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Extracellular laminin regulates hematopoietic potential of pluripotent stem cells through integrin β1-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 β1 (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 α5, β1, and γ1 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 α6β1 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 μM) was purchased from Tocris (Bristol, UK), ILK inhibitor Cpd22 (1 μM) from Calbiochem (Merck, Darmstadt, Germany), Wnt inhibitor Wnt-C59 (1 μM) from Selleck Chemicals (Houston, TX, USA), and SP600125 (10 μM) 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 μg/cm^2, except LM411-E8 (1.0 μg/cm^2), for 1 h at 37°C in CO_2 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 μM Y27632 (Wako, Osaka, Japan). For passaging, the cells were detached by treatment with 0.5 × TrypLE Select (Gibco, Carlsbad, CA, USA) and 5 mM EDTA/DPBS for 4 min at 37°C in a CO_2 incubator and were scraped and then seeded at 0.1 × 10^5 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 × 10^5 cells) of hPSCs treated with 1 mM $CaCl_2$ (Sigma-Aldrich) and 20% KSR (Invitrogen, Carlsbad, CA, USA) or 181 PU/ml Dispase 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 μg/ml ascorbic acid (Sigma-Aldrich), 450 μM α-monothioglycerol (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 CO_2 incubator with hypoxia (5% O_2) on days 1–7. In the revised hPSC-sac method, 50 ng/ml basic FGF (Wako, Osaka, Japan) and 10 μM 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 μM Trolox (Cayman, Ann Arbor, MI, USA), and 50 μM 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 μM Y27632 (Wako), and 15 μM 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 μM SB203580 (Calbiochem), and 30 ng/ml SDF1α (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 $\mu g/cm^2$ 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 $\mu g/cm^2$ 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 GSK3β 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 α6β1 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 α6β1, 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 (Quélo 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 x1/32 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-E8x1/8 (dilution factor = 8, concentration = 0.0625 $\mu g/cm^2$) 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 $\mu g/cm^2$), 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.

