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Article

Generation of liver bipotential organoids with a small-molecule cocktail

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Understanding the mechanism of how cholangiocytes (liver ductal cells) are activated upon liver injury and specified to hepatocytes would permit liver regenerative medicine. Here we achieved long-term *in vitro* expansion of mouse liver organoids by modulating signaling pathways with a combination of three small-molecule compounds. CHIR-99021, blebbistatin, and forskolin together maintained the liver organoids in bipotential stage with both cholangiocyte- and hepatocyte-specific gene expression profiles and enhanced capacity for further hepatocyte differentiation. By employing a chemical approach, we demonstrated that Wnt/β-catenin, NMII–Rac, and PKA–ERK are core signaling pathways essential and sufficient for mouse liver progenitor expansion. Moreover, the advanced small-molecule culture of bipotential organoids facilitates the *ex vivo* investigation of liver cell fate determination and the application of organoids in liver regenerative medicine.

Keywords: liver bipotential organoid, small-molecule cocktail, long-term expansion, progenitor cell, hepatocyte differentiation, liver regeneration

Introduction

The liver is mainly composed of two types of epithelial cells: hepatocytes and cholangiocytes. Hepatocytes are crucial for metabolism and drug detoxification (Itoh, 2016). Upon injury, the liver exhibits an extraordinary regenerative capacity to rescue hepatic mass loss, which is commonly mediated by hepatocyte compensatory proliferation (Michalopoulos and DeFrances, 2005; Michalopoulos, 2018). Besides, cholangiocyte-to-hepatocyte transition/differentiation can be triggered to restore hepatocyte population, especially in case of impaired hepatocyte proliferation (Deng et al., 2018).

Multiple signaling pathways integrate to precisely control the fate determination of cholangiocytes. Notch signaling is required for cholangiocyte specification and maintenance (Tchorz et al., 2009; Zong et al., 2009; Yimlamai et al., 2014). Transforming growth factor-beta (TGF-β) signaling mediates cholangiocyte specification and biliary morphogenesis (Clotman et al., 2005; Schaub et al., 2018). Both Notch and TGF-β prevent cholangiocytes from transiting to hepatocytes upon liver injury. Hippo signaling contributes to ductal reaction for liver regeneration (Pan, 2007; Yimlamai et al., 2014; Pepe-Mooney et al., 2019; Planas-Paz et al., 2019). And PKA signaling promotes cholangiocyte proliferation through activating ERK and β-catenin (Francis et al., 2004; Fanti et al., 2014).

In a three-dimensional (3D) culture system, bile ducts embedded in Matrigel can self-organize into long-term expanding liver ductal organoids in defined medium consisting of the Wnt agonist R-spondin1, EGF, FGF10, and HGF (REFH), among others. Notably, the cholangiocytes in ductal organoids can differentiate into hepatocytes following the withdrawal of Wnt stimulation and the inhibition of TGF-β and Notch signaling (Huch et al., 2015; Broutier et al., 2016), which well mimics the *in vivo* cholangiocyte-to-hepatocyte transition. These organoids not only accelerate liver disease modeling and drug discovery (Broutier et al., 2017; Nuciforo et al., 2018) but also enable the dissection of how hepatocyte specification is regulated.

Long-term *ex vivo* stabilization of cell identity and functionality in organoids is highly dependent on precise spatiotemporal gene expression regulation by extrinsic and intrinsic signals. For liver organoids, extrinsic growth factors (REFH) activate multiple downstream pathways, including Wnt/β-catenin, non-canonical Wnt, Akt, MAPK, ERK, Rac, and PKA (Ciardiello and Tortora, 2008; Avraham and Yarden, 2011; Gherardi et al., 2012; Watson and

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Francavilla, 2018) and even more through signaling crosstalks. It is therefore critical to determine the basic intrinsic signals sufficient for liver progenitor expansion. We postulated that the screening of a minimal combination of small molecules could yield hits if we focus on desired outcome (e.g. long-term expansion of liver organoids) even without *a priori* knowledge on the native stimuli.

Here, we reported a chemical method to generate liver organoids from cholangiocytes. We found that targeting glycogen synthase kinase 3 (GSK3β), nonmuscle myosin II (NMII), and adenylate cyclase by a small-molecule cocktail maintains liver organoids in bipotential stage with both cholangiocyte- and hepatocyte-specific gene expression profiles and enhanced capacity for further hepatocyte differentiation. This study lays a foundation for liver cell plasticity research and provides promising approaches for liver regenerative medicine.

Results

A chemical strategy to generate mouse liver organoids from cholangiocytes

To develop a chemical strategy for mouse liver organoid culture, we sequentially replaced the essential growth factors (REFH) with small molecules. As shown in Figure 1A, freshly isolated cholangiocytes gave rise to fast-proliferating liver organoids within 5 days upon REFH treatment, while R-spondin1 withdrawal led to dramatically decreased organoid formation and expansion. Interestingly, although R-spondin1 stimulates both Wnt/β-catenin and Wnt/non-canonical signaling, sustaining Wnt/β-catenin hyperactivation by GSK3β inhibitor CHIR-99021 (Chir) efficiently rescued the organoid culture in the absence of R-spondin1 (Figure 1A).

However, we encountered unexpected difficulties replacing the other three growth factors. Withdrawal of EGF and FGF10 resulted in rapid decay of cholangiocyte cultures, which could not be blocked by chemically activating EGF receptor with NSC228155 and FGF receptor with SUN11602. Besides, FH1, an HGF agonist, could not mimic the function of HGF in maintaining liver organoids (Figure 1A). To overcome these barriers, we screened a 303-stem cell signaling compound library for liver organoid formation and expansion in RH (R-spondin1 and HGF) and REF (R-spondin1, EGF, and FGF10) conditional media. We found that blebbistatin (Bleb), a NMII heavy-chain ATPase inhibitor, efficiently restores liver organoid culture in the absence of EGF and FGF10. Moreover, forskolin (Fors), an adenylate cyclase activator, replaced HGF in supporting liver organoid expansion (Figure 1A). This incomplete correspondence between growth factors and functional replacing chemicals suggests that extrinsic signals might undergo tissue-specific crosstalk to produce intrinsic signaling output and consequent liver organoid expansion.

We then set out to determine whether combination of Chir, Bleb, and Fors could achieve long-term liver organoid maintenance instead of growth factors. Indeed, as observed in REFH, Chir+Bleb+Fors small-molecule cocktail successfully generated liver organoids from cholangiocytes, which could be passaged without losing expanding ability (Figure 1B). In an effort to further downsize the cocktail, we found that removing either Fors or Bleb led to primary organoid formation failure, while removing Chir led to significant reduction in organoid expansion after passage (Figure 1B). These data demonstrated that Chir+Bleb+Fors is the minimal combination essential and sufficient for the chemical culture of liver organoids.

