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Nutritional modulation of mouse and human liver bud growth through a branched-chain amino acid metabolism

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ABSTRACT

Liver bud progenitors experience a transient amplification during the early organ growth phase, yet the mechanism responsible is not fully understood. Collective evidence highlights the specific requirements in stem cell metabolism for expanding organ progenitors during organogenesis and regeneration. Here, transcriptome analyses show that progenitors of the mouse and human liver bud growth stage specifically express the gene branched chain aminotransferase 1, encoding a known breakdown enzyme of branched-chain amino acids (BCAAs) for energy generation. Global metabolome analysis confirmed the active consumption of BCAAs in the growing liver bud, but not in the later fetal or adult liver. Consistently, maternal dietary restriction of BCAAs during pregnancy significantly abrogated the conceptus liver bud growth capability through a striking defect in hepatic progenitor expansion. Under defined conditions, the supplementation of L-valine specifically among the BCAAs promoted rigorous growth of the human liver bud organoid in culture by selectively amplifying self-renewing bi-potent hepatic progenitor cells. These results highlight a previously underappreciated role of branched-chain amino acid metabolism in regulating mouse and human liver bud growth that can be modulated by maternal nutrition *in vivo* or cultural supplement *in vitro*.

KEY WORDS: Liver bud, Metabolism, Branched chain aminotransferase 1, L-valine, Induced pluripotent stem cells

INTRODUCTION

Organ bud progenitor cells, which have a remarkable capacity for rapid cell growth and differentiation into multi-lineage cells, play important roles in organ development. Hepatic progenitor cells (HPCs), or liver bud progenitors, specify from foregut endoderm at embryonic day (E)9.5 in mice, followed by massive HPC expansion with a 10^4-fold increase in population, doubling from E9.5 to E13.5 in mice (Koike et al., 2014; Takebe et al., 2013). This tremendous growth is regulated by signals secreted from neighboring mesenchyme such as hepatocyte growth factors (Matsumoto et al., 2001), bone morphogenetic proteins (Rossi et al., 2001) and fibroblast growth factors (Serls et al., 2005), and by transcriptional networks that act intrinsically in the HPCs, such as Tbx3 (Suzuki et al., 2008), Smad2/3 (Weinstein et al., 2001) and beta-catenin (Micsenyi et al., 2004). Yet, the mechanism regulating this intensive and transient amplification in developing liver bud is largely unknown.

Emerging studies of stem cell metabolism have elucidated a role for cell type-specific metabolic pathways that are modulated during proliferation, differentiation or reprogramming processes (Ito and Suda, 2014; Shyh-Chang et al., 2013a). For instance, embryonic stem cells (ESCs) generally utilize the glycolytic system as a self-renewing propagation process (Kim et al., 2006), whereas differentiating ESCs shift their metabolism to oxidative phosphorylation (Folmes et al., 2012). Consistently, during the cellular reprograming process to induced pluripotent stem cells (iPSCs), glycolytic metabolism transitions from dependence on oxidative phosphorylation in adult fibroblast (Bukowiecki et al., 2014). The pivotal role of unique amino acid metabolism is also reportedly shown to maintain the pluripotency. Indeed, starvation of Thr, Met, Cys or Gln abolished mouse or human iPSC growth in culture (Shiraki et al., 2014; Shyh-Chang et al., 2013b; Tohyama et al., 2016). Thus, the mechanism of energy creation is diverse, depending on each cell or tissue type and its differentiation state. However, little is known about the metabolic dynamics during the early growth phase of hepatogenesis.

The aim of this study was to identify the metabolic demands of the rapid hepatic progenitor growth phase during mouse liver bud development and study their modulatory effects on *ex vivo* expansion by defined culture medium. Furthermore, in order to address the human-specific metabolic dynamics during the early growth phase of hepatogenesis, we took advantage of our recently developed liver bud organoid model from human iPSC (hiPSC) (Takebe et al., 2013) so as to determine if the implied metabolic mechanism is conserved in humans.