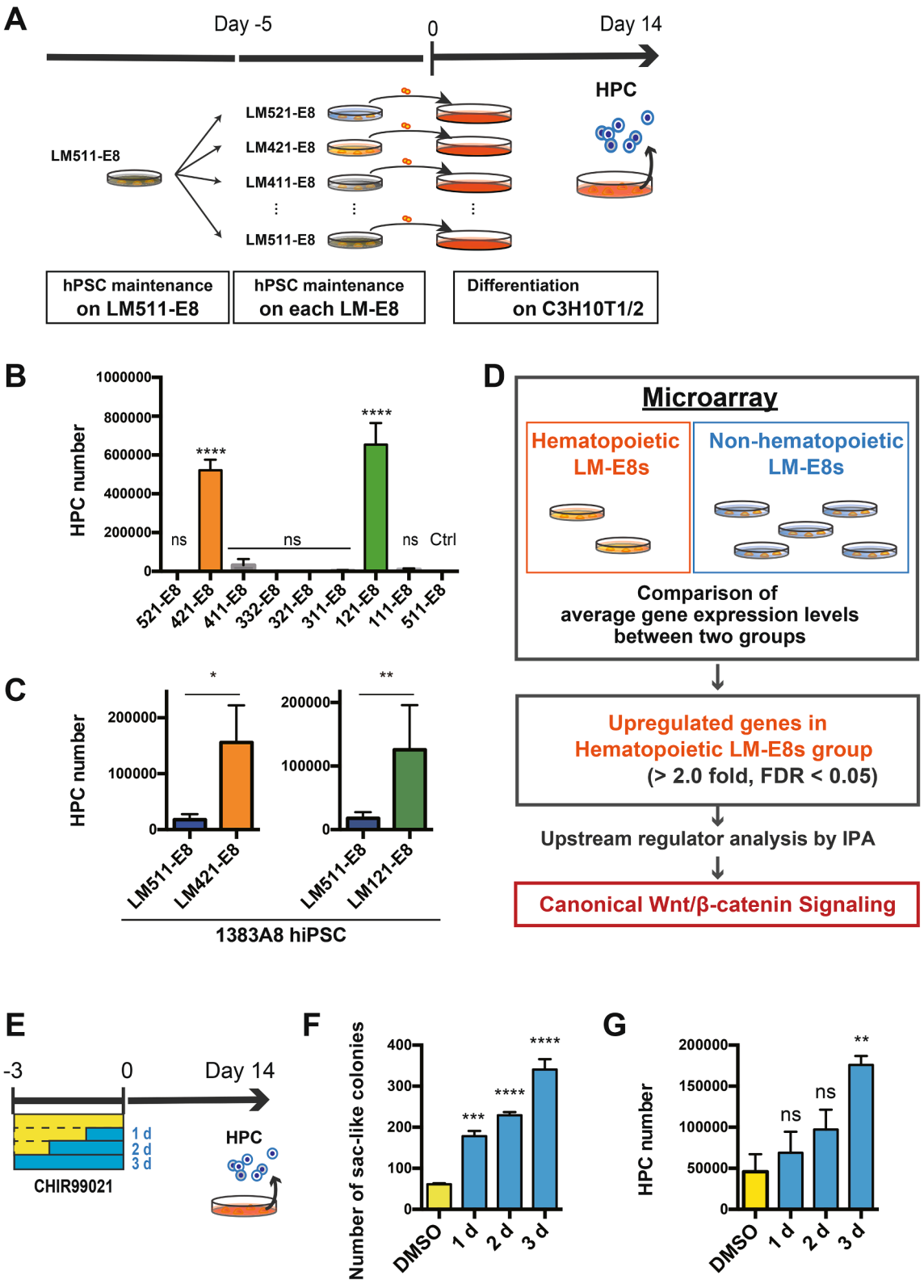

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Fig. 1. Extracellular laminin scaffolds determine the hematopoietic potential of hPSCs by controlling the canonical Wnt/β-catenin signaling pathway. (A) Schema of the experiments for comparing the effect of LM-E8s on the maintenance of hPSCs for HPC differentiation (basic conditions = 0.5 μg/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 CD34$^+$CD43$^+$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≧3, mean ± SEM. ****$P < 0.0001$ vs. control LM511-E8 (Ctrl) by Dunnett's test. n.s., not significant. (C) The number of CD34$^+$CD43$^+$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≧3, mean ± 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≧3, mean ± SEM. ***$P < 0.001$ and ****$P < 0.0001$ vs. DMSO by Dunnett's test. (G) The number of CD34$^+$CD43$^+$HPCs on day 14 differentiated from 0.7 to 0.8 × 10^5 hPSCs in (E). n = 3, mean ± 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 CD34$^+$CD43$^+$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 CD34$^+$CD43$^+$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 CD34$^+$CD43$^+$HPCs on day 14 more than 50-fold (average ± SEM; 19.11 ± 7.245 × 10^5 vs. 0.36 ± 0.095 × 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 CD31$^+$CD117$^+$CD309$^+$CD73$^-$CD34$^+$CD144$^+$CD43$^-$CD45$^-$CD41$^-$ 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 CD34$^+$CD43$^+$HPCs (Fig. 7B and Supplemental figure S6A). As for erythroid differentiation by the revised method, the calculated count of CD235$^+$ 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 CD41$^+$CD42b$^+$ megakaryocytes and platelets (Fig. 7D, Supplemental figure S6D and E) from the same number of CD34$^+$CD43$^+$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 μg/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