The chemical-cultured liver organoids (Chem-Orgs) could be maintained *in vitro* for >20 passages (4 months) with unattenuated expanding and colony-forming capacity (Figure 2A and B). Confocal image section stained for the adhesion junction marker E-cadherin and the proliferation marker Ki67 revealed that the Chem-Orgs are single-layered and fast-proliferating (Figure 2C and D). Sox9 and CK19 staining confirmed that the Chem-Orgs retained the expression of cholangiocyte signature genes, comparable to growth factor-cultured ductal organoids (GF-Orgs) (Figure 2C; Supplementary Figure S1). Notably, the ductal phenotype was retained, and fluorescein diacetate transport assay showed that Chem-Orgs retained the bile duct function as well as GF-Orgs (Figure 2E).

Chemical culture maintains mouse liver organoids in bipotential stage

The gene expression patterns of GF-Orgs and Chem-Orgs over passages were assessed by mRNA sequencing. Surprisingly, Chem-Orgs showed significantly upregulated expression of hepatocyte signature genes, represented by *Alb*, *Hpx*, *Sult1a1*, *Tat*, and cytochrome P450 genes, as compared to GF-Orgs (Figure 3A). Notably, this hepatocyte-like gene expression profile was maintained stably after passages (Figure 3A). Gene ontology (GO) term analysis of differentially expressed genes (DEGs) also indicated the hepatocyte-specific functions ‘sulfotransferase activity’ and ‘glucuronosyltransferase activity’ (Figure 3B). In addition, GSEA analysis revealed that both hepatocyte-enriched genes (Hu et al., 2018) and hepatocyte signature genes (Kim et al., 2017) were significantly enriched in Chem-Orgs rather than GF-Orgs (Figure 3C). Quantitative real-time PCR (qRT-PCR) further validated that Chem-Orgs had dramatically enhanced expression of hepatocyte marker genes (*Afp*, *Ttr*, *Alb*, *Cyp3a11*, and *Sult1a1*) but comparable expression of cholangiocyte marker genes (*Krt7*, *Krt19*, and *Sox9*) (Figure 3D). These data suggest that the Chem-Orgs tend to be in a bipotential status, in which both cholangiocyte and hepatocyte signature genes expressed.

We then characterized the cell identity in Chem-Orgs employing immunofluorescence. As shown in Figure 3E, cholangiocytes in GF-Orgs specifically expressed Sox9, but no hepatocyte marker Albumin was observed. In contrast, a Sox9$^+$ population in Chem-Orgs flooded Albumin in the cytoplasm, showing the identity of cholangiocyte-to-hepatocyte transiting liver progenitor cells. The Chem-Orgs were also subjected to hepatocyte function test, which showed that Chem-Orgs were more competent for low-density lipoprotein (LDL) uptake than GF-Orgs (Figure 3F).

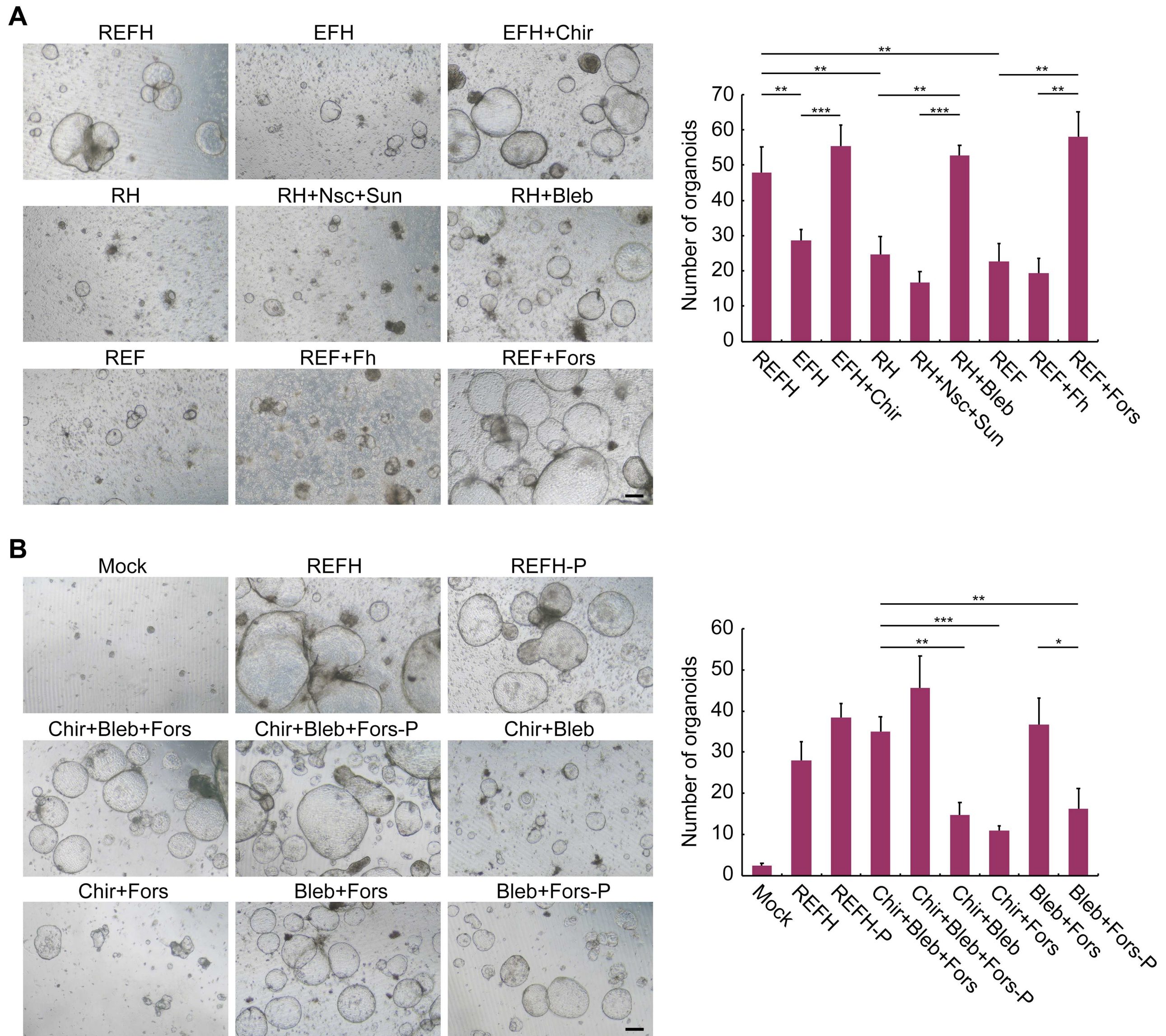

Figure 1 Establishment of 3D chemical culture system of murine liver organoids. (**A**) Bright-field images of liver organoids cultured with various combinations of growth factors and small molecules (left) and the number of organoids per well of different combinations (right). (**B**) Bright-field images of liver organoids under different culture conditions (left) and the number of organoids in fixed-size field (right). Scale bar, 200 μm. $n = 3$ independent experiments, mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. R, R-spondin1; E, EGF; F, FGF10; H, HGF; Sun, SUN11602; Nsc, NSC228155; Fh, FH1; P, passaged.

