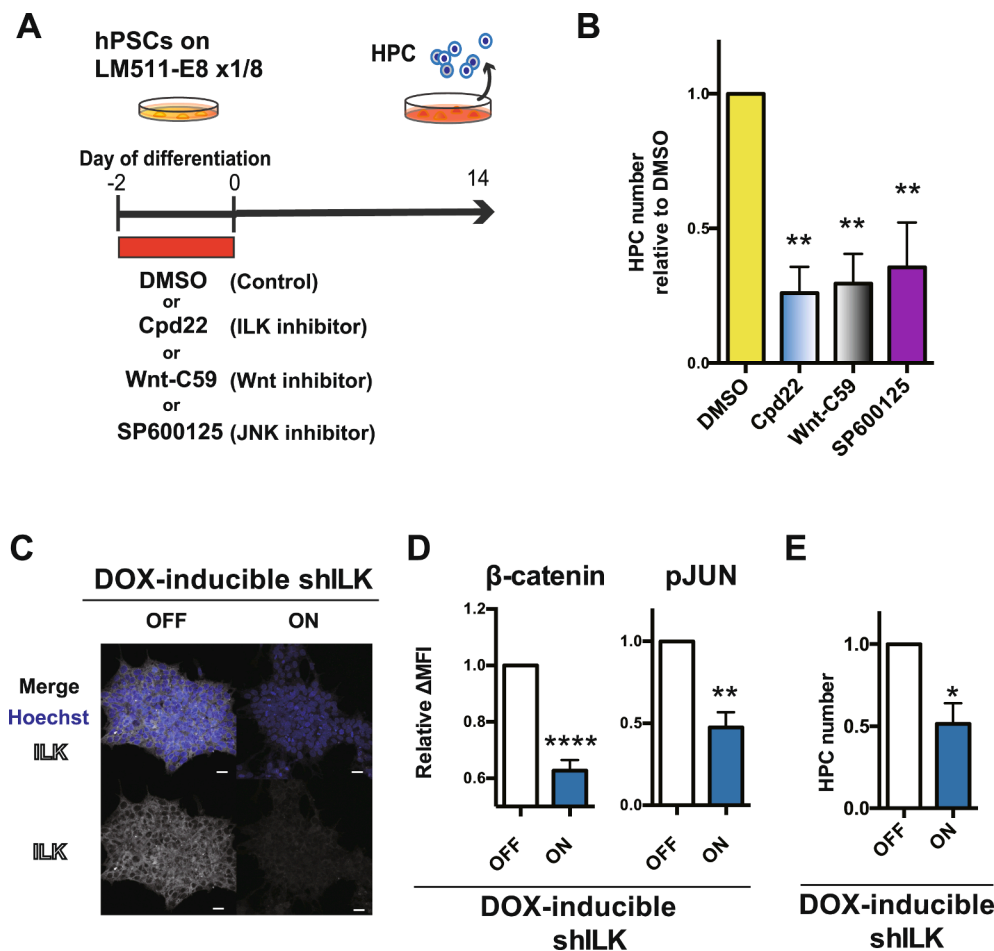


Fig. 4. The ITGB1-ILK- β -catenin-pJUN axis is crucial for the hematopoietic differentiation of hPSCs. (A) Schema for pre-treating 1383A8 hiPSCs with the indicated compounds for two days and then differentiating to HPCs with the hPSC-sac method. (B) Number of CD34⁺CD43⁺HPCs on day 14 differentiated from 0.7 to 0.8×10^5 hPSCs relative to the DMSO pretreatment condition. Inhibitors of ILK, the canonical Wnt/ β -catenin signaling pathway, and Jun all lowered the numbers of CD34⁺CD43⁺HPC on day 14. $n = 3$, mean $\pm$ SEM. $^{**}P < 0.01$ vs. DMSO by Dunnett's test. (C-E) 1383A8 hiPSCs maintained on diluted LM511-E8 were transfected with doxycycline (DOX)-inducible shRNA for ILK. hPSCs under DOX-OFF and DOX-ON conditions were analyzed by fluorescence microscopy for Hoechst and ILK after 3 days (C; scale bars = 25 μ m), by flow cytometry analysis for the intracellular expression of β -catenin and phosphorylated JUN (pJUN) as Δ MFI relative to secondary conjugated antibody staining after 3 days (D), and for the relative CD34⁺CD43⁺HPC yield after 14 days (E). $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ for D, E. t test.

cascade in the hPSC-sac method, we could enhance the hematopoietic commitment of hPSCs maintained on LM511-E8 by simply diluting LM511-E8 to $0.0625 \mu\text{g}/\text{cm}^2$, yielding a higher number of HPCs. These results were conserved in several hPSC lines (Supplemental figure S4C-G).

Several hematopoietic differentiation protocols for hPSCs contain CHIR99021 in the early phase to enhance the efficiency of differentiation (Kitajima et al., 2016; Sturgeon et al., 2014; Zhang et al., 2019). However, our hPSC-sac method does not add a canonical Wnt/ β -catenin signaling pathway activator, thus likely revealed the variable hematopoietic potential of hPSCs is due to differences in canonical Wnt/ β -catenin signaling pathway activation caused by the extracellular matrix used in the maintenance culture. Our study may also contribute to clarifying previously unknown roles of extracellular environmental laminins on signaling events that regulate cell growth dynamics during gastrulation (White et al., 2017).

Several reports have attributed the heterogeneous differentiation capability of individual hPSC clones to intrinsic elements such as DNA methylation remnants from before or during the reprogramming (Huang et al., 2014; Koyanagi-Aoi et al., 2013; Nator et al., 2012; Polo et al., 2010). Genetic abnormalities (Kajiwar et al., 2012) and the expression levels of factors influencing hematopoiesis, i.e., IGF2 (Nishizawa et al., 2016), have also been considered. In the present paper, we demonstrated the extracellular matrix is an extrinsic factor that has an important effect on the differentiation potential of undifferentiated hPSCs, possibly through the $\alpha 6 \beta 1$ integrin dimerization complex, a major laminin receptor. LM511, the most ubiquitously expressed laminin in the human body together with LM521, is commonly used as an *in*

vitro scaffold for feeder-free maintenance (Doi et al., 2014; Nakagawa et al., 2015; Sasaki et al., 2015; Shibata et al., 2018; Takebe et al., 2017; Umekage et al., 2019). It also tightly binds to integrin $\alpha 6 \beta 1$, the most dominant integrin complex on hPSCs (Nishiuchi et al., 2006). Laminins with the $\alpha 1$ subunit, such as LM121, are expressed in various tissues such as the eyes, liver and kidney (Ekblom et al., 2003; Virtanen et al., 2000), and for *in vitro* differentiation, LM121 is used to promote neurite extensions (Sasaki et al., 2010). Although LM121 also has a high affinity for integrin $\alpha 6 \beta 1$ (Sasaki et al., 2010), we found the hPSC binding capacity of LM121-E8 was significantly lower than that of LM511-E8 (Fig. 2D). LM421 is expressed on endothelial cells and kidney cells, suggesting roles for renal and synaptic functions and inflammation (Hansen and Abrass, 2003; Patton et al., 2001; Susek et al., 2018). We found that the E8 fragment of LM421 and LM121 induces hematopoietic commitment, but the E8 fragment of LM511 does not (Fig. 1B). LM421- and LM121-E8 share $\beta 2$ and $\gamma 1$ chains. So too does LM321-E8, which does not show hematopoiesis (Fig. 1B), suggesting the mere combination of $\beta 2$ - $\gamma 1$ chains is not sufficient.

In those contexts, each laminin isoform has been thought to have a specific effect on stem cell differentiation and cell behavior according to the location it is expressed (Yap et al., 2019). Our experiments demonstrated that optimal adhesive avidity is required for the hematopoietic differentiation potential (Fig. 3D). These results also showed that weaker adhesive avidity promotes a higher expression of ITGB1 on hPSCs (Fig. 3B and 3C). Therefore, we propose that the weak laminin scaffold stimulates hPSCs to express ITGB1 molecules in order to bind to LM-E8s. However, our microarray data for hPSCs on several LM-E8s (Fig. 1D and 1E) did not directly reveal the upregulation of ITGB1 or