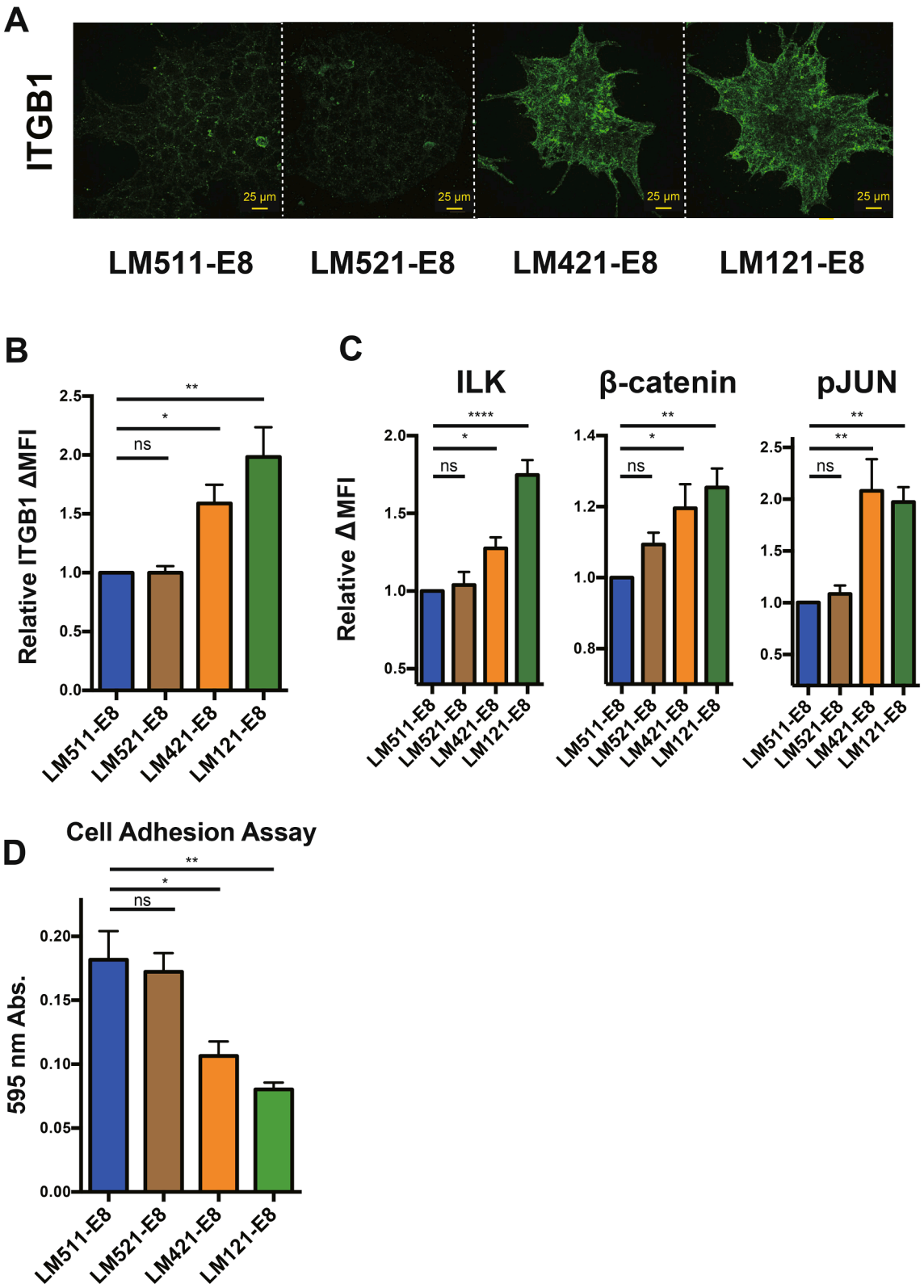

Fig. 2. Hematopoietic laminins have weaker binding avidity for hPSCs, resulting in higher ITGB1 expression level. (A) Fluorescence microscopy images of 1383A8 hiPSCs maintained on LM-E8s. hiPSCs on LM511-E8 and LM521-E8 express lower levels of ITGB1. (B) 1383A8 hiPSCs maintained on different LM-E8s were compared for ITGB1 expression measured by flowcytometry (values normalized to LM511-E8). n≧3, mean ± 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). ΔMFI represents mean fluorescence intensity (MFI) levels subtracted by the MFI of secondary conjugated antibody staining only. ΔMFI of LM511-E8 was set to 1. hPSCs on LM511-E8 express lower levels of ITGB1, ILK, and β-catenin, similar to LM521-E8. n≧3, mean ± 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 ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LM511-E8 by Dunnett's test. n.s., not significant.