Taken together, we demonstrate that the small-molecule cocktail achieves long-term culture of liver bipotential organoids, through maintaining the cholangiocyte-to-hepatocyte transiting liver progenitor cells.

Liver bipotential organoids derived from mouse cholangiocytes have enhanced capacity for hepatocyte differentiation

We probed the cell of origin for liver progenitor cells in Chem-Orgs. To rule out the possibility that a small number of freshly isolated hepatocytes survived in chemical cocktail to generate liver progenitor cells, we generated primary liver ductal organoids with growth factors and switched to chemical culture upon passage. As shown in Figure 4A, chemical switch led to cholangiocyte-to-hepatocyte transition of liver ductal organoids, represented by increased expression of *Afp*, *Ttr*, *Alb*, and *Cyp3a11*, which indicated that liver progenitor cells in Chem-Orgs originated from cholangiocytes.

We next examined the hepatocyte differentiation ability of these liver bipotential organoids. We found that either OSM + dexamethasone (hepatocyte maturation inducers) treatment or viral delivery of Hnf4α (master transcriptional factor for hepatocyte specification) led to upregulation of hepatocyte lineage signatures (Figure 4B and C). These data demonstrated that Chem-Orgs could further differentiate into hepatocytes.

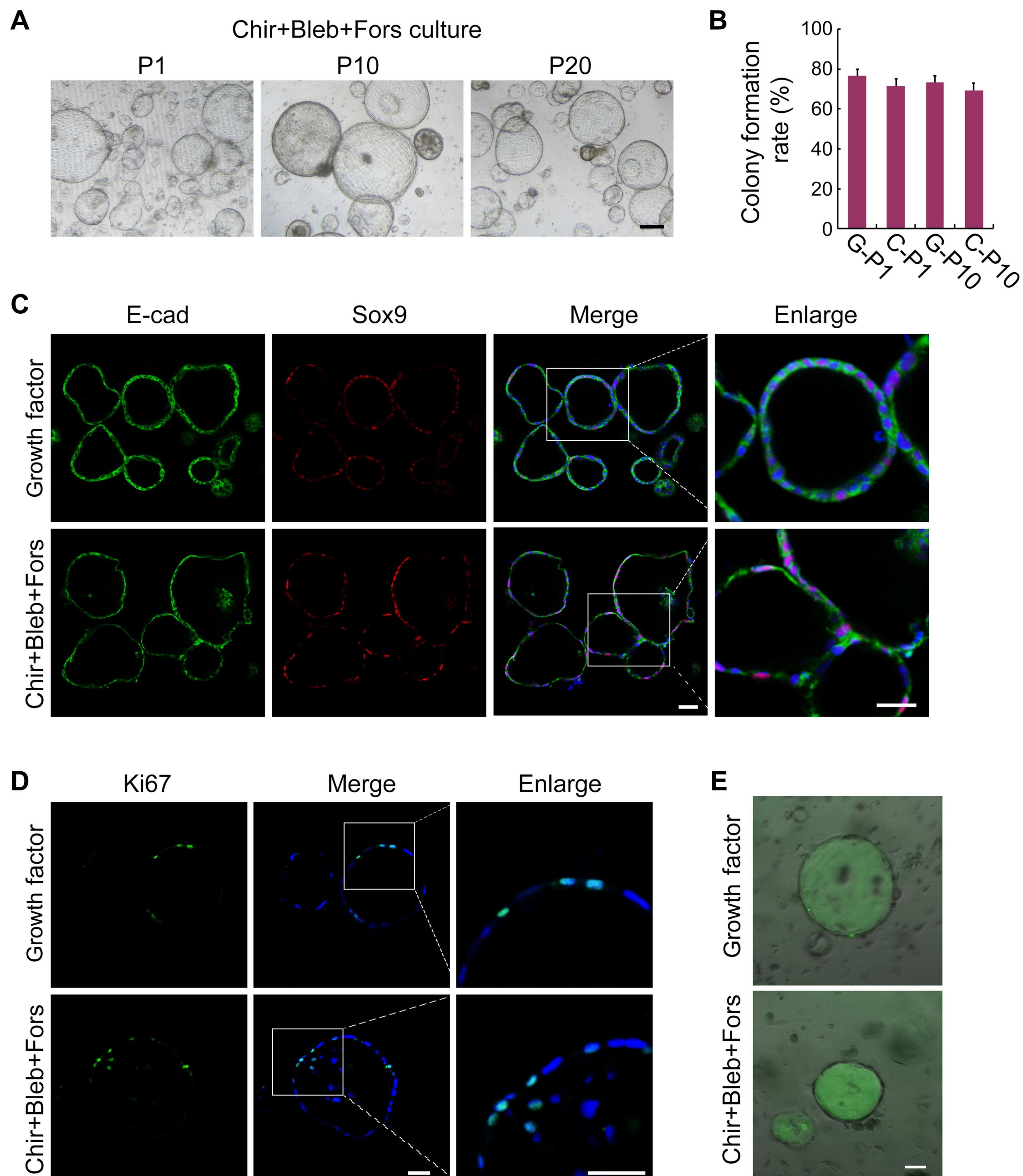

Figure 2 Characterization of Chem-Orgs during expansion. (**A**) Representative bright-field images of Chem-Orgs from P1, P10, and P20. Scale bar, 200 μm. (**B**) Colony formation assay of GF-Orgs and Chem-Orgs at P1 and P10. Cloning efficiency was evaluated by formation/seeding ratio. $n = 3$ independent experiments, mean ± SEM. (**C** and **D**) Confocal images of GF-Orgs or Chem-Orgs at P5. Scale bar, 50 μm. (**E**) Representative images demonstrating the fluorescent substrate detected in the lumen of cystic organoids. Scale bar, 50 μm.

Particularly, Chem-Orgs showed improved hepatocyte differentiation compared to GF-Orgs (Figure 4B), suggesting that chemical-induced liver progenitor cells have higher plasticity than cholangiocytes.

It has been reported that blocking TGF-β signaling by A83-01 promotes the cholangiocyte-to-hepatocyte transition. We optimized the cocktail and found that adding A83-01 further boosted the generation of liver progenitor cells in Chem-Orgs (Figure 4B), but had no obvious effect on the long-term maintenance of colony formation and expansion ability (Supplementary Figure S2). Of special note, Chir+Bleb+Fors+A83 cocktail-cultured bipotential organoids exhibited greatly enhanced hepatocyte differentiation ability (30.56-fold Alb induction and 367.95-fold Cyp3a11 induction relative to the standard hepatocyte differentiation of GF-Orgs) upon OSM + dexamethasone treatment (Figure 4B).

