Modelling human hepato–biliary–pancreatic organogenesis from the foregut–midgut boundary

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Organogenesis is a complex and interconnected process that is orchestrated by multiple boundary tissue interactions1–7. However, it remains unclear how individual, neighbouring components coordinate to establish an integral multi-organ structure. Here we report the continuous patterning and dynamic morphogenesis of hepatic, biliary and pancreatic structures, inhibiting in a three-dimensional culture of human pluripotent stem cells. The boundary interactions between anterior and posterior gut spheroids differentiated from human pluripotent stem cells enables retinoic acid-dependent emergence of hepato-biliary-pancreatic organ domains specified at the foregut–midgut boundary organoids in the absence of extrinsic factors. Wherefore transplant-derived tissues are dominated by midgut derivatives, long-term-cultured microdissected hepato-biliary-pancreatic organoids develop into segregated multi-organ anlagen, which then recapitulate early morphogenetic events including the invagination and branching of three different and interconnected organ structures, reminiscent of tissues derived from mouse explanted foregut–midgut culture. Mis-segregation of multi-organ domains caused by a genetic mutation in HES1 abolishes the biliary specification potential in culture, as seen in vivo8,9. In sum, we demonstrate that the experimental multi-organ integrated model can be established by the juxtapositioning of foregut and midgut tissues, and potentially serves as a tractable, manipulatable and easily accessible model for the study of complex human endoderm organogenesis.

The hepato-biliary-pancreatic (HBP) anlage, which is demarcated by HHEX (haematopoietically expressed homeobox protein) and PDX1 (pancreatic and duodenal homebox 1) expression, is first specified at the boundary between the foregut and midgut10 and forms an epithelial vesicle that invaginates ventrally from the primitive gut11–13. The disruption of boundary-defining genes around this area, such as BMPR1A (bone morphogenetic protein receptor, type IA)14, HLX (H2.0-like homeobox)15, CDX2 (caudal-type homeobox 2)16, NKX3-2 (NK3 homeobox 2)17, HHEX18, PDX1 and SOX9 (SRY-related HMG-box 9)19, markedly alters balanced organogenesis along the stomach–HBP–intestine in vivo1–7. Subsequent diversification of HBP lineages is probably mediated by adjacent mesenchymal bone morphogenetic protein (BMP) at their boundaries by indirectly repressing SOX9 in the posterior liver bud cells20. Thus, contiguous, dynamic organogenesis occurs in a complex environment and is probably driven by successive neighbouring-tissue interactions15,16,17. However, patterning and balanced HBP organogenesis has not been successfully modelled in tissue culture owing to technical difficulties, hindering detailed mechanistic studies15,16,17.

Here, we use a three-dimensional differentiation approach using human pluripotent stem cells (PSCs) to specify gut spheroids with distinct regional identities comprising both endoderm and mesoderm. We show that anteroposterior interactions recapitulate the foregut (marked by SOX2 (SRY-related HMG-box 2)) and the midgut (marked by CDX2) boundary in vitro, modelling the inter-coordinated specification and invagination in the human HBP organoid (HBPO).

To determine the source of HHEX- and PDX1-expressing cells, we used unlabelled anterior and GFP-labelled posterior spheroids. We found that both HHEX- and PDX1-expressing cells at the boundary from three different PSC lines (Extended Data Fig. 1c–e). HBP progenitor induction critically requires the cell-to-cell contact between the anterior and posterior spheroids (Extended Data Fig. 2a, b). PDX1-expressing cells (Fig. 1c) comprised 5% of cells in the boundary region, 0% of cells in the anterior region and 1% of cells in the posterior region (Fig. 1d). PDX1-expressing cells were observed in anterior–posterior (AP) and posterior–posterior (PP) spheroid combinations. By contrast, HHEX-positive cells were only detected in AP and not in anterior–anterior (AA) or PP spheroid combinations (Fig. 1e, Extended Data Fig. 2c), indicating that balanced induction of the HBPO progenitors requires AP fusion.