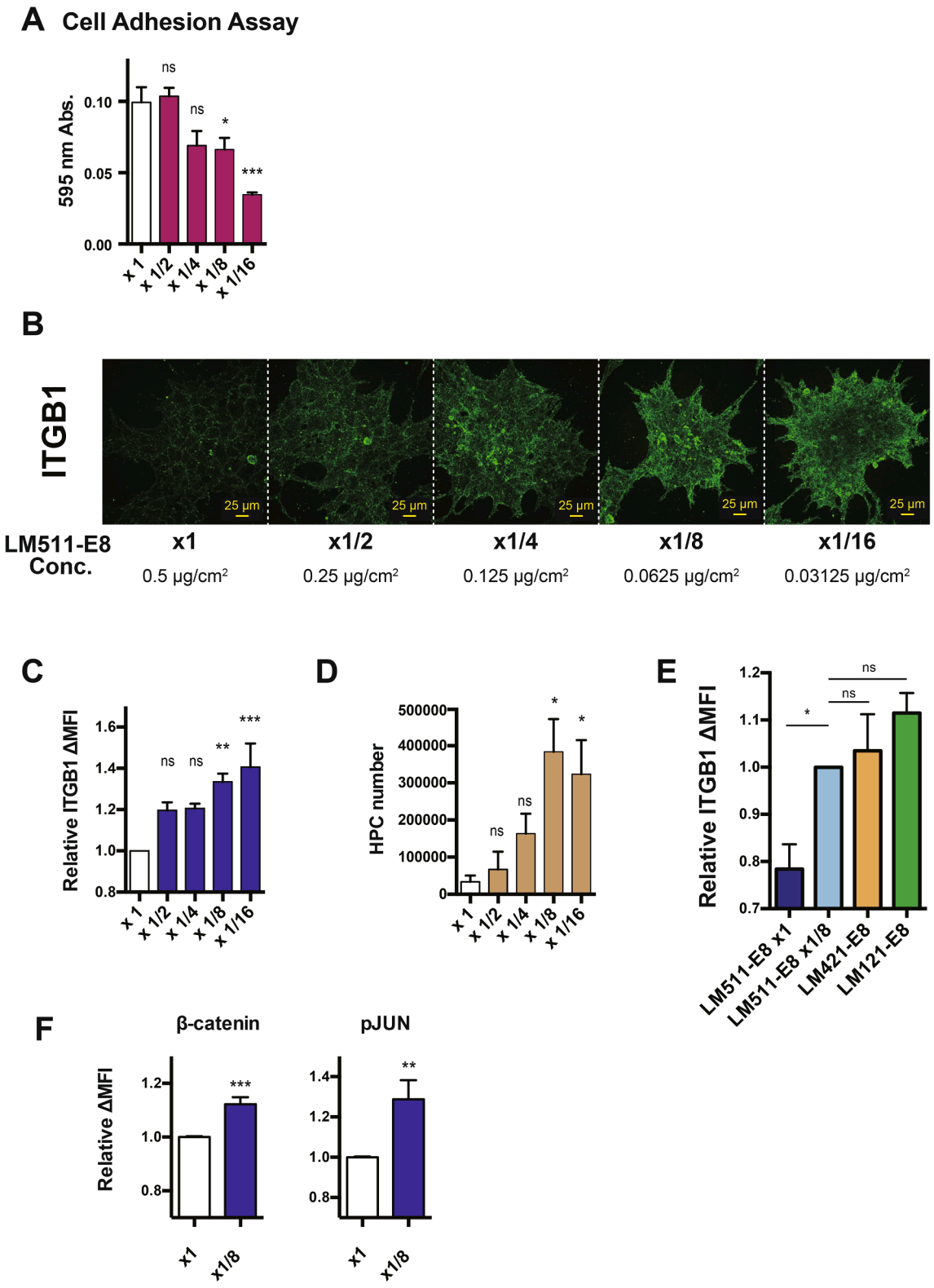

Fig. 3. Low concentration of non-hematopoietic LM511-E8 leads to increased ITGB1 expression and the hematopoietic differentiation of hPSCs. (A-D) 1383A8 hiPSCs maintained on serially diluted LM511-E8 were analyzed for their binding avidity (A), ITGB1 expression by fluorescence microscopy (B), normalized 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 ± 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≧3, mean ± 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 μg/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≧3, mean ± SEM. **$P = 0.0093$ and ***$P = 0.0004$ by *t* test.

