Terminal proliferation and differentiation of CFU-E is stimulated by EPO, whereas BFU-E, which are more immature than CFU-E, respond to stem cell factor (SCF), insulin-like growth factor (IGF)-1, corticosteroids, interleukin (IL)-3 and IL-6, in addition to EPO [15]. Although FL is the most active organ for erythropoiesis, little is known about how erythropoiesis is regulated in that tissue.

Here, in order to identify niche cells for erythropoiesis, we used flow cytometry based on surface molecule expression to separate cells in early FL into hepatoblasts (HBs), sinusoid endothelial cells (SECs) and hematopoietic cells (HCs), and then evaluated cytokine expression in each fraction.

Materials and methods

Animals. ICR and C57BL/6J mice were purchased from Nihon SLC (Hamamatsu, Japan) and Kyudo (Tosu, Japan), respectively. *Map2k4*$^{+/-}$ mice were provided by RIKEN BioResource Center (Tsukuba, Japan). Noon of the day of the plug was defined as 0.5 day post-coitum (dpc). Embryos at 12.5 and 14.5 dpc were dissected in PBS under a stereomicroscope. Animals were handled according to Guidelines for Laboratory Animals of Kyushu University.

Flow cytometry. For hepatoblasts and sinusoid endothelial cells, fetal livers at 12.5 and 14.5 dpc were digested in 1mg/mL collagenase (Washington Biochem Co., Freehold, New Jersey) in alpha-MEM containing 20% FBS, filtered through 40-μm nylon mesh, and washed once with PBS. Cells were stained with a FITC-conjugated anti-mouse Dlk-1 Ab (MBL, Nagoya, Japan), a PE-conjugated anti-mouse Lyve-1 Ab (MBL), an APC-conjugated anti-mouse CD31 Ab (Biolegend, San Diego, CA), a PE-Cy7-conjugated anti-mouse CD45 Ab (eBioscience, San Diego, CA), and a PE-Cy7-conjugated anti-mouse Ter119 Ab (eBioscience).

Real time-PCR. RNA was extracted from sorted and fetal liver samples using a RiboPure™ kit (Life Technologies, Carlsbad, CA) and mRNA was reverse transcribed using a High-Capacity RNA-to-cDNA kit (Life Technologies). cDNA synthesis quality was evaluated by amplifying mouse *β-actin* by PCR. Thirty thermal cycles were employed as follows: denaturation at 95°C for 10 seconds, annealing at 60°C for 20

seconds, and extension at 72°C for 20 seconds. Gene expression levels were measured by real time-PCR with TaqMan® Gene Expression Master Mix and StepOnePlus™ real time PCR (Life Technologies). All probes (*Flt3-L, TPO, EPO, SCF, IL-3, IL-6, IL-11, G-CSF* and *GM-CSF*) were from TaqMan® Gene Expression Assays (Life Technologies). All samples were assayed in triplicate wells. mRNA levels were normalized to *β-actin* and the relative quantity (RQ) of expression was compared with a reference sample.

Enzyme-Linked Immunosorbent Assay (ELISA). Lysates of sorted cells were obtained using a cell lysis buffer (M-Per® Mammalian Protein Extraction Reagent, Thermo Fisher Scientific, Waltham, MA) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma Aldrich, St. Louis, MO). The sample was centrifuged at 14000xg at 4°C for 15 minutes. Supernatants containing soluble protein were collected and protein concentration was estimated by measuring absorbance at 280 nm (NANODROP 2000C, Thermo Fisher Scientific). SCF and EPO in sorted cells were assayed using an ELISA kit (Mouse SCF Immunoassay and Mouse EPO Immunoassay, R&D systems) according to the manufacturer's instructions. Experiments were performed in duplicate. The O.D. was measured using a Thermo Multiskan EX plate reader (Thermo Fisher Scientific).

Immunohistochemistry. Dissected ICR mouse embryos were fixed in 2% paraformaldehyde in PBS overnight at 4°C and washed in PBS three times. After 27% sucrose infusion, embryos were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen (vapor phase). Frozen embryos were sectioned at 20μm, transferred onto glass slides (Matsunami, Osaka, Japan), and dried. After blocking in 1% BSA in PBS, sections were incubated with primary antibodies overnight at 4°C. Anti-mouse Dlk-1 Ab (MBL), anti-mouse Lyve-1 Ab (MBL), anti-mouse c-Kit Ab (R&D Systems), anti-mouse SCF Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse EPO Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse Ki-67 Ab (Dako Corporation, Carpinteria, CA) served as primary antibodies. After washing in PBS three times, sections stained with Dlk-1, Lyve-1, c-Kit or Ki-67 antibodies were incubated with secondary antibodies and TOTO-3 (Life Technologies) for nuclear staining. In samples stained with SCF or EPO antibodies, the TSA Biotin System (PerkinElmer, Covina, CA) was used to amplify the signal. Donkey anti–rabbit IgG-Alexa555, Donkey anti–goat IgG-Alexa488, Donkey anti–rat IgG-Alexa488 and Alexa546 and Streptavidin Alexa546 (all from Life Technologies) served as secondary antibodies. Coverslips were mounted using fluorescent mounting medium (Dako Corporation, Carpinteria, CA). Slides were observed using a FV-1000 confocal microscope (Olympus, Tokyo, Japan).