To determine the source of HHEX- and PDX1-expressing cells, the fused spheroids were established using unlabelled anterior and GFP-labelled posterior spheroids. We found that both HHEX and PDX1 expression overlapped with GFP, suggesting that the HBP progenitors originate from the posterior gut (Extended Data Fig. 3a). RNA-sequencing (RNA-seq) analysis of day 8, 9, 11 and 12 micro-dissected anterior, boundary and posterior regions showed that the boundary domains at day 11 and day 12 progressively expressed the range of HBP specification markers, whereas anterior or posterior regions gained foregut or midgut–hindgut identity, respectively (Fig. 1f). In sum, these results show that the AP fusion strategy orchestrates autonomous patterning of HHEX- and PDX1-positive HBP progenitors in the absence of exogenous inductive factors.

Next, we established a reporter human induced PSC (iPSC) line to track the fate of progenitor cells by visualizing a tdTomato reporter of a common progenitor marker of the liver, bile duct and pancreas—prospero-related homeobox 1 protein (PROX1)—using CRISPR–Cas9 genome editing (Extended Data Fig. 4a). Similar to HHEX and

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PDX1, PROX1 started to be expressed at the boundary on day 10 and expression subsequently increased (Fig. 2a, Extended Data Fig. 4b). The emergence of PROX1 expression was specific to the AP spheroid combination, and was absent in AA and PP spheroid combinations (Fig. 2b). AP spheroid fusion assays indicated that PROX1-positive cells also originated from the posterior gut cells (Extended Data Fig. 3b, c).

To delineate the self-inductive mechanism of the HBP progenitor, we evaluated the boundary-specific expression profiles of known inductive signalling pathways. RNA-seq identified that retinoic acid signals—but not FGF, BMP, Hedgehog or Notch signals—were activated predominantly at the boundary, but not in the anterior or posterior regions (Extended Data Fig. 4c, Supplementary Tables 1, 2). Consistent with these results, the retinoic acid receptor antagonist BMS493 strongly suppressed gene expression of both HHEX and PDX1 (Fig. 2c). Animal studies have suggested that retinoic acid signalling has an important role in lineage specification into HBP domains21,22. Lateral-plate mesoderm acts as an activator for retinoic acid signalling during the specification in vivo23–25. To identify the cellular source of retinoic acid in our model system, expression of retinoic-acid-signalling-related genes was assessed in isolated epithelial and non-epithelial populations. Fluorescence-activated cell sorting (FACS) analysis showed that 90.3% of the anterior gut cells at day 7 were EpCAM+ epithelial cells, whereas the EpCAM+ ratio decreased to 70.7% in the anterior gut cells by day 12 owing to expansion of non-epithelial cells (Fig. 2f, Extended Data Fig. 1a). Notably, only the anterior non-epithelial cells highly expressed the retinoic-acid-synthesis gene aldehyde dehydrogenase 1 family member A2 (ALDH1A2), as shown in previous animal studies (Fig. 2f). Furthermore, exposing only the posterior spheroid and not the anterior spheroid to BMS493 before fusion suppressed protein-level induction of HHEX and PDX1 (Extended Data Fig. 5a). An E9.0 Prox1::eGFP reporter mouse embryo, cultured with BMS493, also displayed significant inhibition of PROX1-expressing cells after two days (Extended Data Fig. 5b, c). These results show that HBP progenitor self-specification in the boundary model system is regulated by retinoic acid, potentially supported by co-differentiating anterior non-epithelial, probably mesenchymal lineages.

Stem-cell-derived embryonic endodermal cells are highly plastic and usually generate intestinal tissues18,26. To examine whether the fused spheroids have the ability to form HBP tissues in vivo, we transplanted human PROX1+ spheroids into immunodeficient mice. One month after transplantation, the transplant-derived tissues exhibited the small intestinal tissue markers keratin 20, CDX2 and EpCAM, but negligible expression of the other HBP markers (Extended Data Fig. 4d). In
addition, the expression patterns of duodenum marker receptor accessory protein 6 (REEP6, also known as DP1L1) and SOX9 in the transplants were similar to those in the duodenum (Extended Data Fig. 4d).

Even following transplantation of a microdissected PROX1+ domain, the cells did not maintain the HBP fate (Extended Data Fig. 6a), as seen in the graft of mouse E9.0 PROX1-expressing portion (Extended Data Fig. 6b). These results indicate that, despite the presence of HBP progenitors, ectopically transplanted organoids tend to develop into intestinal tissues in vivo.