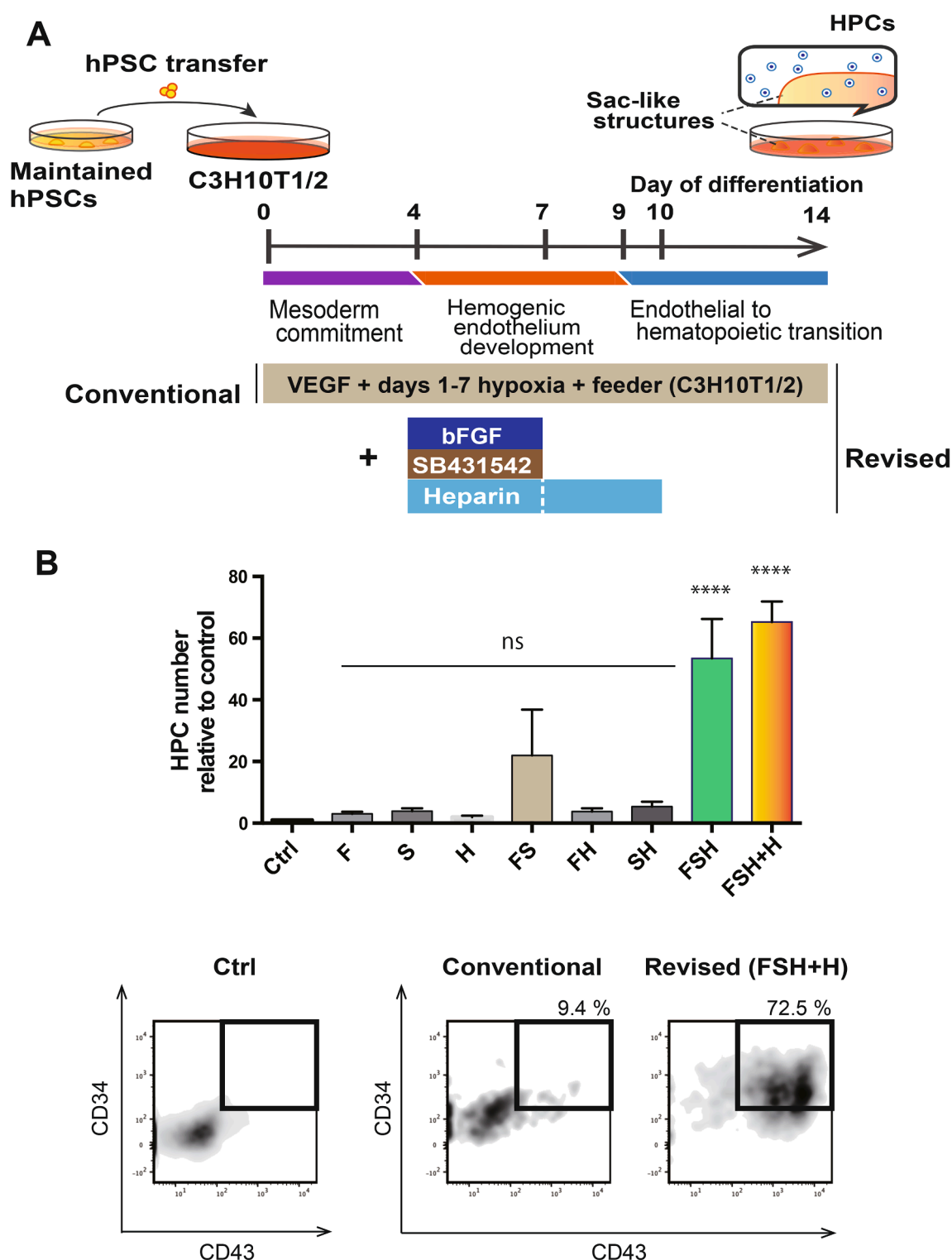


Fig. 5. Revised hPSC-sac differentiation method increases the CD34⁺CD43⁺ HPC yield. (A) Schema of the conventional hPSC-sac method and the revised method, which adds 3 new factors. (B) Relative number of CD34⁺CD43⁺HPCs on differentiation day 14 from KhES-3 hESCs with the conventional hPSC-sac method plus additional factors (Ctrl: Control, F: bFGF added on days 4–7, S: SB431542 added on days 4–7, H: heparin added on days 4–7, FS/FH/SH: two factors added on days 4–7, FSH: all three factors added on days 4–7 FSH, and FSH + H: FS added on days 4–7 and heparin on days 4–10) ($n \geq 3$, mean $\pm$ SEM). Bottom shows representative flow cytometry plots with an isotype control plot (Ctrl) for the CD34⁺CD43⁺HPC ratio on day 14. For the control conventional condition, $0.36 \pm 0.095 \times 10^5$ HPCs were collected per 10-cm culture dish, which was originally seeded with $0.7\text{--}0.8 \times 10^5$ hESCs. **** $P < 0.0001$ vs. control by Dunnett's test. n.s., not significant.

of downstream ILK and β -catenin. One possible reason for the observation is post-translation regulation. Another reason may be that these proteins are upregulated to only the 2-fold of the assay (Fig. 2B and C). As such, it is possible that the mRNA expression levels are within the 2-

fold threshold and thus not detected.

Our data also suggest that the ITGB1 expression on undifferentiated hPSCs predicts the HPC differentiation capacity (Supplemental figure S4A). Therefore, hPSCs with higher ITGB1 expression should be adopted



used, such as patient-derived hiPSCs (Chao et al., 2017; Hirata et al., 2013; Takei et al., 2018) or HLA-matched/homozygous stocked hPSCs (Umekage et al., 2019), the hematopoietic potential could be alternatively enhanced by modulating extrinsic factors such as the LM-E8

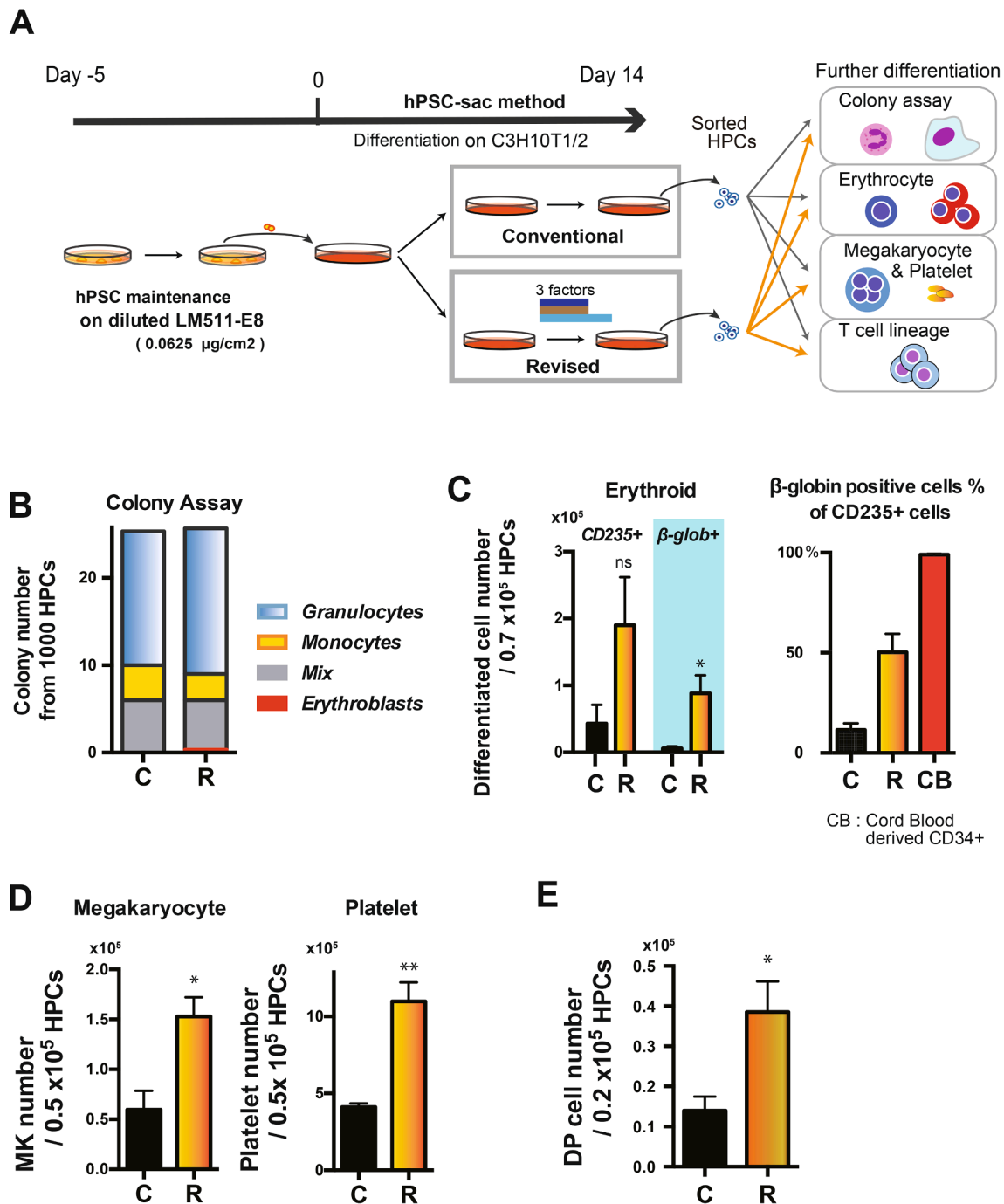


Fig. 7. The revised hPSC-sac method improves the quality and quantity of hematopoietic cells terminally differentiated from hPSCs on low-concentrated LM511-E8. (A) Schematic overview of the experiments in B-F. 1383A8 hiPSCs maintained on low-concentrated LM511-E8 x1/8 were subjected to the conventional or revised hPSC-sac method for CD34⁺CD43⁺HPC differentiation. Each CD34⁺CD43⁺HPC population was sorted by a cell sorter and cultured in various conditions for further terminal hematopoietic lineage differentiation on day 14. (B) Colony forming assays 21 days after differentiation (day + 21) of the same number of CD34⁺CD43⁺HPCs (day 14). n = 3, mean. (C) Left: Erythroid lineage differentiation on day + 21 shown as the calculated number of CD235⁺ and β-globin⁺ cells from the conventional method (n = 3, mean ± SEM, ns: p = 0.1303 and *P = 0.0392 by *t* test). The cells were differentiated from the same number of CD34⁺CD43⁺HPCs by the conventional and revised hPSC-sac methods. Right: Percentage of β-globin⁺ cells within the differentiated CD235⁺ cell population in the left panel and within normal human cord blood (CB) CD34⁺CD133⁺ cells. (D) Number of megakaryocytes and platelets on differentiation day + 10 (n ≥ 3, mean ± SEM, *P = 0.0132 and **P = 0.0016 by *t* test). (E) T cell differentiation on day + 21 for CD4⁺CD8⁺ double positive (DP) cells. n = 3, mean ± SEM, *P = 0.0430 by *t* test.

concentration or by adding canonical Wnt/β-catenin signaling pathway activators.