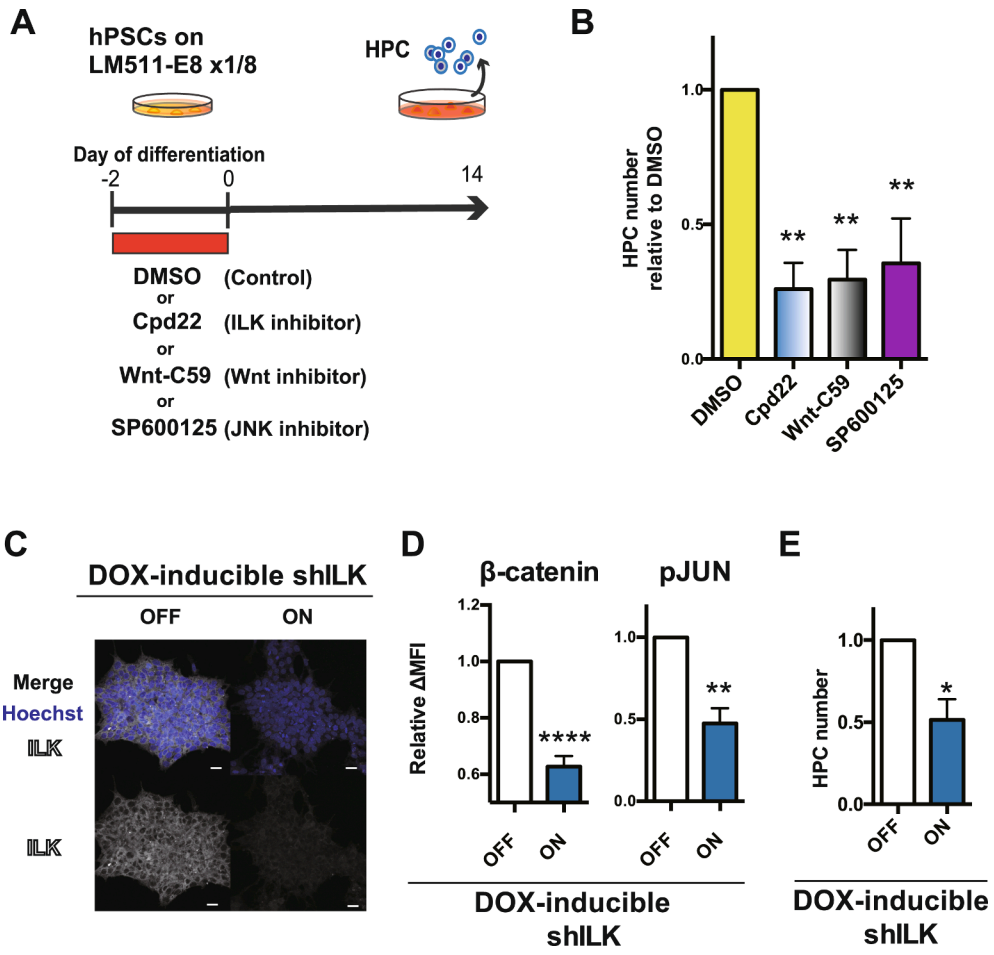

Fig. 4. The ITGB1-ILK-β-catenin-pJUN axis is crucial for the hematopoietic differentiation of hPSCs. (A) Schema for pre-treating 1383A8 hiPSCs with the indicated compounds for two days and then differentiating to HPCs with the hPSC-sac method. (B) Number of $CD34^{+}CD43^{+}$HPCs on day 14 differentiated 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 ± 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 μm), by flow cytometry analysis for the intracellular expression of β-catenin and phosphorylated JUN (pJUN) as ΔMFI 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 μg/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; Nazor et al., 2012; Polo et al., 2010). Genetic abnormalities (Kajiwara 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 α6β1 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 α6β1, the most dominant integrin complex on hPSCs (Nishiuchi et al., 2006). Laminins with the α1 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 α6β1 (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 β2 and γ1 chains. So too does LM321-E8, which does not show hematopoiesis (Fig. 1B), suggesting the mere combination of β2-γ1 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