RESULTS AND DISCUSSION

To clarify a unique metabolic system in HPCs, global transcriptome analysis was performed by comparing E9.5, 10.5, 11.5, 13.5, 15.5, 17.5, 19.5 (just before birth), postnatal day (P)0, P3 and 8-week-old mouse liver cells. Generally, key metabolic genes, including aminoacid, glucose, lipid, bilirubin and urea metabolism, tended to increase in proportion to the developmental stage progression (Fig. 1A). In contrast, branched-chain aminotransferase 1 (*Bcat1*) was strongly expressed in early fetal liver cells (E9.5, 10.5 and 11.5), and suppressed in later developmental stages of the liver (Table S3; Fig. 1B). qRT-PCR analysis confirmed the highest expression of *Bcat1* in HPCs of E11.5 and E13.5 livers (Fig. 1C). Immunofluorescence analysis revealed specific Bcat1 protein localization in liver compared with other tissues at E11.5 (Fig. 1D; Fig. S1). Collectively, our developmental transcriptome data suggested the specific activation of the *Bcat1* gene during the early HPC expansion phase.

Bcats protein are aminotransferase enzymes that catalyze the first step in BCAA (valine, leucine and isoleucine) metabolism to form branched-chain alpha-keto acids (BCKAs) and glutamate (Fig. 2A).

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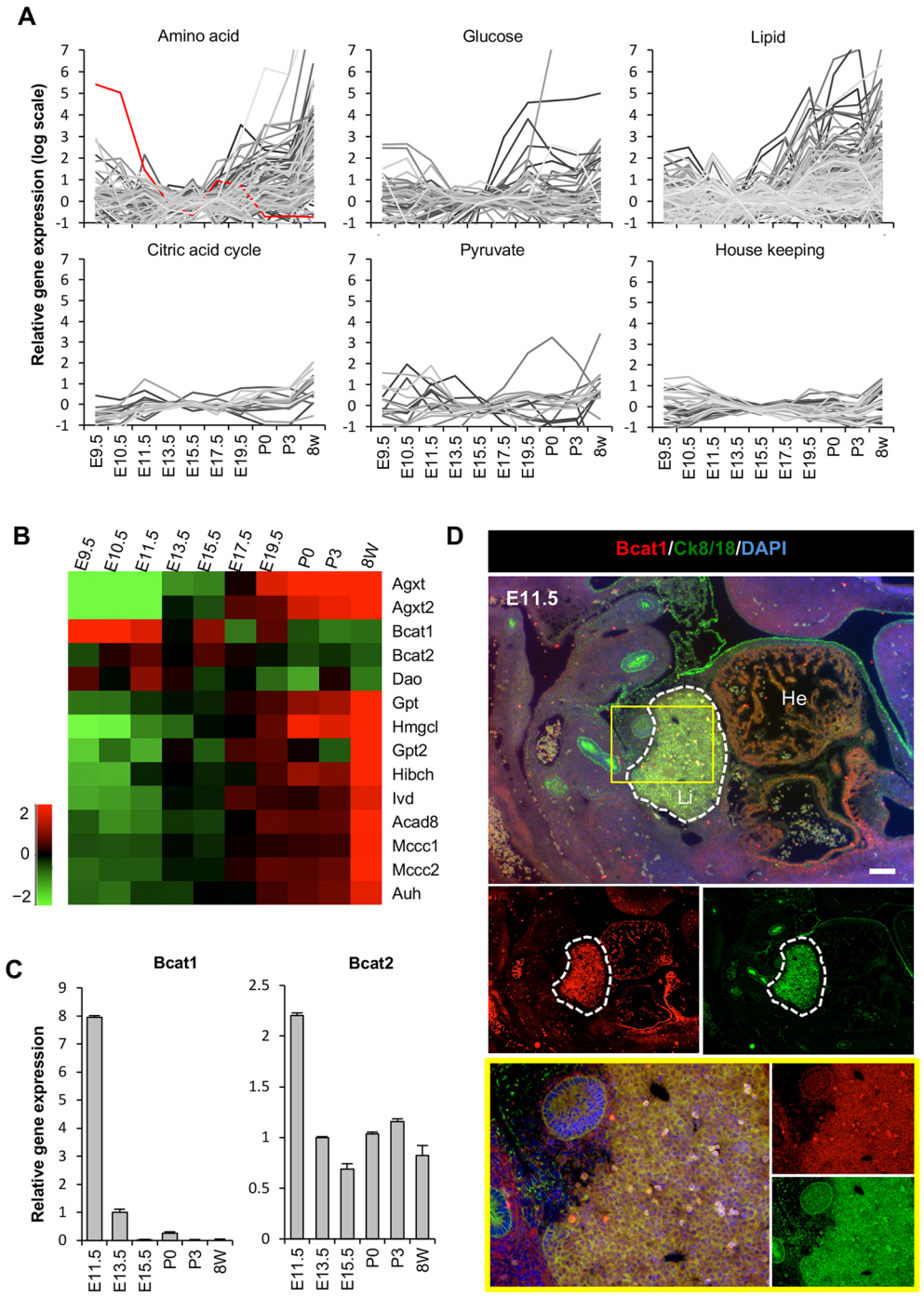