The hPSC-sac method can be applied easily for the *in vitro* hematopoietic differentiation of hPSCs to generate various types of human blood cells, including megakaryocytes for robust platelet production, erythroblasts, neutrophils, monocytes, and T lymphocytes (Hirata et al.,

2013; Ito et al., 2018; Miyauchi et al., 2018; Nishimura et al., 2013). However, the original hPSC-sac method gives a low yield of multipotent HPCs. In the present study, we dramatically improved the hematopoietic differentiation potential of the hPSC-sac method by simply adding bFGF, a TGF-β inhibitor, and heparin to the culture protocol (Fig. 5). Regarding the issue of GMP grade production, the hPSC-sac method uses mouse

C3H10T1/2 cells as feeders, but C3H10T1/2 cells are approved for the manufacturing of clinical products in Japan. FBS bears the same issue, but FBS irradiated at an extremely high dose and human serum are approved for clinical use in the U.S. and Japan, respectively. In combination with low-concentrated LM511-E8 at the undifferentiated maintenance phase of hPSCs, a higher rate of definitive-type hematopoiesis was observed (Fig. 6B, 7C-E). However, the yield was lower than the definitive-type hematopoiesis achieved with cord-blood HPCs, as indicated by the lower level and rate of beta-globin expression in the derived CD235 + erythroid cells. Nevertheless, our differentiation protocol could be used as a tool to elucidate definitive hematopoiesis for further study.

5. Conclusions

We succeeded in improving hematopoietic differentiation and identifying optimal external environmental conditions for the maintenance culture of hPSCs. We found the interaction between extracellular matrix laminin and ITGB1 expressed on the hPSC surface modulates the downstream ILK-canonical Wnt/ β -catenin-pJUN signaling cascade to have a significant effect on hematopoiesis. These results show that the extracellular scaffolds used during the maintenance phase determine the differentiation fate of hPSCs and might explain the discrepancies in experimental results between laboratories that use the same differentiation methods but different extracellular scaffolds in the maintenance culture. Moreover, these findings will be useful for developing individualized medicine by regulating the differentiation capacity of patient-derived hPSCs by simply changing the scaffold without any gene editing. Furthermore, by changing the concentration of the LM511-E8 extracellular environment and also adding bFGF, TGF- β , and heparin reagents to the conventional hPSC-sac method, we succeeded in accelerating the production of definitive hematopoiesis and its yield. In the basic research field of hematology, this study provides an easy and efficient method to obtain HPCs and more definitive hematopoiesis *in vitro*. This study thus should contribute to the development of regenerative medicine using hematopoietic lineages.

Declaration of Competing Interest

A.Y., S.N., K.S., and K.E. have applied for patents related to this manuscript. K.S. is a cofounder and a shareholder of MATRIXOME, Inc. K.E. is a founder of Megakaryon and a member of its scientific advisory board without salary; the interests of K.E. were reviewed and are managed by Kyoto University in accordance with its conflict-of-interest policies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2021.102287>.

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1 **SUPPLEMENTAL METHODS**

2

3 **Flowcytometry analysis**

4 Flow cytometry analysis was performed using FACSVerse (BD Biosciences). To detect cell surface antigens,
5 hPSCs were prepared in PBS containing 3% FBS (staining medium; SM) and stained with antibodies for 30 min
6 at room temperature. Differentiated cells were prepared in SM and stained with antibodies for 30 min on ice. The
7 samples were then washed with SM and analyzed in the presence of propidium iodide (PI). For intracellular
8 staining, Zombie-Aqua dye (Biolegend, San Diego, CA, USA) was used to discriminate live cells. The cells were
9 then fixed in 4% formaldehyde (15 min on ice) and treated with ice-cold 100% methanol for 15 min. The fixed
10 cells were permeabilized with 0.5% saponin for 10 min at room temperature and stained for 60 min at room
11 temperature with primary antibodies (see below for a list of the antibodies) (modified from Ochi et al., 2014). Then
12 the cells were stained with secondary antibodies (see below for a list of the antibodies) at room temperature for 30
13 min. Each step was performed after washing the cells with PBS containing 3% FBS. The obtained data were
14 processed using FlowJo (BD Biosciences).

15

16

17 **Antibodies**

18 The antibodies used are as follows. Anti-hCD34-BV421 (#343610), anti-hCD34-APC/Cy7 (#343614), anti-
19 CD41a-APC (#303710), anti-CD41a-APC/Cy7 (#303716), anti-CD42b-PE (#303906), anti-CD3-Alexa Fluor 488
20 (#300415), anti-CD5-APC (#300612), anti-CD4-PE/Cy7 (#357410), anti-CD8-PE (#344705), anti-CD235ab-
21 Pacific Blue (#306612), anti-hCD117-BV510 (#105839), anti-hCD45-Alexa Fluor 488 (#304017), anti-hCD45-
22 PE/Cy7 (#368531), anti-hCD309-PE (#359904), anti-hCD73-APC (#344006), anti-hCD43-PE/Cy7 (#343207),
23 anti-hCD41a-PE/Cy7 (#303718), mouse IgG1-APC (#400120), mouse IgG1-AF647 (#400130), mouse IgG1-
24 APC/Cy7 (#400127), mouse IgG2a-PE/Cy7 (#400231), mouse IgG2a-BV421 (#400259) and mouse IgG1-AF488
25 (#400132) were purchased from Biolegend Inc.; anti-hCD43-APC (#17-0439-73) from eBioscience (San Diego,

CA, USA); anti-Sox2-V450 (#561610), anti-Oct3/4-AF488 (#560253), anti-Nanog AF647 (#561300), anti-hCD29-PE (#555443), anti-hCD31-FITC (#30884x), anti-hCD144-BV421 (#565671), anti-hTRA-1-60 antigen-AF647 (#560850), mouse IgG1-V450 (#560373) and mouse IgG1-PE (#551436) from BD Biosciences; anti-ILK (#3856S), anti- β -catenin (#19807S) and anti-phospho-c-Jun (#3270S) from Cell Signaling Technologies (Danvers, MA, USA); anti-rabbit-IgG (H+L)Alexa Fluor 555 (#A21428) and -Alexa Fluor 647 (#A21245) goat antibodies from Invitrogen (Carlsbad, CA, USA); and anti- β -globin-AF647 (#sc-21757 AF647) from Santa Cruz Biotechnology (Dallas, TX, USA).

Quantitative RT-PCR

Total RNA was extracted using an miRNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was generated using a ReverTrace Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Quantitative RT-PCR (qPCR) was performed using StepOnePlus (Thermo Fisher Scientific, Waltham, MA, USA). The primer sets used in the assays are shown in Supplemental table 1.

Gene expression microarray analysis

Total RNA was isolated from MK04 hiPSCs maintained on LM-E8s using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). Total RNA (200 ng) was labeled with cyanine 3-CTP and used for the hybridization with SurePrint G3 Human 8x60K slides (Agilent Technologies, Santa Clara, CA, USA). The raw intensity values were imported into GeneSpring GX software (Agilent Technologies) for normalization and statistical analysis. We compared the average gene expression levels in the hematopoietic LM-E8 (LM421-E8, LM121-E8) and non-hematopoietic LM-E8 (LM521-E8, LM411-E8, LM321-E8, LM311-E8, LM111-E8) groups and identified genes that were upregulated in the hematopoietic LM-E8 group > 2-fold and had a false discovery rate (FDR) < 0.05. Ingenuity pathway analysis (IPA, Qiagen Inc.) was used to identify upstream regulators of the observed gene expression changes (see Supplemental figure 1E).

The accession number for the microarray data reported in this paper is NCBI Gene Expression Omnibus: GSE149717.

Immunofluorescence microscopy

Cells were seeded on a LM-E8-coated 35-mm dish (μ -Dish high, Ibidi, Martinsried, Germany), fixed with 4% paraformaldehyde buffer for 15 min, and in some cases permeabilized with 0.1% Triton X-100 for 5 min. The cells were then incubated sequentially with BB515 mouse anti-human CD29 (#564565; BD Biosciences), anti-ILK (#3856S; Cell Signaling Technologies) and Alexa Fluor 647-goat anti-rabbit IgG secondary antibody (#A21245; Invitrogen). Hoechst 33342 was used to stain the nucleus (NucBlue Live ReadyProbes, #R37605, Thermo Fisher Scientific). Images were acquired as Z-stacks with a confocal microscope (A1R MP+; Nikon, Tokyo, Japan) and visualized by the maximum intensity projection. A 40x/1.15 water immersion objective lens (#MRD77410; Nikon) was used. Images were analyzed using Nikon NIS Elements software or ImageJ/Fiji (NIH).

Cell adhesion assay

The protocol for this assay has been described previously (Sasaki et al., 2010; Wong et al., 2007). A total of 0.5×10^5 cells were seeded into 48-well plates coated with LM-E8s at $0.5 \mu\text{g}/\text{cm}^2$ as the default concentration. After 1 h of incubation, non-adherent cells were removed by rinsing twice with DMEM/F12 medium, and the remaining adherent cells were fixed in 4% paraformaldehyde buffer for 15 min and then in 100% ethanol for 5 min. The cells were then stained with 0.4% crystal violet in methanol for 5 min at room temperature. After washing three times with demineralized water, the remaining cells were solubilized by 1% SDS. The optical density at 595 nm was measured using a NanoDrop2000c (Thermo Fisher Scientific).