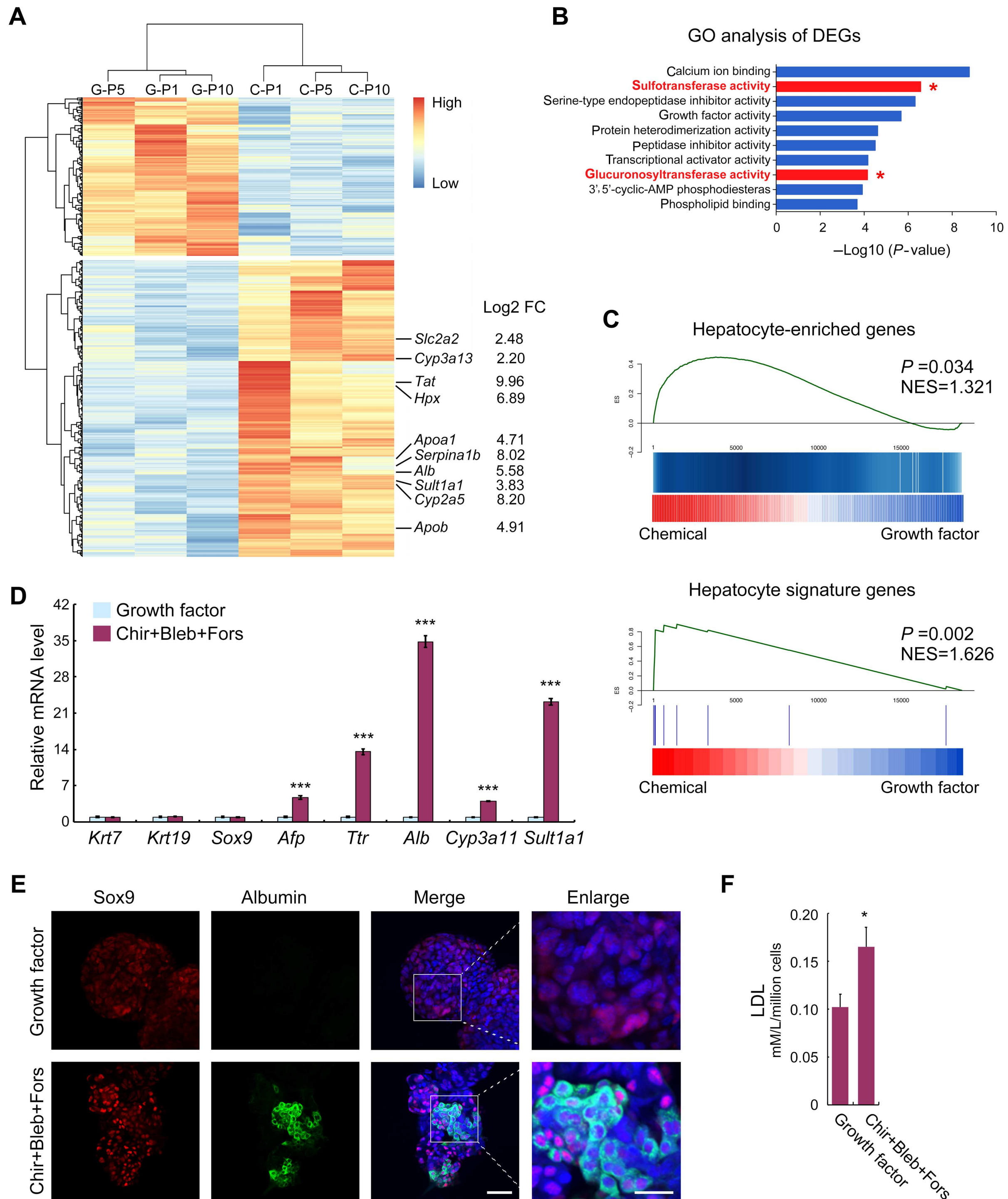

Figure 3 Transcriptional characterization of Chem-Orgs. (**A**) Heatmap of gene expression in GF-Orgs and Chem-Orgs (P1, P5, and P10). (**B**) GO analysis of DEGs in hepatocytes. (**C**) GSEA enrichment analysis of Chem-Orgs vs. GF-Orgs for hepatocyte-enriched genes (upper panel) and hepatocyte signature genes (lower panel). (**D**) Relative mRNA expression of hepatocyte and cholangiocyte/progenitor markers in Chem-Orgs relative to GF-Orgs. $n = 3$ independent experiments, mean ± SEM; $***P < 0.001$. (**E**) Confocal images of GF-Orgs and Chem-Orgs at P5. Scale bar, 50 μm (lower magnification) and 25 μm (higher magnification). (**F**) LDL measured by LDL assay kit. $n = 3$ independent experiments, mean ± SEM; $*P < 0.05$.