Fig. 1. Transcriptome data reveals unique *Bcat1* expression during early stage of liver development. (A) Transcriptome analysis for mouse liver cells at various developmental stages: E9.5, 10.5, 11.5, 13.5, 15.5, 17.5, 19.5, P0, P3 and 8 weeks. Metabolic pathway-related genes were analyzed by microarray and categorized into the following groups: amino acid, glucose, lipid, citric acid cycle and pyruvate, using the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html). Housekeeping genes are shown as control. *n*=3. (B) Heat map showing expression of genes involved in amino acid metabolism during liver development. The colors of the heat map represent: black, mean value; red, increase more than twofold; green, decrease more than twofold. (C) qRT-PCR analysis of mouse *Bcat* gene expression in liver cells from E11.5, 13.5, 15.5, P0, P3 and 8-week-old mice, relative to housekeeping gene *Gapdh*. *n*=3. Data presented as means±s.d. (D) Immunohistochemistry of Bcat1 in E11.5 mouse fetal liver tissue. Bottom image is magnified image of the area surrounded by the yellow box in the top image. Bcat1 protein expression is observed in Ck8/18-positive liver cells. Red, Bcat1; green, Ck8/18; blue, DAPI. He, heart; Li, liver. Dotted white line delineates liver. Scale bar: 150 µm.

Subsequent reactions convert the BCAA derivatives into acetyl-CoA or succinyl-CoA that enter the citric acid cycle. In order to elucidate metabolite production in the BCAA-related pathway, we performed metabolome analysis using E11.5, 19.5 and 8-week-old mouse liver tissues. We found that the actual concentrations of BCAAs at E9.5 were lower than those of 8-week-old mouse liver (Fig. 2A).

Next, pregnant mice were fed BCAA-depleted diets from E8.5 to E13.5 gestation in order to investigate the importance of dietary BCAA supply for early hepatogenesis in the fetus. We found that the relative embryonic liver weight per whole body in embryos from mice fed non-BCAA-containing diets was decreased by 54.0±16.7% (mean±s.d.) of control mice (Fig. 2B,C). Furthermore, similar liver hypoplasia was also observed in embryos from mice fed L-valine-depleted diets (Fig. S2). These results suggest active BCAA metabolism in developing liver bud relative to the more mature stages.

To further dissect the mechanism of liver bud defects, we performed flow cytometry analysis to evaluate the effects on HPCs. Liver cells of E13.5 fetal mice from dams given L-valine-depleted diets were analyzed by detection of hematopoietic cell markers, CD45 (also known as Ptprc) and Ter119 (also known as Ly76) (Kawamoto et al., 2000; Kina et al., 2000) and HPC marker, Dlk1 (Tanimizu et al., 2003). Dlk1+ HPC populations were decreased to 81.7±13.9% of control for L-valine-depleted diets (Fig. 2D,E), whereas the hematopoietic lineages were not affected by this nutrition. These results suggest that activation of dietary BCAA