1 **shRNA-mediated knockdown**

2 The siRNA sequence for ILK (siGENOME, siRNA D-004499-03-0005, Dharmacon, Lafayette, CO, USA) was
3 inserted into a pENTR4-H1tetOx1 vector and then transformed into a doxycycline-inducible CS-CDF-RfA-ETVP
4 vector (both kindly provided by Dr. Hiroyuki Miyoshi, RIKEN) using Gateway LR Clonase (Invitrogen). The
5 shRNA lentiviral supernatant for the ILK vector was generated as previously described (Nakamura, 2014) and
6 added to the hPSC culture. For the mRNA expression and protein expression validation, doxycycline was added
7 3 days before the measurements (day -3). For the differentiation to HPCs, doxycycline was added from day -3 to
8 day 14. Differentiation was initiated using the equivalent number of hPSCs for DOX-OFF and DOX-ON
9 conditions.

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1 **Supplemental Table 1. Sequences of quantitative RT-PCR primers**

2

Target gene		Sequence	
<i>SPI1</i>	Forward	5'-ccactggaggtgtctgacg-3'	
	Reverse	5'-ctggtacaggcggatcttct-3'	
<i>RUNX1c</i>	Forward	5'-gaagtctgaaccagcatagtggcagcag-3'	Woll et al., 2008
	Reverse	5'-gtggacgtctctagaaggattcattccaag-3'	
<i>GFI1B</i>	Forward	5'-gctggagcaggaccagaac-3'	
	Reverse	5'-cagtggagagtcccatcc-3'	
<i>ERG</i>	Forward	5'-aacgagcgcagagtatcgt-3'	Deplus et al., 2017
	Reverse	5'-cgtctggaaggccatattct-3'	
<i>TAL1</i>	Forward	5'-ctatgagatggagattactgatggtc-3'	
	Reverse	5'-gtgtggggatcagcttgc-3'	
<i>ILK</i>	Forward	5'-cacctgctcctatcctactct-3'	
	Reverse	5'-ctgggtccacgacgaaattg-3'	
<i>GAPDH</i>	Forward	5'-gagtcactggcgtcttcac-3'	
	Reverse	5'-gttcacacccatgacgaaca-3'	
<i>NEUROG1</i>	Forward	5'-gcggatgtctcttggctcg-3'	
	Reverse	5'-aaaaggaaaggccgtctagg-3'	
<i>HAPLN1</i>	Forward	5'-gaggagaaagagcgctagctt-3'	
	Reverse	5'-agcccaaagaatcttcttcactt-3'	
<i>ZIC1</i>	Forward	5'-atccacaaaaggacgcacac-3'	
	Reverse	5'-gtcacagccctcaaactcg-3'	
<i>PITX2</i>	Forward	5'-ccttacggaagcccaggt-3'	
	Reverse	5'-ccgaagccattcttcata-3'	
<i>FOXC1</i>	Forward	5'-tgaacgggaatagtagctgtca-3'	
	Reverse	5'-ggacgtgcggtacagagac-3'	
<i>JUN</i>	Forward	5'-ccaaaggatagtgcgagtgtt-3'	
	Reverse	5'-ctgtccctctccactgcaac-3'	
<i>18srRNA</i>	Forward	5'-ccgattggatggtttagtgag-3'	
	Reverse	5'-agttcgaccgtcttctcagc-3'	

3

1 SUPPLEMENTAL FIGURE LEGENDS

2

3 Supplemental Figure 1. hPSC-sac method and gene expression analysis of hPSCs on different LM- 4 E8s (Related to figure 1)

5 (A) Schema of the conventional hPSC-sac method. Small clumps of hPSCs are transferred onto mitomycin-C-
6 treated C3H10T1/2 feeder cells and cultured for 14 days with hematopoietic differentiation medium including
7 VEGF in hypoxic condition for the initial 7 days.

8 (B) Representative bright-field images of 1383A8 hiPSCs maintained on MEFs or LM511-E8 before and after
9 differentiation with the conventional hPSC-sac method. hPSCs maintained on LM511-E8 ($0.5 \mu\text{g}/\text{cm}^2$) rarely
10 formed colonies on day 4 or sac-like structures on day 14 upon differentiation. Scale bars = $100 \mu\text{m}$.

11 (C) The number of $\text{CD34}^+\text{CD43}^+\text{HPCs}$ on day 14 differentiated from 0.7 to 0.8×10^5 1383A8 hiPSCs. 1383A8
12 hiPSCs were maintained on MEFs or LM511-E8 until day 0. $n \geq 3$, mean $\pm$ SEM. $*P=0.0303$ by t test.

13 (D) A heatmap showing log-scaled normalized expression values of upregulated genes in MK04 hiPSCs cultured
14 on the hematopoietic LM-E8s group (LM421 and LM121-E8) compared with the non-hematopoietic LM-E8s
15 (LM111, LM311, LM321, LM411, and LM521-E8) group. Forty-three probe IDs were identified (> 2 -fold
16 change and $\text{FDR} < 0.05$) by microarray analysis. Expression values of each gene were normalized to the mean
17 raw expression value across all samples for each gene. Probes that were not assigned to gene symbols are
18 indicated with probe IDs.

19 (E) Summary of the upstream regulator analysis by Ingenuity Pathway Analysis (IPA). Smaller P values indicate
20 a higher correlation between potential upstream regulators and upregulated genes. Three genes for the
21 “transcription regulator” molecule type had $P < 0.001$ and more than three target molecules each in the dataset.

22 (F) qRT-PCR analysis of 1383A8 hiPSCs for the expression of representative genes normalized to the level of
23 18srRNA expression for validation of the microarray analysis, which indicated CTNNB1 is an upstream regulator,
24 related to supplemental figure S1E. Values are relative to 1383A8 hiPSCs cultured on LM521, which belongs to
25 the non-hematopoietic LM-E8s group. $n=3$, mean $\pm$ SEM.

26

Supplemental Figure 2. Additional channels of immunofluorescence microscopy and pluripotency markers of hPSCs maintained on different LM-E8s. Related to figure 2.

Different assays of 1383A8 hiPSCs maintained on the indicated LM-E8s. (A) Bright-field, ITGB1, Hoechst and merged images. (B) Representative flow cytometry histograms of the intracellular expressions of ILK, β -catenin and pJUN (related to figure 2C). (C) Representative flow cytometry plots of the intracellular expression of pluripotency markers (SOX2, OCT3/4 and Nanog).

Supplemental Figure 3. Additional channels of the immunofluorescence microscopy and pluripotency markers of hPSCs maintained on serially diluted LM511-E8. Related to figures 3.

Different assays of 1383A8 hiPSCs maintained on serially diluted LM511-E8. (A) Bright-field, ITGB1, Hoechst and merged images. (B) Hoechst, ITGB1, ILK and merged images for x1 and x1/8 LM511-E8. Scale bars = 25 μ m. (C) Representative flow cytometry plots of the intracellular expression of pluripotency markers (SOX2, OCT3.4 and Nanog). (D) Representative flow cytometry histograms of the intracellular expression of β -catenin and pJUN (related to figure 3F).

Supplemental Figure 4. Consistent upregulation of ITGB1-ILK- β -catenin-pJUN pathway in different hematopoietic LM-E8 conditions among various hPSC lines. Related to figures 2 and 3.

(A) Four hPSC lines were each maintained on five serially diluted LM511-E8 conditions (from x1 to x1/16 as in figure 3A-D) and assessed for the ITGB1 expression level on day 0 and CD34⁺CD43⁺HPC yield on differentiation day 14. The correlation tendency was predicted from a simple regression analysis. Each plot shows the mean $\pm$ SEM; n=3. $P < 0.0001$.

(B) TkDN-Sev2 hiPSCs maintained on different LM-E8s were compared for ITGB1 expression measured by flow

cytometry (values normalized to LM511-E8). $n \geq 3$, mean $\pm$ SEM; $*P < 0.05$ and $**P < 0.01$ vs. LM511-E8 by Dunnett's test. n.s., not significant. Related to figure 2B.

(C-E) TkDN-Sev2 hiPSCs maintained on default or diluted concentrations of LM511-E8 were compared for the normalized ITGB1 expression by flow cytometry (C), number of CD34⁺CD43⁺HPCs on day 14 differentiated from 0.7 to 0.8 $\times 10^5$ hPSCs (D), and the intracellular expression of β -catenin and pJUN by flow cytometry (E). $n \geq 3$, mean $\pm$ SEM. $*P < 0.05$, $***P < 0.001$ by t test.

(F-G) H1 hESCs maintained on default or diluted concentrations of LM511-E8 were compared for normalized ITGB1 expression by flow cytometry (F) and number of CD34⁺CD43⁺HPCs on day 14 differentiated from 0.7 to 0.8 $\times 10^5$ hPSCs (G). $n \geq 3$, mean $\pm$ SEM. $**P = 0.0011$, $****P < 0.0001$ by t test.