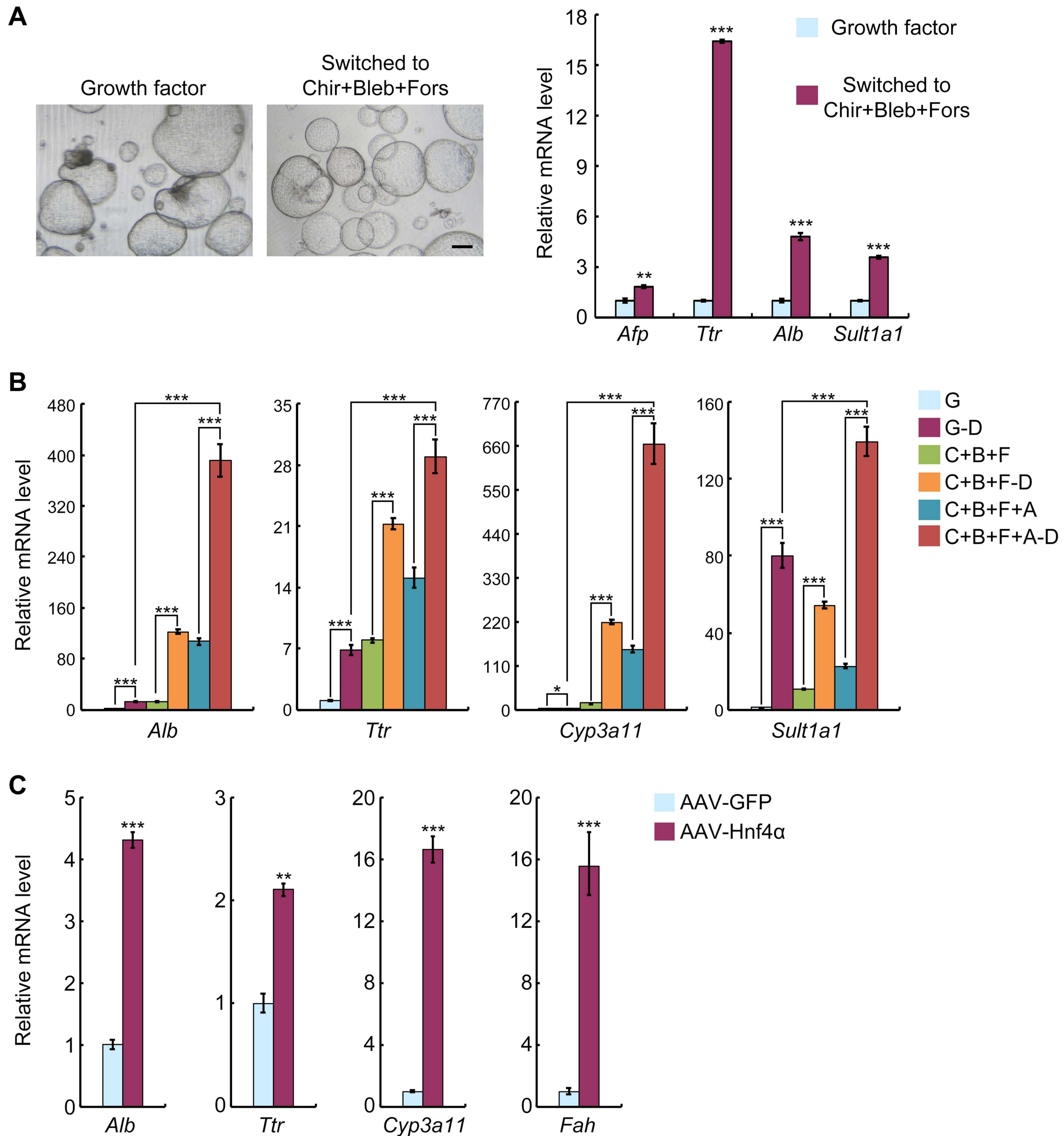

Figure 4 Hepatocyte differentiation potential assay of liver bipotential organoids. (**A**) Bright-filed images (left) and qRT-PCR analysis (right) of GF-Orgs switched to small-molecule culture. Scale bar, 200 μm. (**B**) qRT-PCR analysis of hepatocyte differentiation markers in GF-Orgs and Chem-Orgs (G, growth factors; C, CHIR-99021; B, blebbistatin; F, forskolin; A, A83-01) with or without hepatocyte differentiation inducers (D, differentiation inducers). (**C**) qRT-PCR analysis of hepatocyte differentiation markers in Chem-Orgs and after Hnf4α overexpressing treatment. $n = 3$ independent experiments, mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In summary, we demonstrate that the liver progenitor cells in Chem-Orgs are derived from cholangiocytes and have intensive hepatocyte differentiation capacity.

Wnt/β-catenin, NMII–Rac, and PKA–ERK are signaling pathways essential and sufficient for mouse liver progenitor expansion

Capture of minimal small-molecule combination to generate liver bipotential organoids enables the dissection of basic intrinsic signals sufficient for liver progenitor expansion. According to previous studies, Chir, Bleb, and Fors target Wnt/β-catenin, NMII–Rac, and PKA–ERK signaling pathways, respectively. We found that blocking either of the downstream effectors of these signaling pathways, through the inhibition of TCF/β-catenin-mediated transcription with ICG-001, Rac activity with NSC23766, or ERK activity with SCH772984 (Morris et al., 2013), completely abolished the chemical culture of liver bipotential

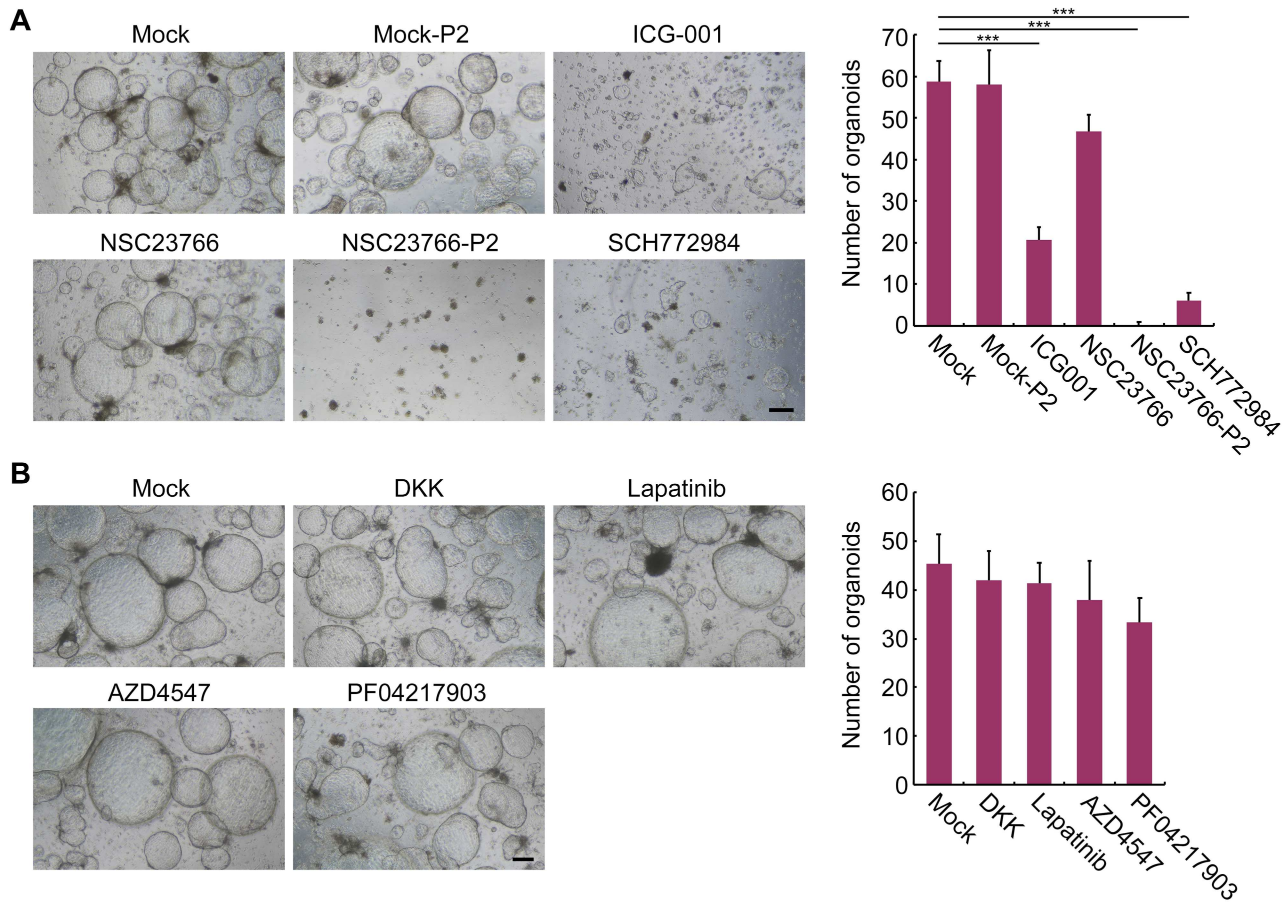

Figure 5 Small-molecule cocktail regulates core signaling pathways for liver progenitor expansion. (**A**) Bright-field images of Chem-Orgs after ICG-001, NSC23766, or SCH772984 treatment (left) and numbers of organoids formed per well (right). (**B**) Bright-field images of Chem-Orgs after DKK, lapatinib, PF04217903, or AZD5447 treatment (left) and organoid numbers formed per well (right, $n = 3$ independent experiments, mean ± SEM). Mock, Chir+Bleb+Fors; P2, passage 2. Scale bar, 200 μm. $n = 3$ independent experiments, mean ± SEM; $^{***}P < 0.001$.

organoids (Figure 5A). These results ultimately confirm that Wnt/β-catenin, NMII–Rac, and PKA–ERK are essential for liver progenitor expansion.