Because the fused spheroids predominantly generated duodenum tissue in vivo, we next excised PROX1-positive regions from the day 13 boundary organoids and cultured them in different formats to effectively model HBP organogenesis (Extended Data Fig. 7a, b). Among the tested culture conditions (Extended Data Fig. 7c), we found that the air–liquid interface system of excised PROX1+ domains continued the morphogenesis and formed a branching structure, hereafter called HBPO (Extended Data Fig. 7b). Time-course imaging showed that the dissected PROX1+ domain changed from an epithelial morphology to a more convoluted structure during two days of culture (Extended Data Fig. 7d). Around day 25, the PROX1+ epithelium in the HBPO started to invaginate and grow outward in multiple directions, progressively forming a branching structure (Fig. 3a, b). The branching structures were not observed in the AA and PP spheroid combinations (Extended Data Fig. 8a–d). Furthermore, the posterior region in day-11 HBPO, which partially expressed PDX1, was not capable of growing invaginating structures at day 30 (Extended Data Fig. 8e).

To determine whether longer-term culture could produce more advanced tissue, we cultured the HBPOs until day 90. The day-90 organoids were morphologically similar to mouse explants isolated from E10.5 embryos and grown for four days in the air–liquid interface system (Fig. 3c). Haematoxylin and eosin staining showed that the long-term cultured HBPOs maintained HBP domains (Fig. 3d). Furthermore, immunofluorescent staining showed expression of the pancreas markers PDX1 and NGN3, the liver marker PROX1 and the bile duct marker CK19 and SOX9 in the organoids (Fig. 3e). Mesenchyme cells expressing α-smooth muscle actin (α-SMA) wrapped around bile duct SOX17+ cells, similar to those in developing gallbladder tissue (Fig. 3e). Immunofluorescent staining with liver markers AFP and albumin, pancreatic markers PDX1, NKX6.1 and GATA4, and bile duct markers DBA and SOX9 confirmed that each lineage of primitive tissues segregated in the HBPOs after 30 days of culture (Fig. 3f–i, Extended Data Fig. 9a). NKX6.1, HNF1B and GATA4 were differentially expressed in the PDX1+ region, and expression of the pancreatic mesenchymal marker NKX6.3 was observed alongside the PDX1+ cells, similar to the expression in in vivo developing pancreas (Fig. 3g, i, Extended Data Fig. 9b). Notably, the bile duct and pancreas domains were directly connected in branching organoids, as shown by whole-mount co-staining of DBA, SOX9 and PDX1, and by the capacity to incorporate fluorescein-labelled bile acid (CLF) (Fig. 3h, Extended Data Fig. 9c). Moreover, at 90 days, the exocrine markers amylase and GATA4 were identified in the pancreatic component of HBPOs (Fig. 3i). On the basis of cholecystokinin A receptor (CCKAR) expression in the HBPOs (Fig. 3k), we analysed the CCK-responsive pancreatic secretory function using an amylase enzyme-linked immunosorbsent assay (ELISA). The ductal structures constricted the day following CCK treatment, and the CCK-treated HBPOs exhibited increased amylase secretion into the supernatants compared with untreated controls (Fig. 3l, m). These results indicate that the boundary-organoid strategy not only generates multiple organ (HBP) domains but also establishes a functional link between the pancreas, especially the exocrine lineage, and the bile duct.