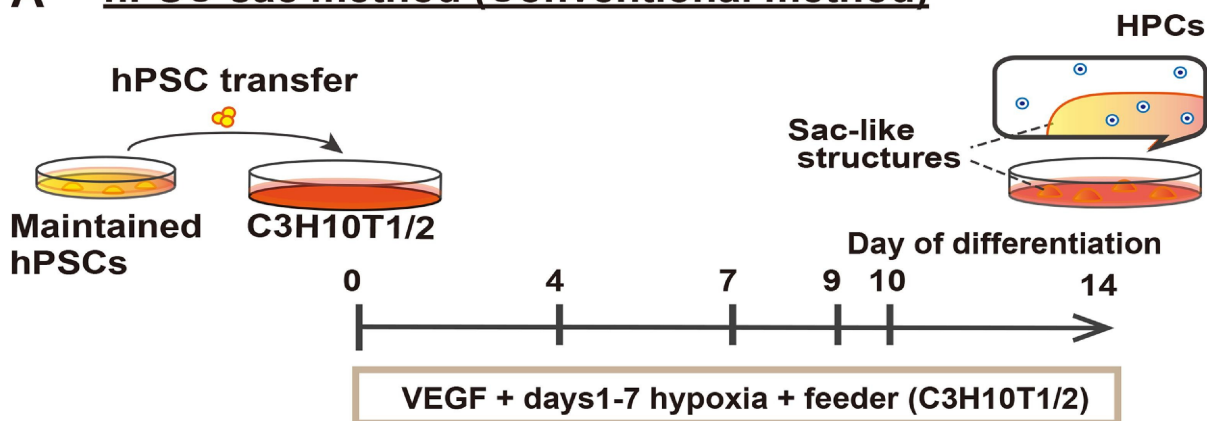
(H-J) 1383A8 hiPSCs maintained on default or diluted concentrations of LM521-E8s were analyzed for the relative ITGB1 expression by flow cytometry (H), the number of CD34⁺CD43⁺HPCs on day 14 differentiated from 0.7 to 0.8 $\times 10^5$ hPSCs (I), and the relative intracellular expression of β -catenin and phosphorylated JUN (pJUN) by flow cytometry (J). $n \geq 3$, mean $\pm$ SEM. $**P < 0.01$ and $***P < 0.001$ by t test. Related to figure 3.

Supplemental Figure 5. DOX-inducible shRNA for ILK system decreases the expression of ILK without affecting survival. Related to figure 4.

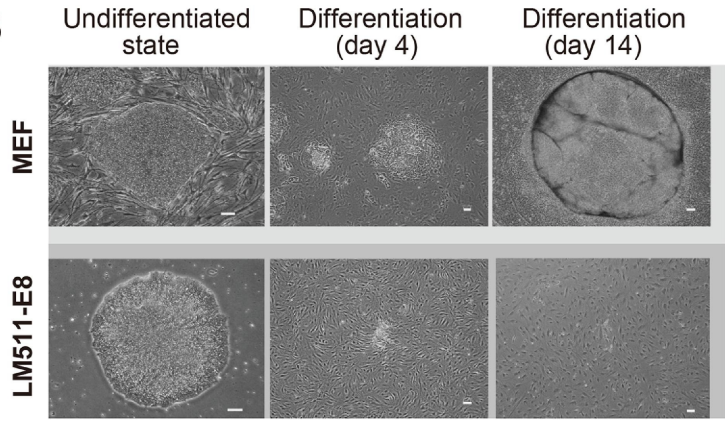
1383A8 hiPSCs maintained on low-concentrated LM511-E8 were transfected with doxycycline (DOX)-inducible shRNA for ILK. hPSCs under DOX-OFF and DOX-ON conditions for 3 days were analyzed by qRT-PCR for the expression of ILK normalized to GAPDH (A) and by flow cytometry for the expression of ILK as Δ mean fluorescence intensity (MFI) relative to secondary conjugated antibody staining only (B). $n = 3$; $***P < 0.001$ and $****P < 0.0001$ by t test. The hiPSCs treated for 3 days with DOX were also analyzed by flow cytometry for the intracellular expression of β -catenin and pJUN (C: representative histograms) and for the relative cell number (D: $n = 3$; ns: $P = 0.579$ by t test).

1 **Supplemental Figure 6. Representative images and immunophenotypes of differentiated**
2 **hematopoietic cells. Related to figure 7.**
3 (A) Representative bright field images of colony forming assays.
4 (B) Representative images of May-Giemsa staining of differentiated erythroblasts and erythrocytes.
5 (C) Representative histogram of β -globin expression on differentiated CD235⁺ cells. CD235⁺ cells differentiated
6 from cord blood CD34⁺CD133⁺ cells show a β -globin positive rate over 99%. Related to figure 7D, right panel.
7 (D) Representative images of May-Giemsa staining of megakaryocytes (left) and bright field images of extending
8 proplatelets (right, arrows).
9 (E) Representative flow cytometry plots of differentiated CD41⁺CD42b⁺ megakaryocytes and platelets (orange
10 dots) and isotype control (gray dots).
11 (F and G) Representative flow cytometry plots of the gating strategy for detecting CD4⁺CD8⁺ T cell progenitors
12 (DP cells) (F) and of cells stained with target antibodies (brown dots) or isotype controls (gray dots) (G).
13

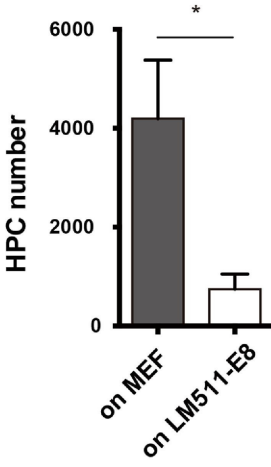
A hPSC-sac method (Conventional method)



B



C



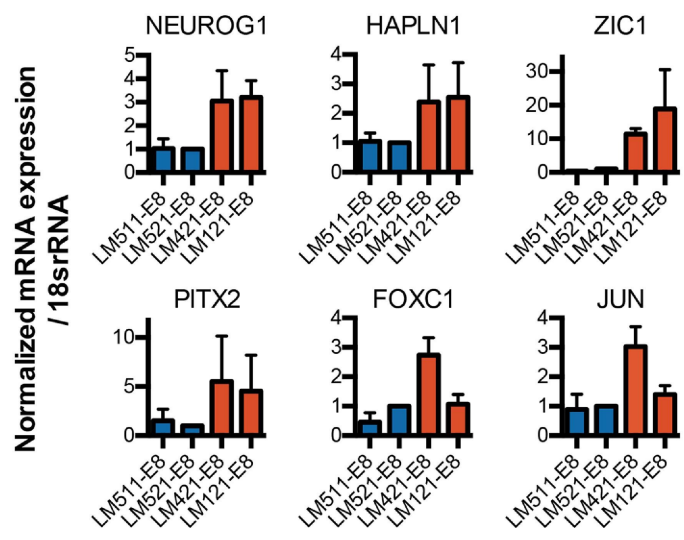
D



E

Upstream Regulator	Molecule Type	p-value of overlap	Target molecules
CTNNB1	transcription regulator	3.54E-08	CTSF, FOXC1, HAPLN1, HHEX, JUN, NEUROG1, PAX7, PITX2, ZIC1
PITX2	transcription regulator	0.000136	HMX1, PITX2, TBX1
SOX2	transcription regulator	0.000662	DLX4, JUN, NEUROG1, PITX2