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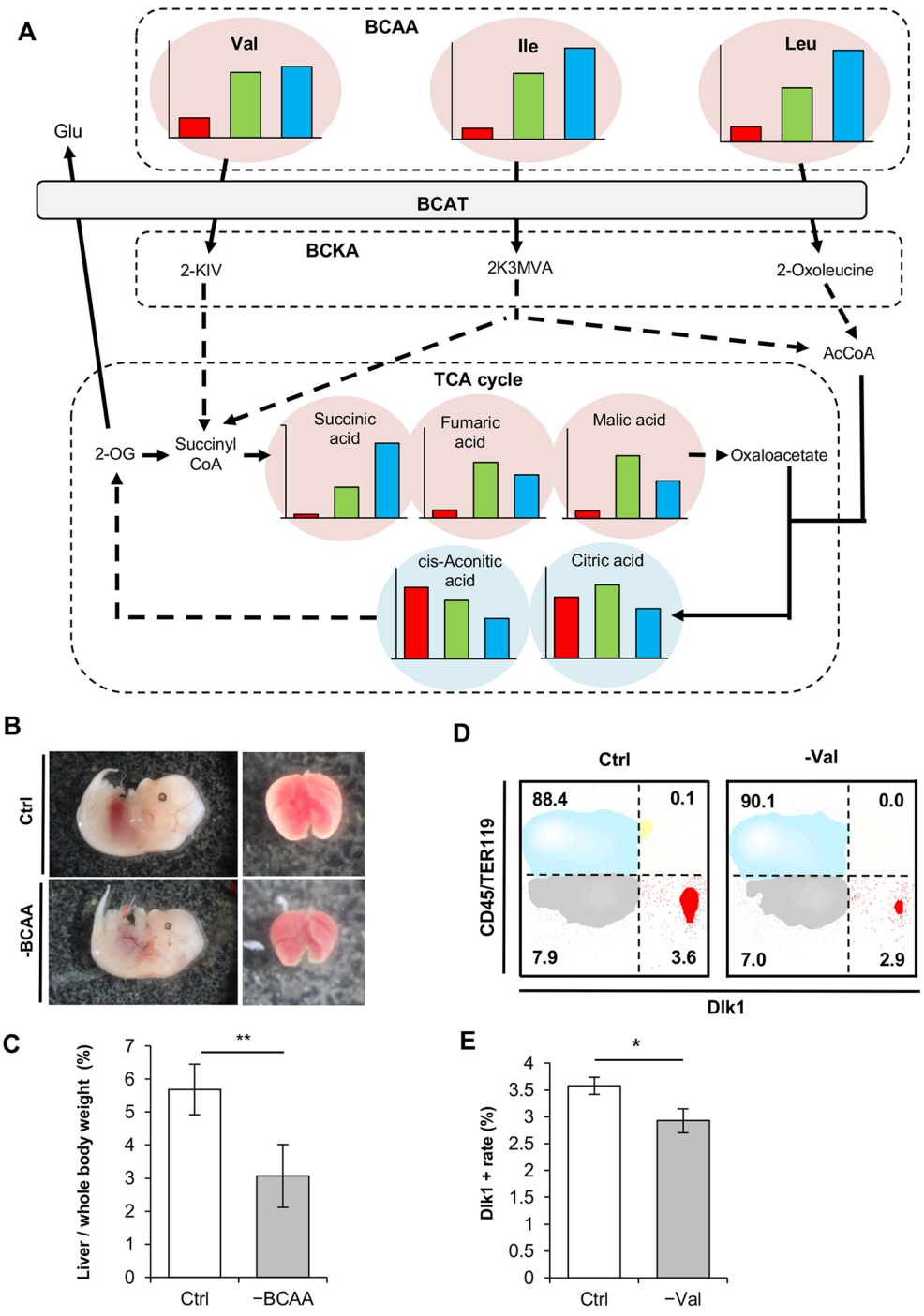

Fig. 2. Branched-chain amino acid metabolism is crucial for fetal liver bud growth. (A) Diagram of the BCAA metabolism pathway. Column charts show that the concentration (nmol/g) of each metabolite in liver cells from E11.5 (red), 19.5 (before birth) (green) and 8 week (blue) mice. The color of the circle behind each chart indicates which developmental stage has the minimum amount of each metabolites (pink, E11.5; blue, 8 weeks). (B,C) The effect of BCAA-depleted diet in mouse liver development. (B) E13.5 mouse embryo and liver after feeding a BCAA-depleted diet to the dam from E8.5. (C) The ratio of fetal liver weight to body weight. n=11 for control and n=3 for BCAA-depleted diet. (D,E) HPC frequency in E13.5 fetal liver after feeding a control or an L-valine-depleted diet to the dam from E8.5. (D) Flow cytometry analysis of E13.5 fetal liver cells. Red, Dlk1-positive CD45- and TER119-negative population; blue CD45- and TER119-positive/Dlk-negative population. (E) Percentage of Dlk-positive cells in the samples analyzed by flow cytometry in D. n=3. Data in C,E presented as means±s.d. *P<0.05, **P<0.01 by Mann–Whitney U-test.

metabolism by *Bcat* genes is essential for fetal liver bud growth, presumably due to failure of HPC expansion.

To test if BCAA affects HPC growth, the proliferative capacity of HPCs from various developmental stages was examined using a clonogenicity assay *in vitro* in the presence of BCAA supplements. Isolated fetal liver cells were attached to laminin-coated plates under clonal conditions to assess their colony-formation capability. Using liver cells from E11.5, 13.5 and 15.5 embryos, we found that highly proliferative colonies comprising >90 cells were 30.9±10.0% increased in E11.5 liver cells by 4.0 mM of L-valine supplementation, but not increased in E13.5 or E15.5 cells. The other types of amino acids or their combinations did not stimulate colony expansion in E11.5 liver bud cells (Fig. 3A,B). A dose escalation study of L-valine demonstrated that the number of highly clonogenic colonies from E11.5 fetal liver proportionally increased 4.0 mM out of 0.4 mM, 0.8 mM and 4.0 mM conditions (Fig. S3). The addition of 4.0 mM of L-valine to medium did not affect the bi-potent differentiation capacity for hepatic lineage cells identified by albumin and Ck7 co-immunostaining and gene expression analysis (Fig. 3C,D). These results suggest that L-valine supplementation promotes murine HPC expansion with sustained bi-directional differentiation capacity.