To further ascertain whether the three signaling pathways are sufficient, we excluded the potential contribution of self-secreted ligands through blocking upstream receptors. As shown in Figure 5B, neither of the receptor inhibitors, including DKK for Wnt receptor, Lapatinib for EGF receptor, AZD4547 for FGF receptor, and PF-04217903 for HGF receptor, interfered with the organoid culture, indicating that the extrinsic signals do function through constituting intrinsic Wnt/β-catenin, NMII–Rac, and PKA–ERK signaling.

These data together demonstrate that Wnt/β-catenin, NMII–Rac, and PKA–ERK are core signaling pathways essential and sufficient for liver progenitor expansion.

Non-canonical Wnt signaling promotes the expansion of human bipotential organoids

We tested whether the small-molecule cocktail could be applied in the maintenance of human liver organoids. The human ductal organoids were generated from primary bile ducts with growth factors (Huch et al., 2015) and then switched to chemical culture upon passage. Unexpectedly, Chir+Bleb+Fors treatment abolished the expansion of human liver organoids, which was efficiently restored by the withdrawal of Chir (Figure 6A). The Bleb+Fors could maintain the organoids in fast-proliferating status for three passages.

It is interesting that R-spondin1 promotes but Chir inhibits the expansion of human liver organoids. As previous studies show that R-spondin1 activates both Wnt/β-catenin signaling and non-canonical Wnt signaling (Ohkawara et al., 2011; Hao et al., 2012), while hyperactivation of Wnt/β-catenin signaling by Chir attenuates non-canonical Wnt signaling (Huang et al., 2016), we set out to examine whether non-canonical Wnt signaling is required for human liver organoid culture. Indeed, stimulating non-canonical Wnt signaling by its ligand Wnt5a (Mehdawi et al., 2016) significantly improved the expanding ability of human liver organoids in presence of Bleb+Fors, especially after three passages when chemical-cultured organoids started to decay (Figure 6B). These results indicate that non-canonical Wnt signaling promotes the expansion of human liver organoids.

Gene expression analysis revealed that the chemical switch led to cholangiocyte-to-hepatocyte transition of human liver ductal organoids, represented by dramatically increased expression

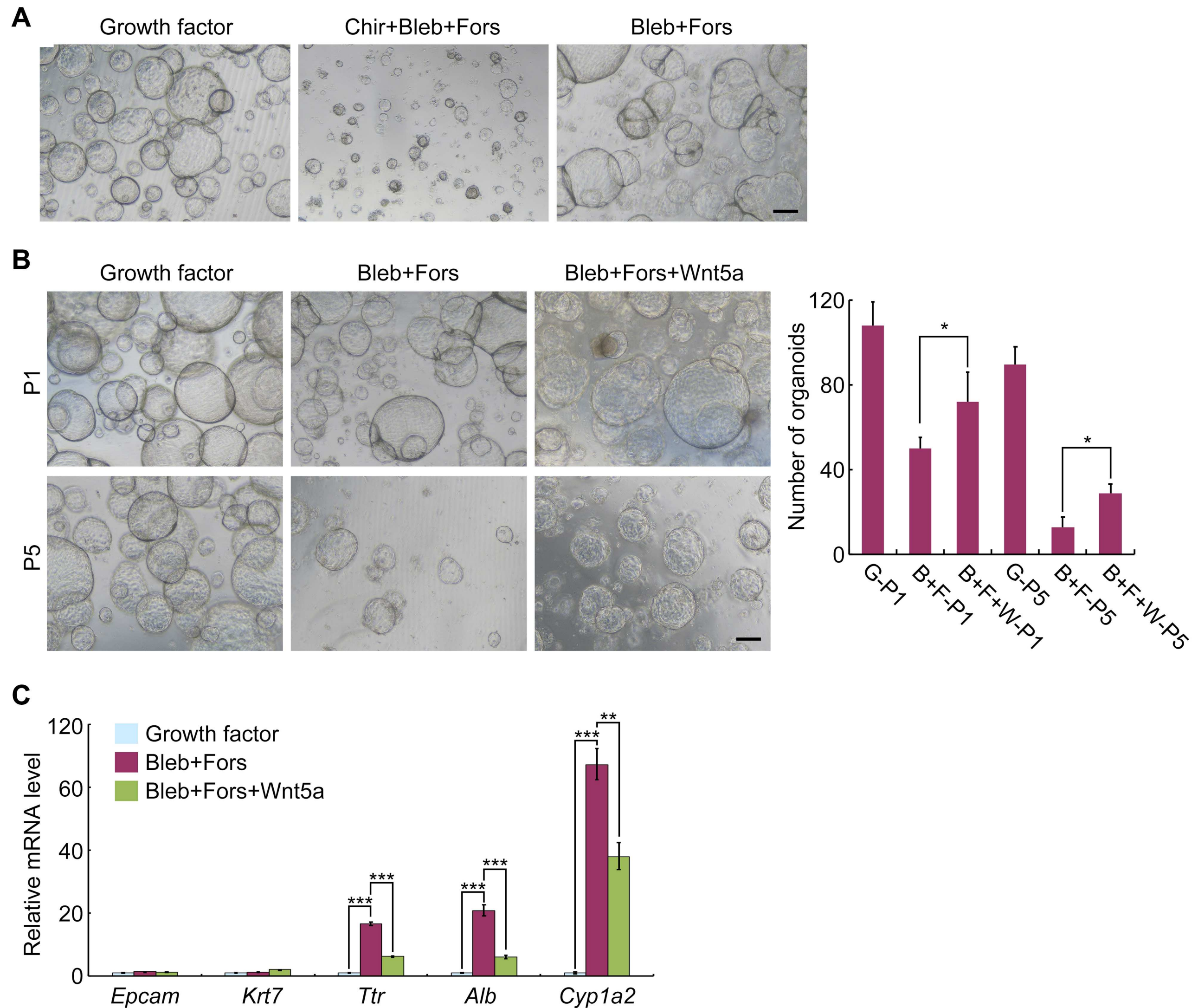

Figure 6 Chemical culture of human adult bile duct-derived bipotential organoids. (**A**) Bright-field images of growth factor- or chemical-cultured human liver organoids. Scale bar, 200 μm. (**B**) Bright-field images of human liver organoids cultured in growth factors or Bleb+Fors with or without Wnt5a at P1 and P5 (left) and numbers of organoids formed per well (right). Scale bar, 200 μm. (**C**) qRT-PCR analysis of hepatocyte and cholangiocyte markers in growth factor- or chemical (Bleb+Fors, with or without Wnt5a)-cultured human liver organoids. $n = 3$ independent experiments, mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of hepatocyte signature genes *Ttr*, *Alb*, and *Cyp1a2* (Figure 6C). These data supported that Bleb+Fors maintained human liver organoids in bipotential stage. Moreover, the addition of Wnt5a restricted the hepatocyte differentiation (Figure 6C), which might benefit the long-term maintenance of human liver organoids.