F



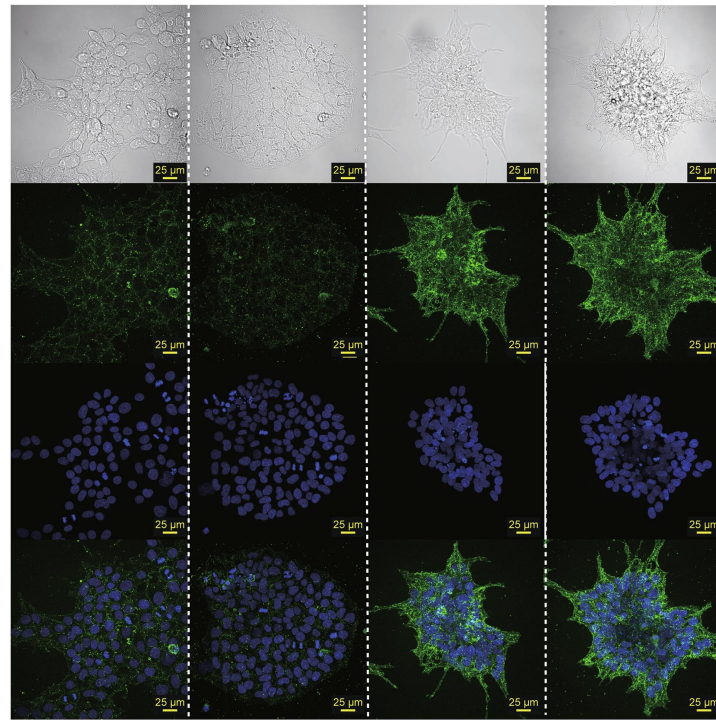
A

Bright-field

ITGB1

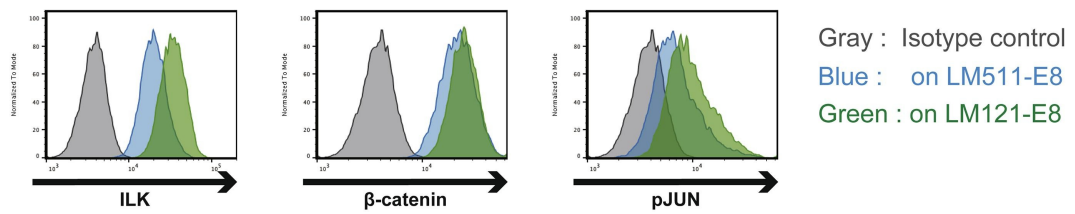
Hoechst

Merge

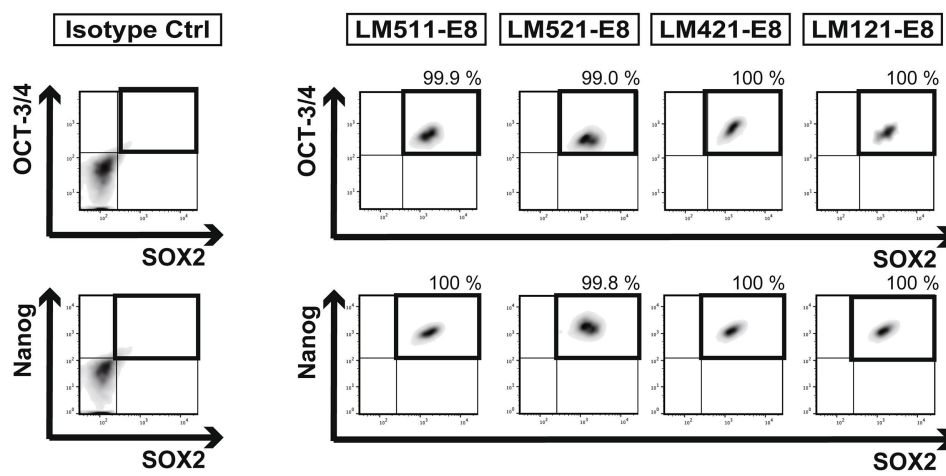


LM511-E8 LM521-E8 LM421-E8 LM121-E8

B



C



A

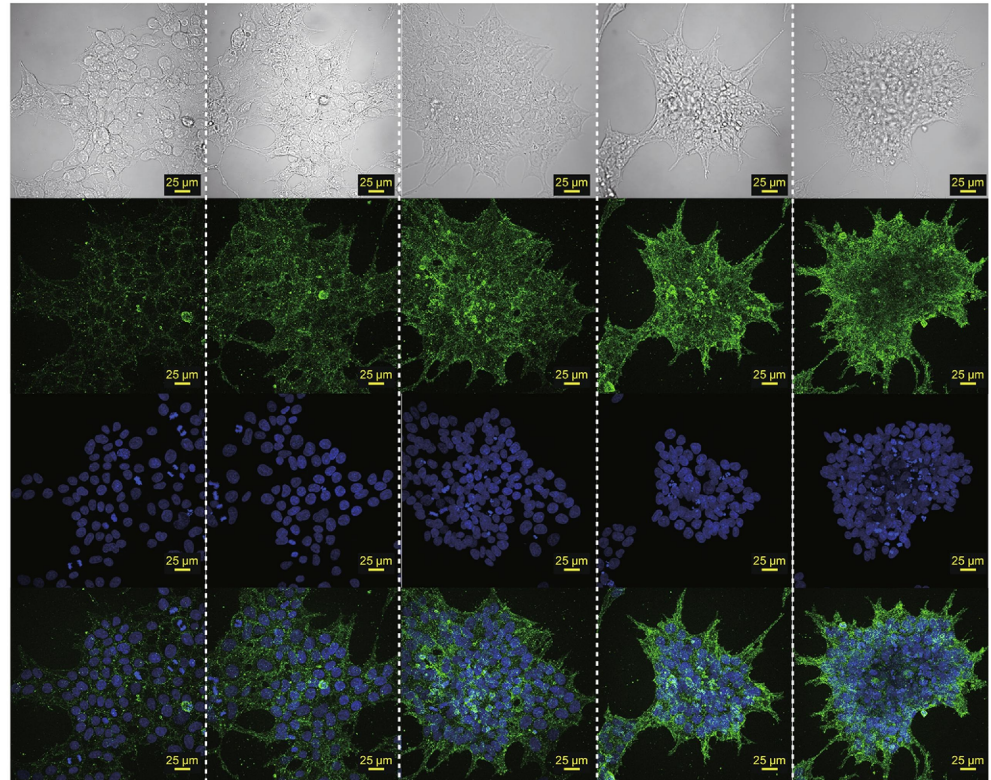
Bright-field

ITGB1

Hoechst

Merge

**LM511-E8
Conc.**



x1

x1/2

x1/4

x1/8

x1/16

0.5 µg/cm²

0.25 µg/cm²

0.125 µg/cm²

0.0625 µg/cm²

0.03125 µg/cm²

B

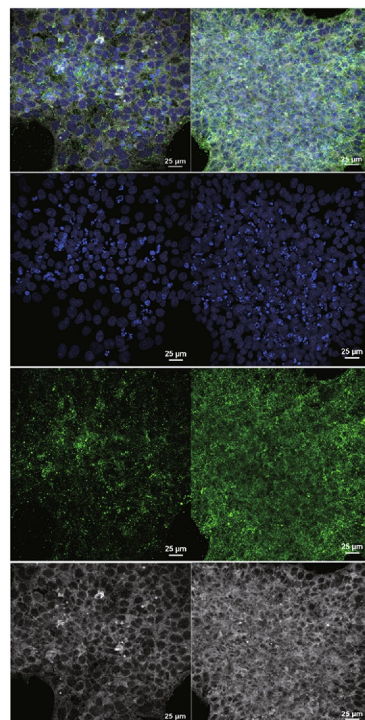
Merge

Hoechst

ITGB1

ILK

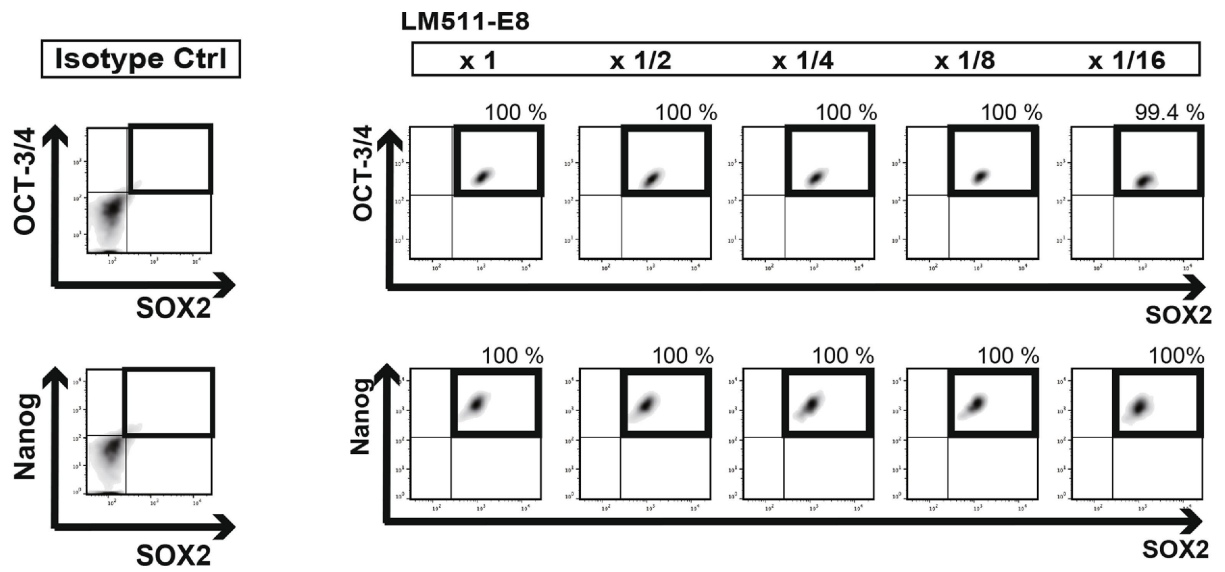
**hPSCs on
LM511-E8**



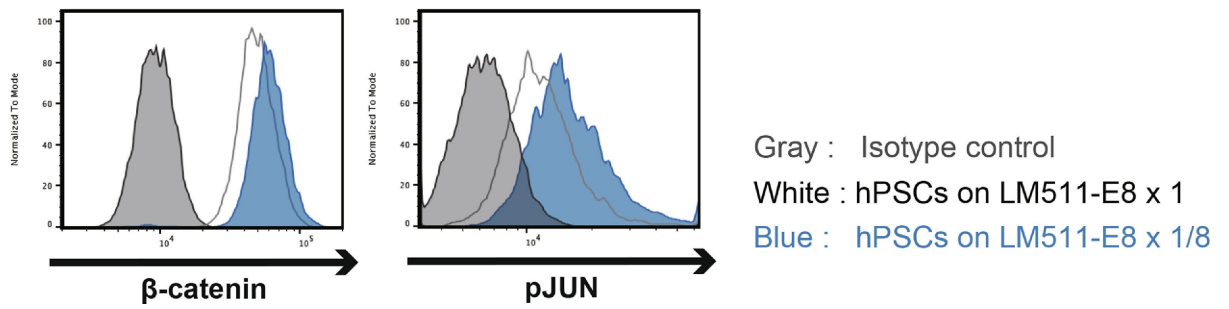
x 1

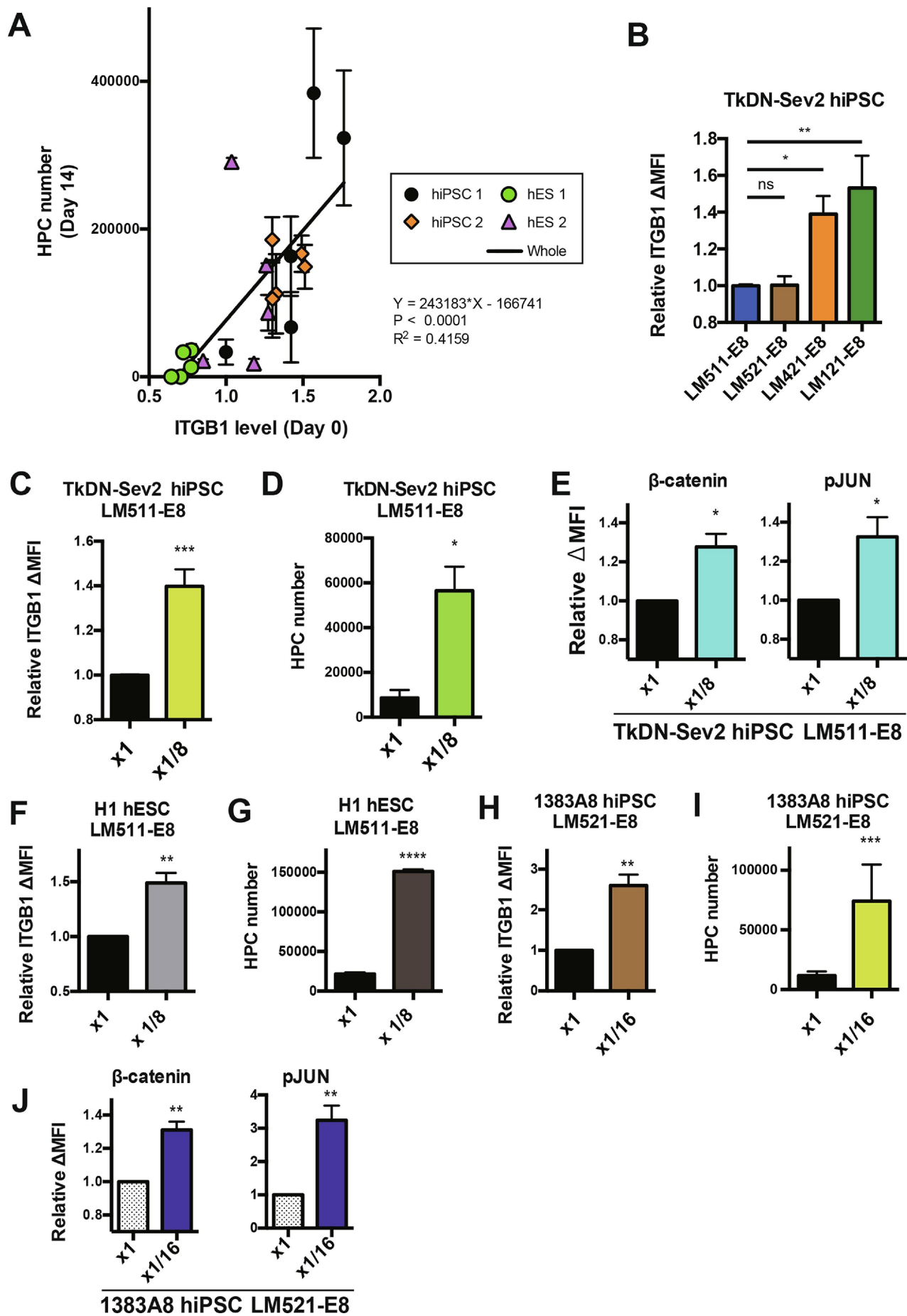
x 1/8

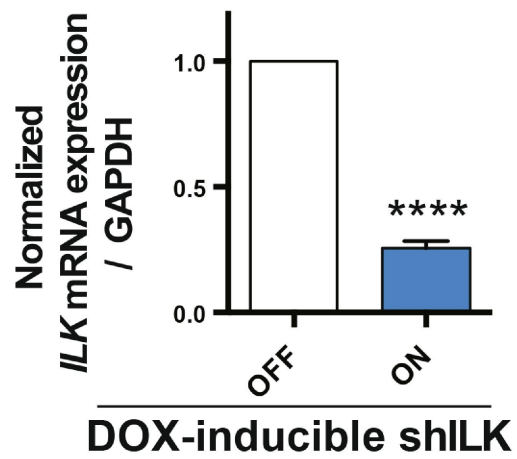