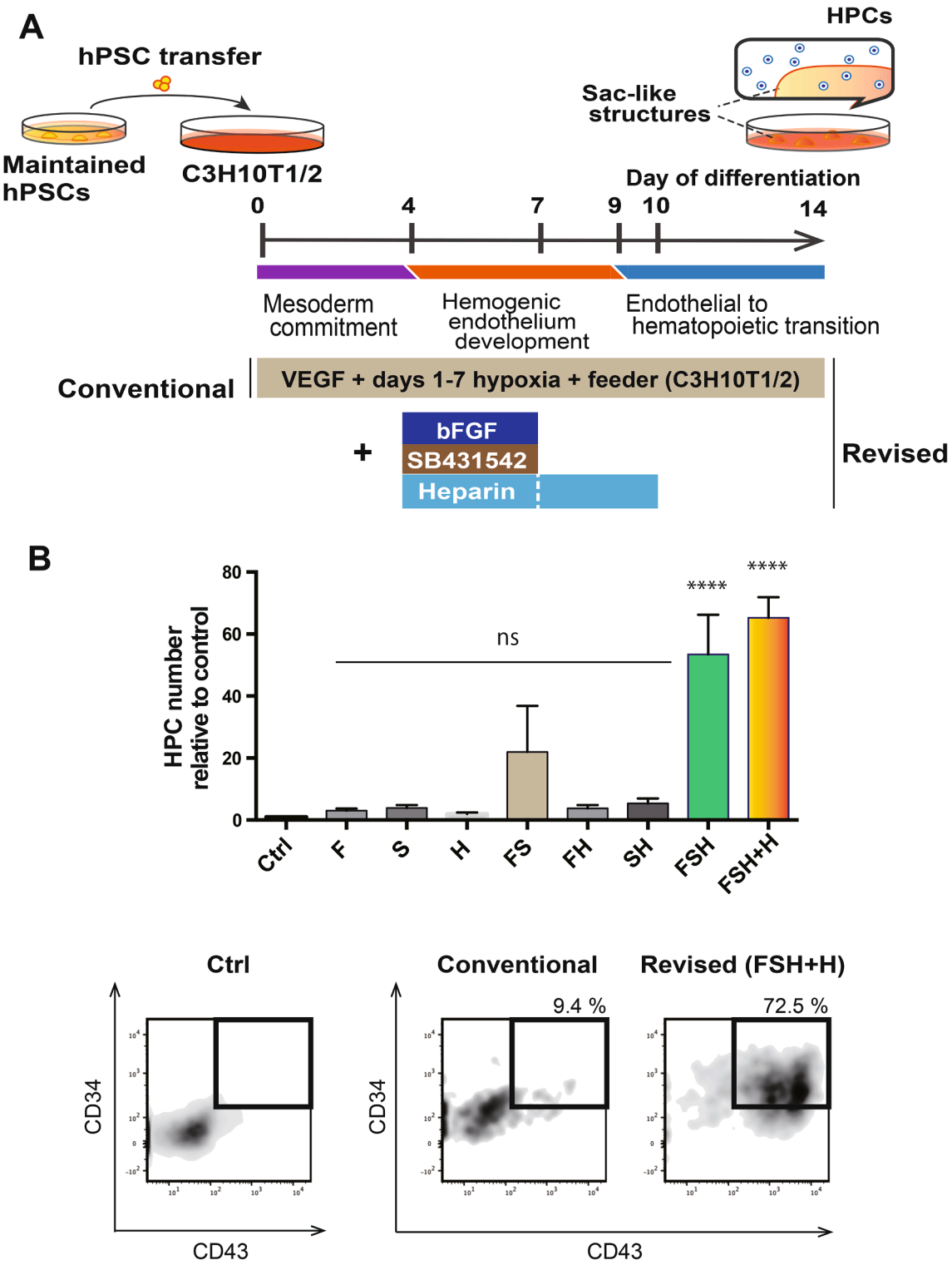

Fig. 5. Revised hPSC-sac differentiation method increases the $CD34^{+}CD43^{+}$ HPC yield. (A) Schema of the conventional hPSC-sac method and the revised method, which adds 3 new factors. (B) Relative number of $CD34^{+}CD43^{+}$HPCs on differentiation day 14 from KhES-3 hESCs with the conventional 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) ($n \geqq 3$, mean ± 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 0.7–0.8 $x10^{5}$ 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