To determine the relevance of L-valine for human liver bud growth, we employed a previously established 3D liver bud (LB) organoid model (Takebe et al., 2013) (Fig. 4A). First, we compared *BCAT1* gene expression by qRT-PCR, showing that

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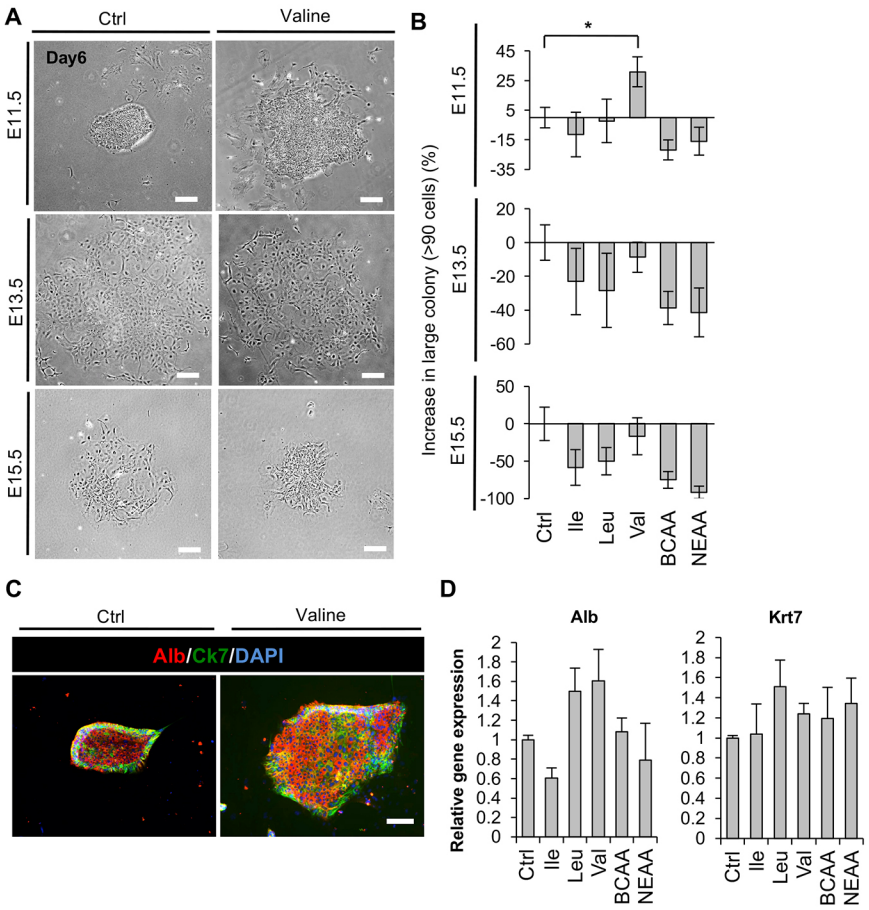

Fig. 3. L-valine supplementation amplifies murine bipotent hepatic progenitor cells. (A) Image of E11.5, 13.5 and 15.5 mouse liver cell-derived colonies after 6 days of culture in control or 4 mM L-valine-supplemented medium. (B) Increasing percentages of large colonies including >90 cells after 6 days of culture in control medium, or medium supplemented with BCAAs (4 mM each of valine, isoleucine and leucine) or 4 mM non-essential amino acids (NEAAs). *n*=12. (C) Immunofluorescence image of E11.5 fetal liver cells derived from a large colony cultured for 6 days in control medium or medium supplemented with 4 mM of valine. Red, Alb; green, Ck7; blue, DAPI. These colonies indicate the bi-potency of hepatocyte and cholangiocyte. (D) Gene expression of *Alb* and *Krt7* in E11.5 fetal liver cells cultured in control medium or medium supplemented with 4 mM of valine, isoleucine or leucine, BCAAs (4 mM each of valine, isoleucine and leucine), or 4 mM non-essential amino acids (NEAAs) for 6 days, relative to housekeeping gene *Gapdh*. *n*=3. Data in B,D presented as means±s.d. **P*<0.05 by Mann–Whitney *U*-test. Scale bars: 100 µm.