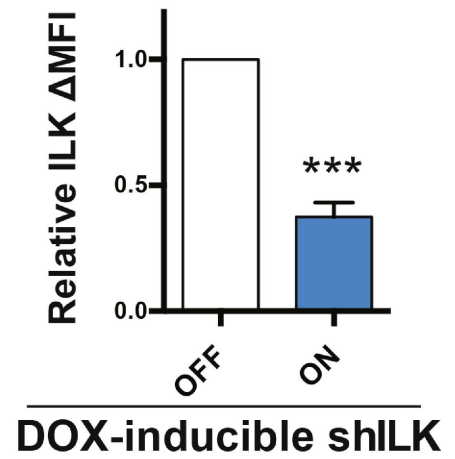
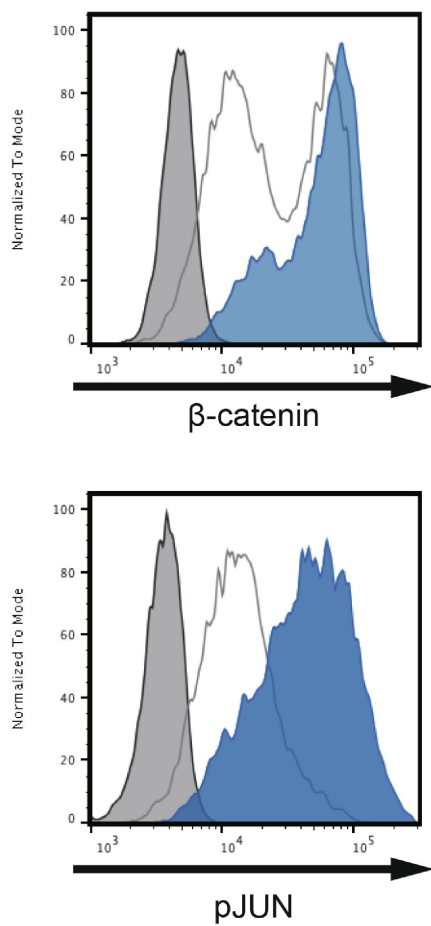
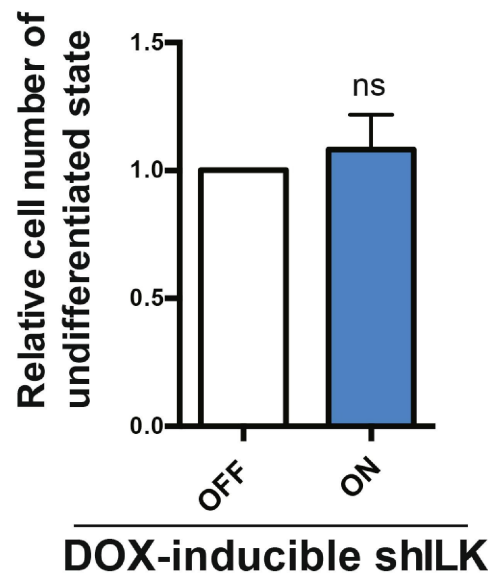
C



D





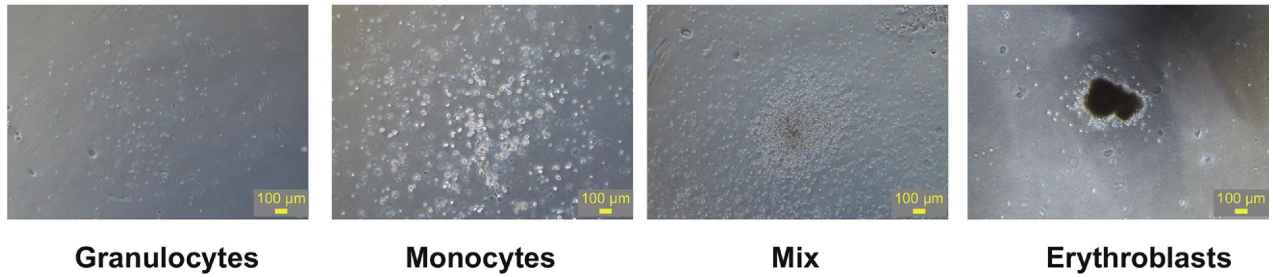
A**B****C****D**

Gray : Isotype control

White : Dox-inducible shILK Dox on

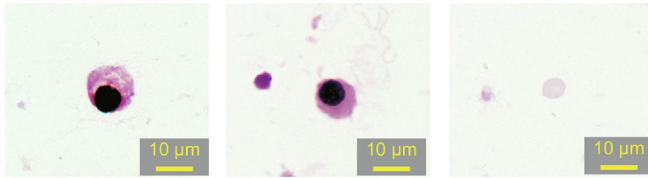
Blue : Dox-inducible shILK Dox off

A

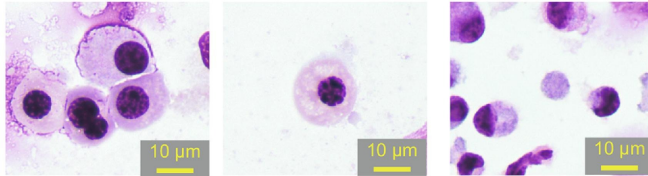


B

Conventional

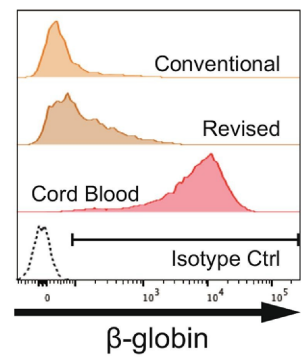


Revised



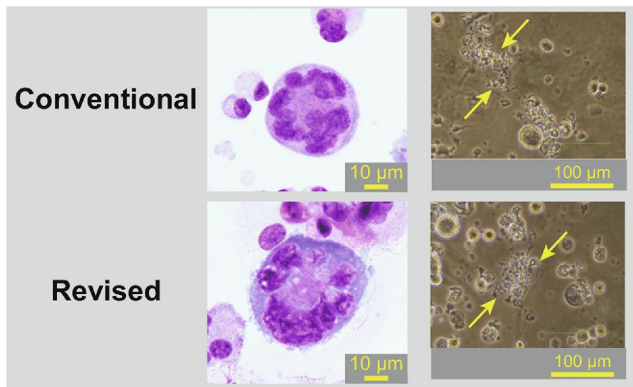
C

β -globin expression histogram of whole CD235+ cells

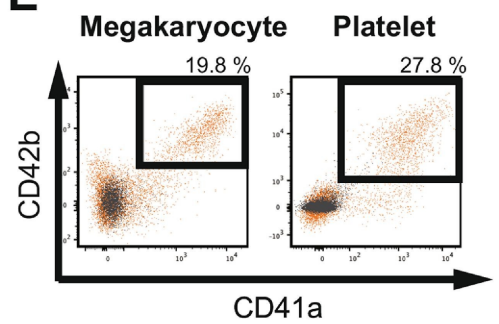


D

Megakaryocyte (MG Staining) Proplatelet formation (Bright field)