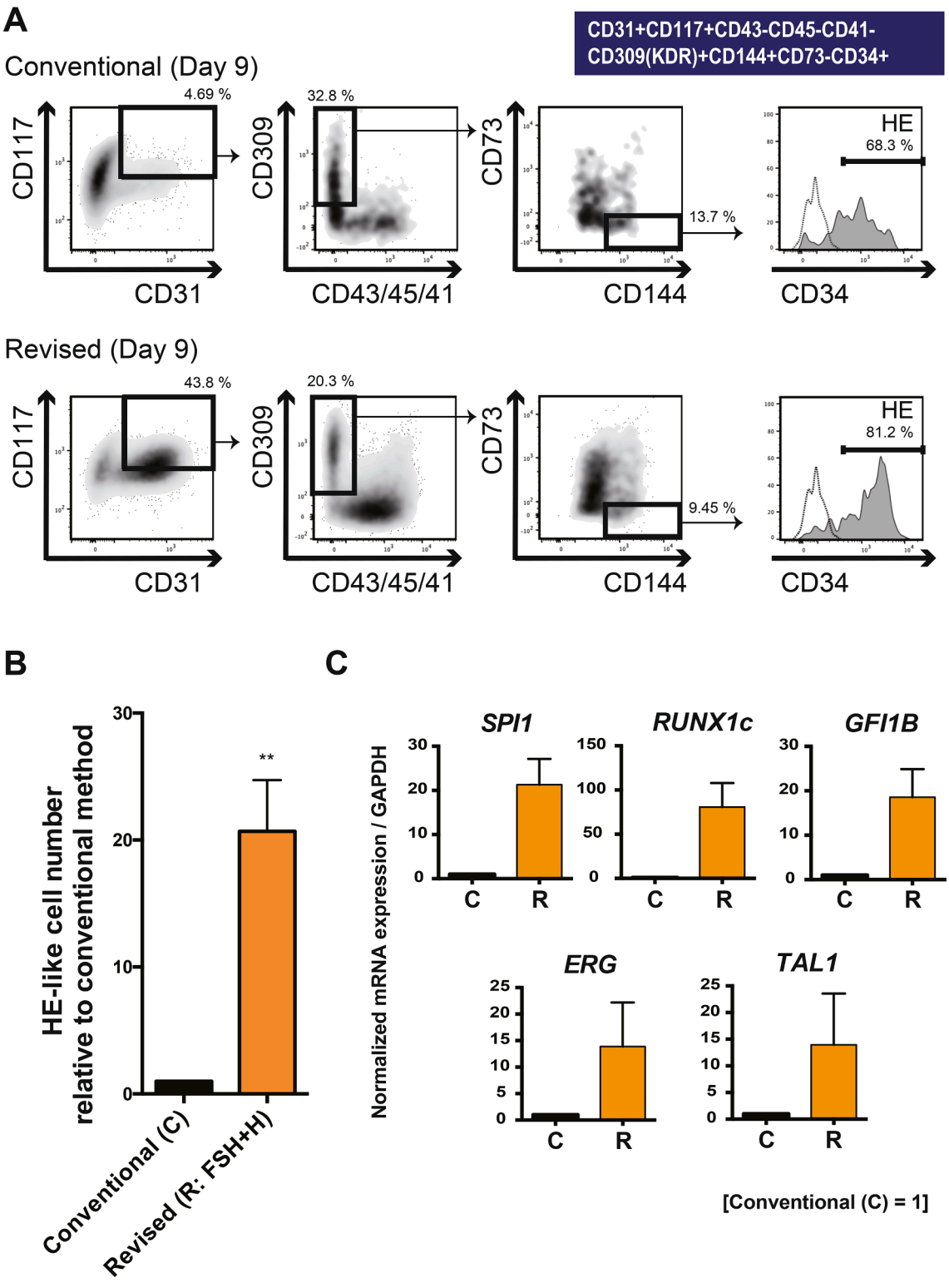

Fig. 6. HE-like cells increase with the revised hPSC-sac differentiation method. (A) Representative flow cytometry plots of HE-like cells differentiated from KhES-3 hESCs. The gating strategy for detecting HE-like cells in the conventional and revised hPSC-sac differentiation methods on day 9. Dotted lines in the histograms represents fluorescence minus one (FMO) and isotype-control stained control. (B) Relative number of detected HE-like cells per equivalent number of hPSCs (KhES-3 hESCs) in a dish on differentiation day 9. n≧3, mean ± SEM. $^{**}P = 0.0028$ by *t* test. (C) qRT-PCR analysis of all collected cells for the expression of hematopoiesis-related genes normalized to the level of GAPDH expression. Values are relative to the conventional hPSC-sac method condition. n≧3, mean ± SEM.

when inducing hematopoietic lineages. Furthermore, it seems that each hPSC line has its own baseline ITGB1 level, and the difference in this expression may be a new factor to explain the heterogenous differentiation capability of hPSCs. In the case when a specific cell line must be 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