its expression significantly peaked at human LB stage but not at less mature or more mature stages, transplanted LB (2 month) or human adult liver tissues (Fig. 4B). Generated LBs were then cultured under growth condition in medium supplemented with various amino acids to test their modulatory effect. Quantitative image-based analysis showed that L-valine supplementation caused a massive volume expansion up to 166.3±27.3% larger in size relative to the control medium condition (Fig. 4C; Fig. S4A), which is estimated to be a ∼twofold increase of hiPSC-derived HPCs in cell number per LB. As the modulatory effects of stromal lineages remain minimal (Fig. S4B), it is suggested that unique L-valine metabolism by *BCAT1* is conserved both in human and mouse during liver bud development, highlighting the potential of an L-valine nutritional approach for modulating human fetal liver bud growth.

Next, we examined whether the BCAA effect is specific to human HPC in LB by assessing an isolated cell culture. To examine the direct effects of amino acids, hiPSC-derived HPCs expressing high levels of *BCAT* genes and HPC marker proteins (Fig. S4C,D) were cultured in the BCAA-supplemented medium (Fig. 4D). Similar to mouse HPC culture, HPCs supplemented with 4.0 mM L-valine significantly increased cell proliferation compared with cells grown under control conditions (Fig. 4E). In contrast, the expression profiles of a number of genes were not affected by L-valine supplementation, including various stage-specific markers, such as *NANOG*, *FOXA2*, *SOX17*, *AFP*, *ALB* and *SOX7*, suggesting maintenance of HPC differentiation status by L-valine supplementation (Fig. S4E). To further investigate whether the L-valine-supplemented culture medium could expand hiPSC-HPCs through several passages, we performed subsequent re-plating culture. L-valine-treated hiPSC-HPCs maintained their rigorous proliferative capacity even after three passages (Fig. 4F). Furthermore, hepatocyte marker genes such as *ALB*, *RBP4* and *ASGR1* were induced after hepatic differentiation of hiPSC-HPCs expanded through several passages. These results suggest that L-valine supplementation effectively propagates hiPSC-HPCs similar to mouse iPSC-HPCs by preserving their bi-potent differentiation capability, leading to a future mass production strategy by expanded HPC with the ability to differentiate into hepatocyte-like cells (Fig. 4G).

Organoid technology has paved the way for modeling human disease and development, and ultimately for regenerative therapy. However, identifying a missing cue required to recapitulate the massive organ bud expansion processes that naturally occur during embryogenesis remains a major challenge. Combined transcriptome and metabolome analysis identified a transient requirement of specific BCAA metabolism for early organ bud development in the liver by potentiating hepatic progenitor cell growth. Equally notable are the possibilities for modulating liver bud growth by a simple nutritional strategy both *in vitro* and *in vivo*. During the early phase of organogenesis, several animal studies indicated the importance of maternal nutrition for fetal stage-critical events regarding gastrointestinal tract development including expansion, differentiation and vascularization. Fetuses from dams subjected to nutrient restriction during early to mid-gestation have decreased growth of the gastrointestinal tract, including liver (Duarte et al., 2013; Wang et al., 2008). In humans, maternal dietary amino acid supplementation to improve fetal growth has been considered as an