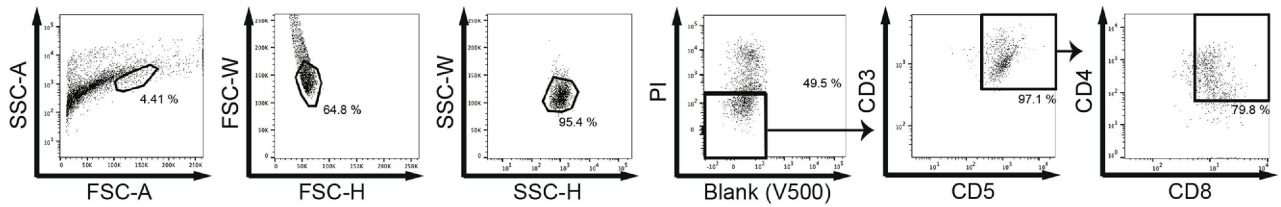


E

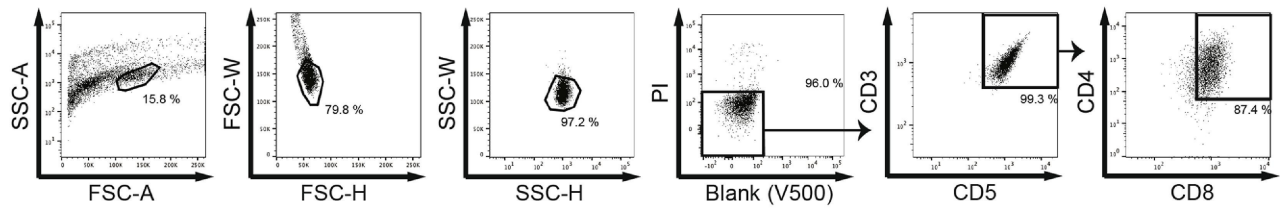


F

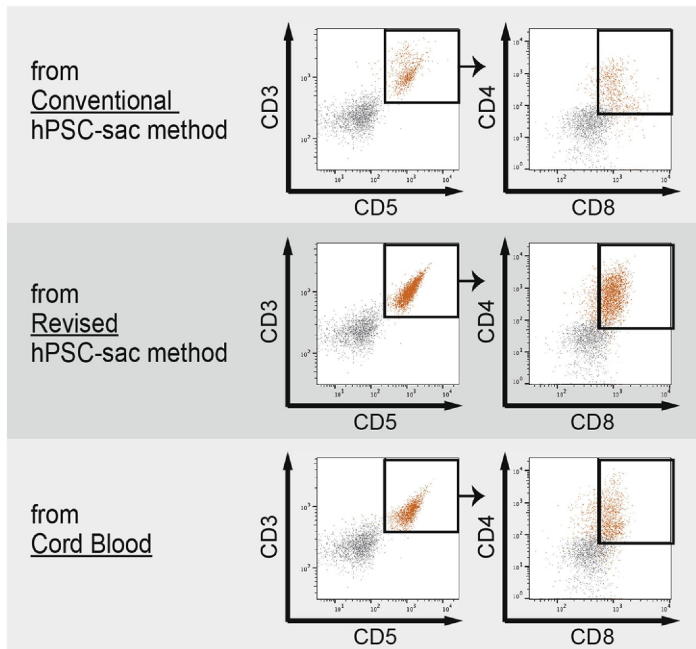
Differentiated T cell plots
by Conventional hPSC-sac method



Differentiated T cell plots
by Revised hPSC-sac method



G T cell plots with Isotype control



Supplemental table 2. Normalized signal intensity

ProbeName	GeneSymbol	MK04 on LM111E8	MK04 on LM311E8	MK04 on LM321E8	MK04 on LM411E8	MK04 on LM521E8	MK04 on LM121E8	MK04 on LM421E8
A_23_P15619	PRAC1	4.424677	7.3243823	7.001653	6.1778355	4.790534	131.86198	29.199741
A_23_P110837	IRX4	22.155985	29.681583	28.353298	13.37456	20.57747	468.05768	241.199
A_33_P3420680	NEUROG1	16.2228	15.40843	6.7323575	6.029263	4.6151423	351.10437	277.82462
A_19_P00320354	XLOC_I2_013542	1258.5055	2368.968	1904.0221	1550.225	1401.9391	4354.485	3154.6106
A_23_P15174	MT1F	263.99185	304.4173	348.4079	164.95064	282.16815	1635.0061	716.6423
A_23_P7727	HAPLN1	140.47797	207.57718	132.842	189.35916	128.32558	1049.6656	771.5305
A_33_P3336257	IRX1	325.5837	685.047	315.56995	134.94691	418.9677	2323.9104	1722.9996
A_33_P3381870	DMTN	19.15571	52.745876	34.25885	34.61202	42.504158	217.85757	114.50772
A_32_P86067	ZIC1	5.510605	30.02395	12.382845	12.228012	4.8547406	104.661674	45.69116
A_23_P167367	PITX2	4.152432	14.690116	6.8070354	6.008787	4.458042	105.603874	37.95158
A_32_P205110	FOXC1	35.889412	33.35466	46.519356	36.648167	42.46129	148.33832	89.03059
A_23_P107283	HOBX2	5.8785796	5.842756	5.97019	5.3034315	3.8449419	176.11801	51.407997
A_33_P3323298	JUN	657.7106	460.37094	451.407	226.12224	432.10168	962.2582	833.02234
A_23_P353667	MIR7-3HG	55.427063	74.556145	63.50844	86.53038	66.94794	95.54192	201.45563
A_33_P3379076	LOC101929056	7.3874	7.1268053	7.3188734	6.6273293	4.760711	113.233894	53.37917
A_19_P00323692	XIST	98.36046	108.619385	59.668247	71.634155	79.53535	237.80257	228.82932
A_23_P171143	TSPAN6	41.671207	139.73099	53.492645	33.189606	17.255695	1181.942	475.98666
A_33_P3299279	ANXA2R	25.57616	38.76481	29.720114	26.636127	24.432898	101.94229	54.15257
A_23_P211345	TBX1	86.90953	81.6705	65.60176	65.77423	60.656013	878.2722	363.81735
A_23_P24433	CTSF	277.12723	626.94305	612.9884	235.58565	399.2508	1419.6655	958.47455
A_23_P164196	DLX4	36.87883	58.940815	31.272913	39.837067	51.633625	78.318474	100.692696
A_19_P00331623	XIST	91.30629	117.14498	63.11245	55.141445	94.62543	245.76634	225.73013
A_33_P3254956	RIPPLY3	82.24856	92.11809	104.11651	81.64996	128.15959	215.56282	207.1161
A_23_P156025	IRX2	711.82935	879.544	499.30566	204.69992	403.23886	2180.502	1345.6245
A_33_P3291579	PAX7	40.04479	53.267696	20.190178	14.769281	36.38111	193.32097	152.3938
A_32_P34920	FOXD1	93.32396	94.194565	83.13608	64.948296	107.20518	756.1005	317.21573
A_32_P174365	SATB2	76.011894	41.559673	51.34267	66.88331	59.841316	174.08728	94.67066
A_23_P421011	KAZALD1	12.799381	17.799051	36.51856	34.320255	31.590776	103.46701	62.60924
A_23_P47034	HHEX	58.209858	79.1611	38.860893	62.318016	64.37768	149.25212	193.6397
A_23_P49539	BAHCC1	20.396791	29.226284	13.57546	6.3558774	18.04976	156.19957	95.38666
A_33_P3328450	HMX1	30.858011	37.44317	23.557756	19.94834	26.373783	123.61863	81.546776
A_33_P3354646	PNLIPRP1	50.644093	61.66199	63.59168	53.57492	73.13135	140.04317	186.87996
A_32_P60606	DKFZp667F0711	18.76472	38.358555	35.866844	9.401775	41.789204	84.16048	118.34189
A_33_P3280646	CASR	3.9612148	6.892587	7.117491	6.3618135	10.43196	128.78842	16.402327
A_19_P00330885		9.502543	8.039175	16.394642	7.208128	24.113358	157.70828	80.59254
A_33_P3395400	LOC100130256	4.384558	7.7795787	8.00519	7.2205358	14.762871	274.72797	106.93262
A_33_P3464749	LINGO1-AS1	29.247524	7.6169205	7.862064	10.58002	4.829571	27.315536	117.168526
A_19_P00800524		172.21007	122.26741	154.17464	108.21132	220.83043	605.74554	355.742
A_19_P00330953		24.28972	33.971897	30.240835	24.01147	18.651712	135.02022	109.17532
A_33_P3316903	Inc-GTF2E2-1	66.27258	76.221535	77.54664	68.16105	88.68541	96.36332	275.88324
A_23_P129144	MYZAP	12.800531	15.571129	7.952176	7.216915	20.68696	211.28343	50.29524
A_33_P3274763		4.3639574	7.724452	7.957499	7.2256026	6.519532	177.99669	54.477608
A_33_P3507384	LOC152274	4.3527474	7.702111	7.9371743	7.223479	19.762934	189.04324	28.08216