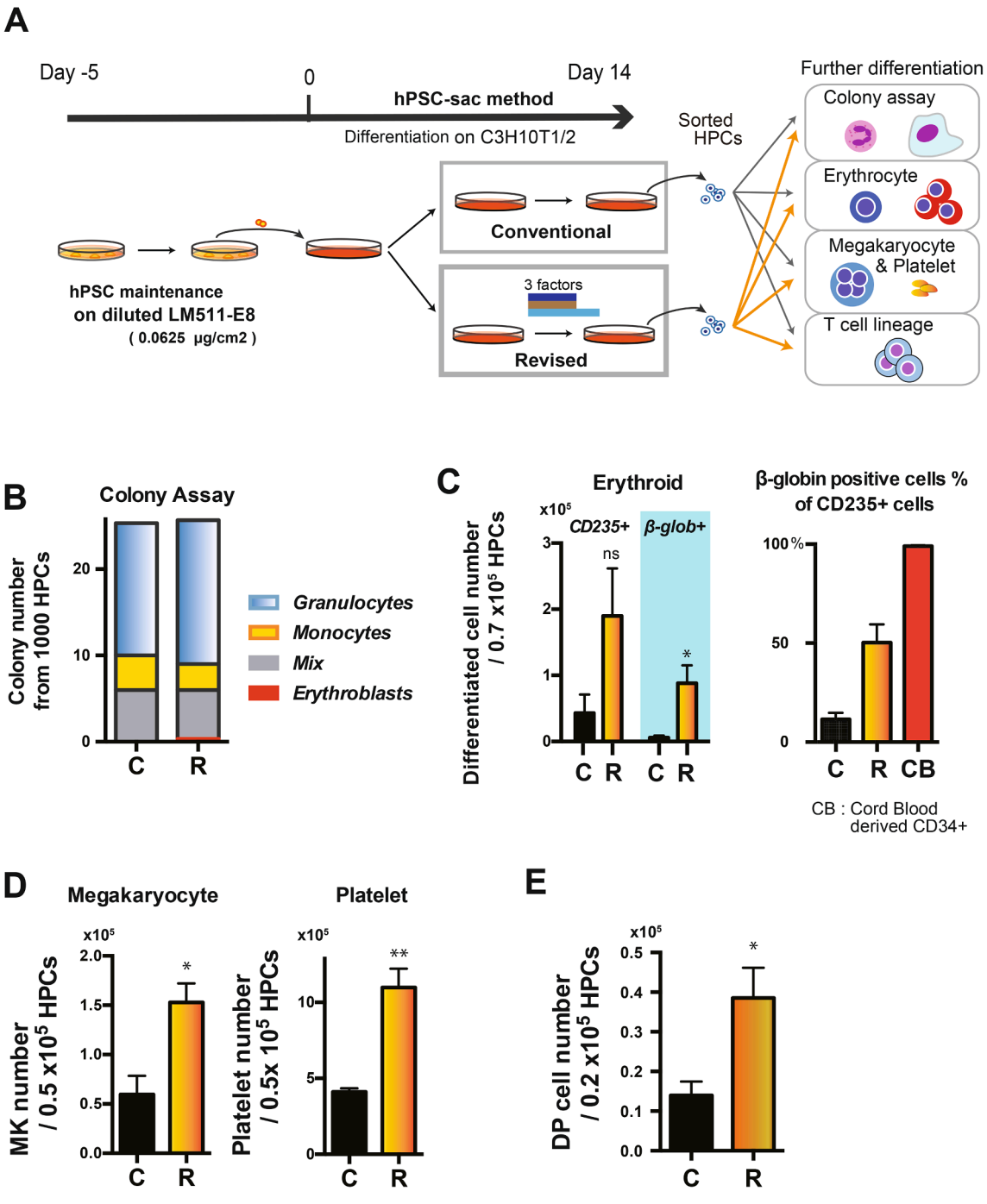

Fig. 7. The revised hPSC-sac method improves the quality and quantity of hematopoietic cells terminally differentiated from hPSCs on low-concentrated LM511-E8. (A) Schematic overview of the experiments in B-F. 1383A8 hiPSCs maintained on low-concentrated LM511-E8 x1/8 were subjected to the conventional 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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