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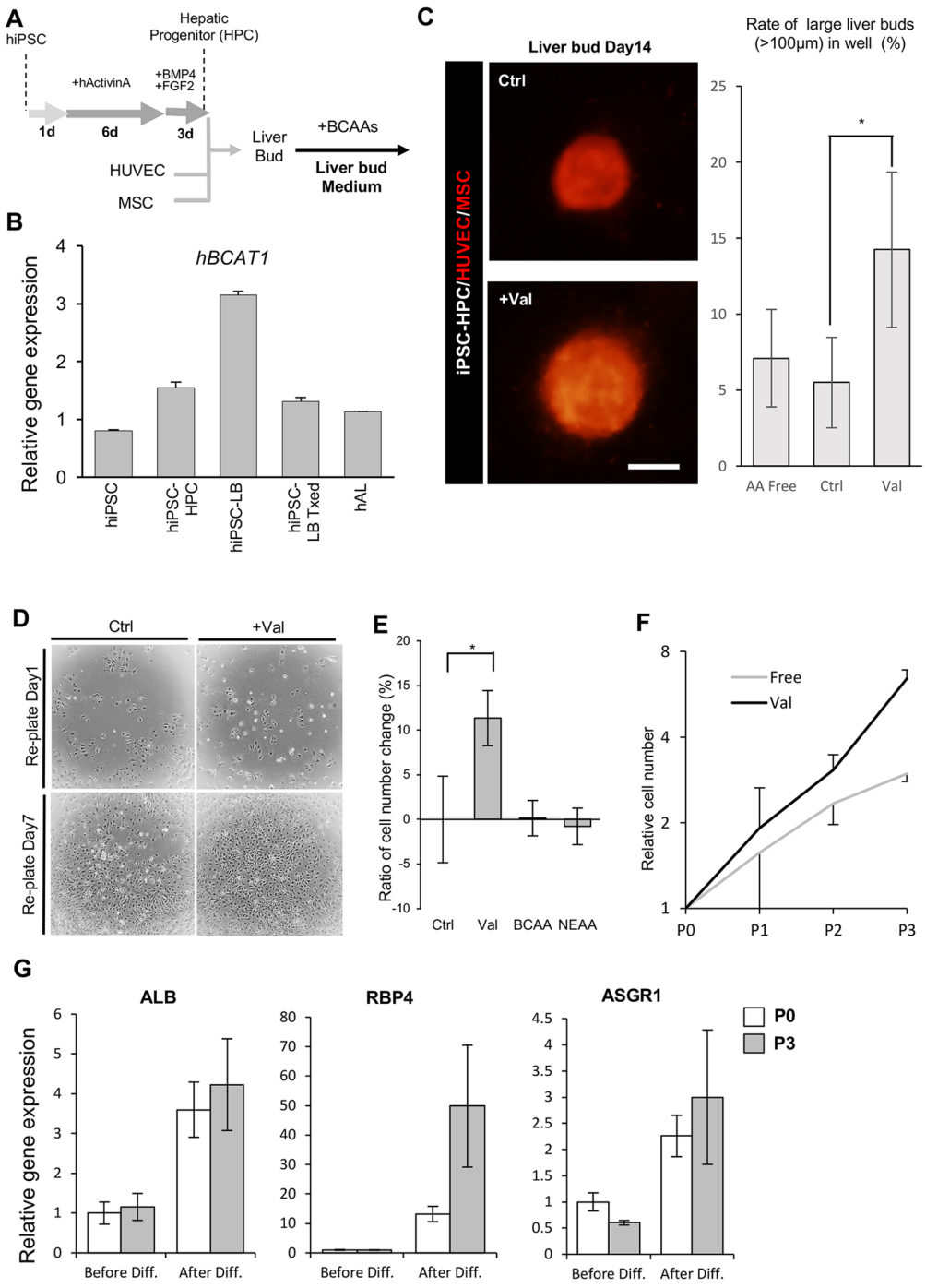

Fig. 4. L-valine supplementation promotes hiPSC liver bud growth through selective HPC expansion. (A) Schematic of the generation and culture method of the liver bud. (B) Stage-specific *BCAT1* expression in various stages of human liver development. (C) Left: Representative fluorescence image of liver bud cultured in the presence or absence of L-valine. Right: The size of cultured liver buds in amino acid-depleted, control or L-valine-enriched condition were measured by imaging cytometer, represented as percentage of large liver buds (>125 μm). *n*=5. (D) hiPSC derived from HPCs cultured for 7 days in control or L-valine-abundant conditions. (E) The increased percentage of hiPSCs derived from HPC cultured for 7 days in control medium, or medium supplemented with BCAAs (4 mM each of valine, isoleucine and leucine) or non-essential amino acids (NEAAs). *n*=3. (F) The change in cell number during serial passages in control or L-valine-abundant medium. Each passage was performed every 7 days. *n*=9. (G) The hepatic differentiation capacity of HPC after serial passaging. *ALB*, *RBP4* and *ASGR1* gene expression was confirmed by qRT-PCR. White columns show gene expression in P0 HPC and gray columns show gene expression in P3 HPC, relative to 18S ribosomal RNA. *n*=8. Data in B,C,E-G presented as means±s.d. **P*<0.05 by Mann–Whitney *U*-test. Scale bar: 100 μm.

option to prevent or treat intrauterine growth restriction, as the specific amino acid transport system can transfer all of the BCAAs (Brown et al., 2011). However, the primary therapeutic time window is significantly limited from mid- to late-gestational stages in humans due to a lack of a human predictive model. As examined in our study, the application of our recently developed human PSC-organoid model will be a novel alternative to assess the benefit of nutritional intervention in culture before entering clinical application. Future studies of BCAA supplementation will aid in developing an effective nutritional strategy for promoting liver bud growth during the early phase of pregnancy.