Single cell preparation and cell counting. To prepare single cell suspensions of HCs from *Map2k4*$^{-/-}$ and wild-type FL at 12.5 dpc, dissected FL was crushed on the 40μm nylon cell strainer (BD Falcon, Bedford, MA) with the inner of 2.5 mL syringe. Cells were washed in PBS and collected into the tube. The number of living cells was counted after Trypan Blue staining.

Results

Cytokine expression in hepatoblasts

We examined expression of several cytokine genes in FL tissue samples at 12.5 and 14.5 dpc using real-time PCR. Significant expression of *FMS-like tyrosine kinase 3 ligand* (*Flt3l*), *thrombopoietin* (*TPO*), *erythropoietin* (*EPO*) and *interleukin-6* (*IL-6*) was seen in FL (Fig. 1A). *Dlk* (Delta-like 1 homolog) is a marker of hepatoblasts (HBs) in FL [16], while Lyve-1 (lymphatic vessel endothelial hyaluronan receptor 1) marks sinusoid endothelial cells (SECs) [17]. As shown in Figure 1B, fractions of HBs, SECs and hematopoietic cells (HCs) were isolated from mouse FL at both 12.5 and 14.5 dpc by flow cytometry based on expression of the following surface markers: (1) HBs, CD45–/Ter119–/Dlk-1+; (2) SECs, CD45–/Ter119–/Lyve-1+/CD31+; and (3) HCs, CD45+/Ter119+. To determine which component contributes to cytokine production, we used real-time PCR to examine *EPO, Flt3l, IL-6, SCF* and *TPO* expression in isolated HBs, SECs and HCs (Fig. 1C). *EPO* and *TPO* were expressed predominantly in HBs both at 12.5 dpc and 14.5 dpc, suggesting that erythropoiesis and megakaryopoiesis are activated by HBs in FL. Levels of *SCF* mRNA were higher in HBs than in SECs. Expression of *Flt3l* and *IL-6* was predominantly detected in HCs, suggesting that HSCs and HPCs expand via an autocrine mechanism. To investigate expression of the cytokine proteins EPO and SCF, we undertook Enzyme-Linked ImmunoSorbent Assay (ELISA) and found that EPO protein was predominantly detected in HBs (63.8 pg/mL/10000 cells) but was not detected in SECs and HCs (Fig. 2A). SCF protein, however, was detected in all fractions (Fig. 2B). In agreement with

SCF mRNA expression, HBs expressed SCF protein at a higher level (7.01 pg/mL/10000 cells) than did SECs (2.38 pg/mL/10000 cells) or HCs (0.025 pg/mL/10000 cells). When we compared expression levels of EPO and SCF proteins in each fraction, EPO expression was highest in HBs, suggesting that HBs secrete EPO to regulate FL erythropoiesis (Fig. 2C). To confirm EPO and SCF protein localization in FL, we performed immunohistochemistry and found that EPO protein was expressed primarily in Dlk-1-expressing HBs (Fig. 2D), in agreement with ELISA analysis. Staining of FL with anti-SCF antibody revealed SCF protein in both SECs expressing Lyve-1 and HBs expressing Dlk-1 (Fig. 2E-F).

Fetal liver of Map2k4$^{-/-}$ *mouse embryos*

To characterize HB function in FL, we analyzed *Map2k4* (*mitogen-activated protein kinase kinase 4*, formerly known as *Sek1* and *MKK4*)$^{-/-}$ mouse embryos, which lack FL HBs [18-20]. Real-time PCR analysis of whole FL from mutant and wild-type embryos showed that among cytokine-encoding genes, *EPO* and *SCF* mRNAs were down-regulated in the mutant mice, implying an impairment in hematopoiesis, particularly erythropoiesis, in *Map2k4* mutant mice (Fig. 3A). To evaluate potential alterations in hematopoiesis, we performed immunohistochemistry and found that the number of c-Kit expressing cells which represent HSCs and HPCs in the FL at this stage, decreased in a FL field from *Map2k4*$^{-/-}$ mouse embryo relative to wild-type embryo (Fig. 3B; green) [23]. In addition, the number of cells expressing Ki-67, a marker of cell proliferation, decreased in a FL field from *Map2k4*$^{-/-}$ mouse embryos

compared to wild-type embryos (Fig. 3B; red). In agreement with the Ki-67 staining, there were fewer FL cells seen in *Map2k4*$^{-/-}$ versus wild-type embryos (Fig. 3C).