Discussion

Liver diseases negatively impact the quality of life and survival of patients and often require liver transplant among liver cancer patients and those with liver failure whose conditions cannot be treatment. Hence, understanding the cellular and molecular mechanisms of how liver ductal cells differentiate into hepatocytes would be essential in liver regenerative medicine. The emergence of liver organoids provides great opportunities to study liver cell plasticity and tissue regeneration. In the present study, we developed a chemical culture that generates 3D liver organoids *in vitro* by using a small-molecule cocktail. The Chem-Orgs was maintained in a bipotential status with both cholangiocyte and hepatocyte signature and could further differentiate into hepatocytes. We demonstrate that Wnt/β-catenin, NMII–Rac, and PKA–ERK are core signaling pathways essential and sufficient for liver progenitor expansion. The extrinsic signals do function through constituting intrinsic Wnt/β-catenin, NMII–Rac, and PKA–ERK signaling (Figure 7).

It has previously been reported that liver regeneration through hepatocyte proliferation and hepatocyte or cholangiocyte reprogramming to bi-phenotypic cells responds to liver regeneration

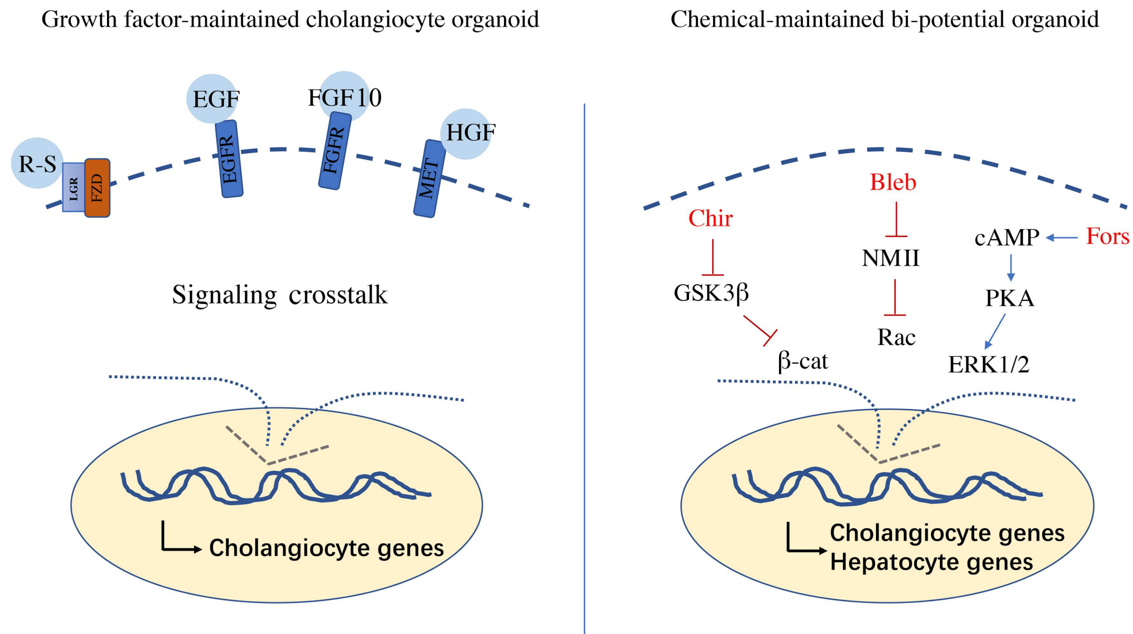

Figure 7 The model of how extrinsic signals maintain the expansion of liver organoids *in vitro*. Growth factors (REFH) activate multiple downstream pathways, and the signaling crosstalk can maintain GF-Orgs with cholangiocyte-specific gene expression (left). Chir, Bleb, and Fors function through Wnt/β-catenin, NMII–Rac, and PKA–ERK signaling pathways, respectively, which are core signaling pathways essential and sufficient for the expansion of Chem-Orgs in cholangiocyte-to-hepatocyte bipotential status.

(Miyajima et al., 2014; Deng et al., 2018). Liver organoids could model the liver regeneration process in response to damage. In 2013, Han Clevers and his colleagues isolated bile ducts and showed that they can self-organize into 3D structures with sustained long-term expansion. These organoids tend to possess characteristics similar to cholangiocytes or liver progenitor cells and retain the ability to differentiate into hepatocyte-like cells (Huch et al., 2013). Recently, the use of growth factors and small-molecule combinations to culture hepatocyte organoids that mimic the *in vivo* liver hepatocyte proliferation in response to injury has been reported (Hu et al., 2018; Peng et al., 2018). Besides, some efforts have focused on the establishment of chemical-defined culture medium and have found application in the culture of intestinal organoids, hematopoietic stem cells, and progenitor cells (Jiang et al., 2018; Li et al., 2018). Notably, small-molecule cultures could be used in the propagation of reprogramed liver progenitor-like cells (Katsuda et al., 2017; Fu et al., 2019), or hepatocytes in 2D (Zhang et al., 2018; Xiang et al., 2019) and can serve as an efficient tool in virus cell biology and drug screening. One key novelty of our method is that Chem-Orgs were maintained in a bipotential status with both cholangiocyte and hepatocyte signatures and had enhanced capacity for further hepatocyte differentiation. Of note was Bleb, a basic molecule in our culture medium which we have previously reported to mechanistically improve the survival of intestinal stem cells and the growth of intestinal organoids by inhibiting of NMII and activating Akt through Rac1 and PAK1 (Zhao et al., 2015). Bleb was also found to act as a specific activator that enhances kidney organoid differentiation from human pluripotent stem cell (Czerniecki et al., 2018). Here we further demonstrated that Bleb is also beneficial for the survival and proliferation of liver progenitor cells and can be employed in the establishment of an efficient and cost-effective culture for liver organoids *in vitro*.

Gene expression analysis of Chem-Orgs revealed that the hepatocyte fate genes such as Alb and Serpina1b tend to decrease during passage (Figure 3A); not coincidentally, recent work from Lijian Hui's lab also showed that *in vitro* cultured human hepatocytes had decreased hepatocyte fate gene expression during amplification (Zhang et al., 2018). We hypothesized that an appropriate reduction in hepatocyte differentiation during passage was beneficial for the long-term maintenance of liver organoids.

Previous studies have reported the role of Wnt signaling in bile duct differentiation. Stabilization of β-catenin together with HNF1β, HNF6, and Notch pathway signals in hepatoblast promotes bile duct development and differentiation (Decaens et al., 2008; Cordi et al., 2016). However, at the last maturation stage of the biliary tree, SOX17, an antagonist of the Wnt signaling pathway, is highly expressed (Merino-Azpitarte et al., 2017). During chronic liver damage, Lgr5-mediated Wnt signaling pathway is not required for ductal reaction in response to liver injury (Planas-Paz et al., 2019). In organoid culture, the Wnt activator R-spondin was essential for the growth of bile ductal organoids *in vitro* (Huch et al., 2013, 2015). Interestingly, our data showed that Wnt/β-catenin signaling activation by Chir could maintain mouse liver ductal organoids in long-term culture, while Chir repressed the growth of human liver ductal organoids. However,

Wnt5a, a ligand of the non-canonical Wnt signaling, could promote the expansion of human ductal organoids. Similar reports have earlier been illustrated and support our observation that the non-canonical Wnt signaling pathway was essential to maintain human ductal organoids *in vitro* (Sampaziotis et al., 2017). These results taken together suggest the difference response to Wnt signaling in human and murine liver ductal cells.