One remaining question in our studies is whether the importance of the BCAA nutritional effect for progenitor growth is specific to HPC. Compared with our results demonstrating impairment of HPC expansion capability by direct BCAA deprivation *in vitro*, the effect of *in vivo* maternal dietary deprivation seemed considerably greater. This observed discrepancy could be attributed to: (1) secondary effects of the other HPC supportive progenitors affected *in vivo*, or (2) the culture system not accurately reflecting the physiological condition *in vitro*. Previous rat studies confirmed the former interpretation because fetal systemic growth could be affected by administration of BCAA combined with caloric restriction (Brown et al., 2011). Additionally, collective evidence suggests that amino acid or glucose metabolism is important for maintaining hematopoietic lineage growth (Takubo et al., 2013;

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Yu et al., 2013). The promotional effects of hematopoietic progenitors for hepatoblast proliferation have been demonstrated through paracrine oncostatin M support in various reports (Kamiya et al., 1999; Kinoshita et al., 1999). Recent reports have revealed that adult hematopoietic stem cell components crucially require a valine supply to maintain their stem cell pool (Taya et al., 2016). The investigation of fetal hematopoietic progenitor dysfunction by BCAA deprivation and secondary impairments in HPC proliferation will be a rigorous topic to further dissect the supportive mechanism of liver bud development.

Indeed, supplementing with BCAAs both *in vitro* and *in vivo* consistently promotes the expansion of HPCs on a cellular, organoid and organism level. This is important to note because from a regenerative medicine perspective, a patient would theoretically require about 10^{10} hepatocytes in therapeutic applications (Takebe et al., 2013). There are several similar approaches available for obtaining enriched target populations from human PSCs such as elimination of ESCs (Wang et al., 2009) and purification of cardiomyocyte populations with glucose- and glutamate-depleted medium (Tohyama et al., 2016, 2013). These novel approaches are expected to aid in establishing a safer cell source free of undesired lineages from hPSCs, which enhances safety during stem cell therapy with a simple and low-cost method (Tohyama et al., 2016). Collectively, such a distinguishing feature of HPC metabolism will be able to promote the pluripotent stem cell-derived HPC expansion for economical mass production of human liver buds, thereby, facilitating a realistic application of iPSC-derived organoids towards therapy.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from Japan SLC (Japan). All animal experiments in this study were performed under approval from the institutional animal care and use committee of Yokohama City University (Approval Number: 11-63). Further details can be found in the supplementary Materials and Methods.

Cell culture

hiPSC (TkDA3-4; kindly provided by Dr Nakauchi at the University of Tokyo) maintenance and organoid formation was performed as described previously (Takebe et al., 2013). Briefly, to induce human liver bud formation *in vitro*, hiPSC-derived HPCs, HUVECs and human MSCs were co-cultured in the presence of indicated BCAAs and plated on EZSPHERE plate (AGC Techno Glass Co, Japan). Further details can be found in the supplementary Materials and Methods.

Staining and image analysis

The fluorescent LB size was analyzed from whole-well scanned image using an INCell analyzer 2000 (GE Healthcare). The antibodies used in this study are listed in Table S1. Further details can be found in the supplementary Materials and Methods.

Transcriptome and metabolome analysis

Acquisition of transcriptome and metabolome profiles was performed as described previously (Takebe et al., 2013). The primers used in this study are listed in Table S2. Further details can be found in the supplementary Materials and Methods.

Statistical analysis

Data are expressed as the means±s.d. from the number of repeated independent experiments described in each figure legend. Comparisons between three or four groups were analyzed using the Mann–Whitney *U*-test. Two-tailed *P*-values of <0.05 were considered significant.

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Competing interests

T.T. and H.T. have served on a scientific advisory boards for Healios Inc. T.T. and H.T. have also granted a license to Healios through Yokohama City University over their inventions that relate to the subject of this manuscript. T.T. and H.T. are conducting research under the collaborative research agreement with Healios and the subject of the research also relates to the subject of this manuscript.

Author contributions

H.K. and T.T. analyzed data, designed this study and prepared the manuscript. H.K., Y.U. and R.-R.Z. performed experiments. T.T., K.S., Y.-W.Z. and H.T. were involved in study design and supervised experiments. All authors discussed the results and commented on the manuscript.

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Data availability

Microarray data are available at Gene Expression Omnibus under accession number GSE46631.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.143032.supplemental

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