Discussion

In FL, HSCs differentiate into mature HCs, particularly erythroid cells [9,10]. SCF and EPO are representative cytokines that regulate erythropoiesis [14,15]. Expression levels of *SCF* and *EPO* genes were higher in whole FL tissue than in adult BM and kidney, suggesting that FL primarily functions in erythropoiesis [24]. Recently, using a competitive repopulating assay, Chou and Lodish reported that FL stromal cells expressing both SCF and Dlk-1 support HSC maintenance [25]. However, it remained unclear which cells secreted EPO in FL. HBs are regarded as common progenitors of hepatocytes and biliary epithelial cells and thought to support liver construction through formation of a mesh-like structure [16,26]. Our results are strongly indicative of an additional role of HBs in producing erythropoietic cytokines SCF and EPO. Although gene expression of *IL-6* was observed in FL at 12.5 dpc, that was not detected in cell fractions of HBs, SECs and HCs (Fig. 1). As shown in Fig. 1B, we could observe unclassified cell fraction (CD45–/Ter119–/Dlk-1–/Lyve-1–/CD31–), based on surface marker expression, implying that this unclassified cell fraction may express *IL-6* gene. It will be further necessary to clarify roles of the unclassified cells in FL hematopoiesis in the future.

The c-Jun NH_2-terminal protein kinases (JNKs) are members of the mitogen-activated protein kinase (MAPK) family. Map2k4 is a MAPK kinase that directly activates JNKs in response to extracellular and intracellular stresses, and its deficiency leads to abnormal hepatogenesis, resulting in loss of HBs [20,27-29]. In situ hybridization shows that expression of *Map2k4* transcripts is detected in FL by 12.5 dpc and then up-regulated until 16.5 dpc, at which time expression is down-regulated [30]. When *Map2k4*$^{-/-}$ FL cells were transplanted into *rag1*$^{-/-}$ adult mice, both B cells and T cells were generated, demonstrating that *Map2k4*$^{-/-}$ FL cells contain HSCs and/or HPCs [28]. In addition, normal hematopoiesis reportedly occurs in the *Map2k4*$^{-/-}$ YS at both 9.5 and 10.5 dpcs, based on erythroid, myeloid and mixed colony formation [28]. Taken together, hematopoietic potential of *Map2k4*$^{-/-}$ FL looked normal compared to wild-type mice. Therefore, the decrease of hematopoietic cells were likely due to lack of HBs, but not due to alteration of hematopoietic potential in *Map2k4*$^{-/-}$ FL. Recently, Hikita et al. reported a novel mouse model lacking both HBs and hepatocytes using an *Alb* Cre driver [31]. This mutant mouse, generated by crossing *Alb Cre* mice with both *bcl-xl*$^{flox/flox}$ mice and *mcl-1*$^{flox/flox}$ mice, showed a decreased number of hepatocytes at 18.5 dpc. Future studies will be required to address the function of HBs and hepatocytes in FL erythropoiesis using this model.

Conclusion

Hepatoblasts comprise a niche for erythropoiesis through cytokine secretion.

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Authorship contributions

D. Sugiyama designed the research, performed research, analyzed data and wrote the paper. K. Kulkeaw performed research and analyzed data. C. Mizuochi, Y. Horio and S. Okayama performed research.

Conflict of Interest Disclosures

The authors have no conflict of interest to declare.

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Figure Legends

Fig. 1. Cytokine gene expression in fetal liver. (A) *FMS-like tyrosine kinase 3 ligand (Flt3l), thrombopoietin (TPO), eryrhropoietin (EPO), stem cell factor (SCF), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-11 (IL-11), granulocyte-colony stimulating factor (G-CSF)* and *granulocyte/macrophage-colony stimulating factor (GM-CSF)* mRNAs were examined in FL samples at 12.5 and 14.5 dpc by real-time PCR. Note high expression of *Flt3l, TPO, EPO* and *IL-6* in FL. (B) A single cell suspension was obtained from FL at 12.5 dpc and expression of CD45/Ter119, Dlk-1, Lyve-1 and CD31 was analyzed by flow cytometry. (1) CD45–/Ter119–/Dlk-1+ defines HBs; (2) CD45–/Ter119–/Lyve-1+/CD31+ defines SECs; and (3) CD45+/Ter119+ defines HCs. (C) Expression of *Flt3l, TPO, EPO* and *IL-6* was examined by real-time PCR in HBs, SECs and HCs sorted by flow cytometry, according to gates defined in Figure 1B. *EPO* and *TPO* expression was high in HBs at both 12.5 dpc and 14.5 dpc. *SCF* expression was higher in HBs than in SECs. Expression of *Flt3l* was high in HCs. Expression of *IL-6* was detected only in HCs.

Fig. 2. Expression of EPO and SCF protein in fetal liver. (A) EPO protein in sorted cells was assayed by ELISA. EPO was detected in HBs (63.8 pg/mL/10000 cells) but not in SECs and HCs at 12.5 dpc. (B) SCF protein in sorted cells was assayed by ELISA. SCF was detected in HBs (7.01 pg/mL/10000 cells), SECs (2.38 pg/mL/10000