In conclusion, we found that liver organoids could be maintained in long-term cultures with chemical-defined medium *in vitro*. Our results extended the understanding of the essential signaling pathways in liver ductal organoid expansion. In additional, Chem-Orgs owned obvious hepatic characteristics and the ability to further differentiate, which further provides an advantage for the study of liver lineage transition. Chem-Orgs can also serve as an economical and a convenient model in researches involving disease modeling, genetic manipulation, toxicity testing, and regenerative medicine.

Materials and methods

Mice and human biopsy

The C57BL/6 mice were purchased from Shanghai Research Center for Model Organism. All animal studies were performed in accordance with the relevant guidelines and under the approval of the Institutional Animal Care and Use Committee of Fudan University. The human liver biopsies were obtained and used for research purpose under the approval of the Medical Ethical Council of Zhongshan Hospital.

Liver ductal organoid culture

Biliary ducts were isolated from 6 to 8-week-old mice. Briefly, the liver tissue was cut into 0.5 mm^3 pieces and washed with cold PBS for three times. Then, the pieces were performed with pre-warmed digestion medium, containing collagenase (Sigma) and dispase II (Sigma) dissolved in wash medium, for 2 h on shaker at 37°C. The supernatant was centrifuged at 300 *g* for 5 min, discarded, and then washed with wash medium (DMEM supplemented with 1% penicillin/streptomycin and 1% fetal bovine serum) for three times and finally washed with cold PBS. After centrifuged at 200 *g* for 5 min, bile ducts were collected and embedded in Matrigel (R&D) and then seeded on a 24-well plate.

Mouse liver expansion medium consists of advanced DMEM/F12 (supplemented with penicillin/streptomycin, GlutaMAX-I, N2, B27, and N-acetylcysteine) plus 10 mM nicotinamide, 500 ng/ml R-spondin1 (R&D), 10 nM recombinant human [Leu15]-gastrin I (Sigma-Aldrich), 50 ng/ml recombinant mouse EGF (Invitrogen), 100 ng/ml recombinant human FGF10 (PeproTech), and 50 ng/ml recombinant human HGF (PeproTech). The medium was refreshed every 3 days.

Chemical-defined medium consists of advanced DMEM/F12 (supplemented with penicillin/streptomycin, GlutaMAX-I, N2, B27, and N-acetylcysteine) plus 10 mM nicotinamide, 3 μM CHIR-99021 (Selleck), 10 μM blebbistatin (Selleck), and 10 μM forskolin (Selleck). The medium was refreshed every 3 days. For liver organoid differentiation, chemical-defined medium was supplemented with 20 ng/ml oncostatin M (R&D) and 3 μM dexamethasone (Sigma-Aldrich).

Immunofluorescence

For whole-mount liver organoid staining, organoids were fixed in 4% paraformaldehyde for 30 min at 4°C, washed with PBST (0.1% Tween in PBS) for three times, and permeabilized with PBS containing 0.2% Triton X-100 (Sigma) for 15 min. Organoids were then washed with PBST for three times and blocked by 5% BSA for 1 h at room temperature. Organoids were incubated with primary antibodies at 4°C overnight, washed with PBST for three times, and incubated with secondary antibody and DAPI for 1 h at room temperature. Organoid imaging was performed on confocal microscope (OLYMPUS, FV3000). The following antibodies were used: goat anti-Albumin (Bethyl), rabbit anti-Sox9 (Santa Cruz), mouse anti-E-cadherin (BD), mouse anti-Ki67 (BD), rabbit anti-CK19 (Proteintech), Cy3-conjugated donkey anti-rabbit, FITC-conjugated donkey anti-goat, Cy3-conjugated donkey anti-mouse, Cy5-conjugated donkey anti-mouse, and FITC-conjugated donkey anti-rabbit (Jackson Lab).

Functional studies

For fluorescein diacetate transport assay, Chem-Orgs or GF-Orgs were loaded with 5 μM fluorescein diacetate (Santa Cruz) for 30 min at 37°C and then washed with advanced DMEM/F12 medium (Life Technologies) for three times. Following completion of the third wash, images were taken using a fluorescence microscope. To assess LDL uptake, liver organoids grown in growth factor medium or chemical medium for 14 days were tested using the LDL assay kit (Nanjing Jiancheng), following the manufacturer's instructions.

Adeno-associated virus (AAV) infection

As described before (Wei et al., 2019), the liver bipotential organoid pellet was suspended with chemical-defined medium. The pre-titrated AAV was added into the medium with gentle mix. The organoid-AAV mixture was transferred to the Matrigel precoated plate and incubated at 37°C for 12 h. Then, the organoids were embedded with Matrigel and seeded on a 24-well plate cultured with chemical-defined medium or differentiation medium.

qRT-PCR

Total RNA was isolated from organoids by RNeasy Mini kit (Qiagen). One microgram of RNA was reverse-transcribed into cDNA with M-MLV Reverse Transcriptase (Invitrogen). qRT-PCR was performed with SYBR Green Mixture (Promega) in triplicates on CFX96 Touch System (Bio Rad). Primers used were listed in Supplementary Table S1.

RNA sequencing and analysis

Total RNA was extracted from liver organoids by RNeasy Mini kit (Qiagen) following manufacturer's instructions and reverse-transcribed into cDNA libraries using the Ovation® RNA-Seq System V2 kit (NuGEN). Samples were sequenced with

paried-ends reads (PE150) using Illumina NovaSeq 6000 platform. The quality control (QC) analysis of the RNA sequencing data was performed using FastQC. The raw sequencing reads were pre-processed as follows: (i) removing adapter sequences and (ii) removing reads with $>$20 bp of low quality (Phred quality score $<$20). The filtered clean reads were aligned to mouse reference genome (mm10) using Tophat2, and then the uniquely mapped reads were assigned to each annotated genes using featureCount. Statistical significance test of DEGs was performed by DESeq2 with R. Genes with absolute log2-transformed fold changes $>$2 were regarded as DEGs, and a threshold of P-value $<$0.05 was used. Hierarchical clustering of log2-transformed RPKMs was generated by R. Gene set enrichment analysis was performed with GSEA v3.0 software (available from the Broad Institute). The raw NGS data were deposited to the NCBI SRA database under accession number (SRP229423).

Statistical analysis

All values are represented as mean $\pm$ SEM. Student's *t*-test and two-way ANOVA test were used to compare difference between two groups as indicated in the figure legends, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Statistical analysis was performed with the SPSS software. Each experiment was independently repeated at least three times.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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