Hes family bHLH transcription factor 1 (HES1) is a transcription factor that regulates the posterior foregut lineage. In Hes1-knockout mice, conversion of the biliary system to pancreatic tissue occurs owing to failed pancreato-biliary segregation. To elucidate whether the HBPO recapitulates the HES1-mediated developmental process, we established HES1-knockout PROX1-reporter iPSCs using the CRISPR–Cas9 system (Extended Data Fig. 10a–c), and confirmed the absence of HES1 gene expression in HES1−/− iPSC-derived organoids (Extended Data Fig. 10d), HES1−/− HBPOs retained PROX1-reporter
Modelling human HBP organogenesis. a, Morphogenetic change of dissected PROX1+ domain over 30 days of air–liquid interface culture. b, Stereomicroscope image of day-37 organoids. c, Boundary organoid has PROXI-expressing HBP domains branching to form putative pancreatic tissue, similar to cultured mouse HBP tissue. Left, mouse E10.5 embryonic tissue after 4 days of culture. Right, PROX1-tdTomato HBP domain at day 90. d, Left, illustration of invaginating liver, bile duct and pancreas in relation to intestine. Right, H&E staining in day-90 boundary organoid. e, f, Immunostaining of CK19 and PDX1 (e, left), PROX1, SOX9 and NGN3 (e, right (main)), α-SMA and SOX17 (e, right (inset)) and AFP, EpCAM, and α-SMA (f). g, h, Whole-mount staining of PDX1, NKX6.1 and GATA4 (g, h, left (main; inset shows magnified view of outlined region)), and PROX1, DBA (g, h, right, top; bottom shows magnified view of outlined region), and NKX6.1 and HNF1B (h, right, top; bottom shows magnified view of outlined region). i, j, k, Immunostaining of NNX6.1 and HNF1B (i, j, left (main; inset shows magnified view of outlined region)) or NR5A2 (j, right, top; bottom shows magnified view of outlined region), and amylase and CCKAR (g, h, right). I, CCK treatment response in putative biliary structure. m, Hormone-induced secretory function of exocrine pancreatic domain. ELISA of amylase before and after three days of CCK treatment. Box plots show the mean, box edges show the 25th and 75th percentiles and whiskers show the range of values. n = 10 independent experiments; unpaired two-tailed t-test. Images are representative of two (b), three (d-f, h-k), four (c, g, i) or six (a) samples showing similar results. Scale bars, 100 µm (h, j), 200 µm (a, c-g, i), 1 mm (b), 500 µm (k) and 50 µm (l).

Multiple-organ integration in stem cell culture is a critical unmet challenge. Here we have demonstrated the generation of a human three-dimensional anteroposterior boundary system that leads to structurally and functionally integrated HBPOs developed at the foregut–midgut boundary. Further HBP organogenesis—that is, the liver budding process, including delamination of the hepatic epithelial sheet by disruption of the laminar layer—requires further investigation that potentially requires additional stromal cell components, such as septum transversum mesenchyme and/or endothelial progenitors.

Nevertheless, the in vitro contiguous specification and early morphogenesis of HBP subdomains provide an opportunity for study of these complex interactions at the boundary and for generating interconnected, multi-organ structures in personalized human organogenesis and disease model systems in vitro, ultimately towards therapy.

Online content
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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Maintenance of PSCs. Human PSC lines were maintained as previously described. Undifferentiated human PSCs were maintained on feeder-free conditions in mTeSR1 medium (StemCell Technologies) or StemFit medium (Ajinomoto) on plates coated with either Matrigel Growth Factor Reduced (Corning) at 1:30 dilution or iMatrix-511 (Nipi) at 0.25 µg cm⁻² in an incubator with 5% CO₂, 95% air at 37 °C. The TkdA3 human iPSC clone (RIRD-CVCL, RJ54, passage number 37 to 47) used in this study was provided by K. Eto and H. Nakauchi. The human 72_3 iPSC (passage number 44 to 46) and human H1 ESC (passage number 55 to 60) were provided from Pluripotent Stem Cell Facility in our institute. Cell lines have been tested for mycoplasma contamination regularly.

Differention of PSCs into anterior and posterior gut spheroid. Differentiation of human PSCs into definitive endoderm was induced using previously described methods with modifications. In brief, colonies of human iPSCs were isolated in Accutase (Thermo Fisher Scientific) and 150,000 cells per ml were plated on Matrigel coated tissue culture plate (VWR Scientific Products). Medium was changed to RPMI 1640 medium (Life Technologies) containing 100 ng ml⁻¹ activin A (R&D Systems) and 50 ng ml⁻¹ bone morphogenetic protein 4 (BMP4; R&D Systems) at day 1, 100 ng ml⁻¹ activin A and 0.2% fetal calf serum (FCS; Thermo Fisher Scientific) at day 2 and 100 ng ml⁻¹ activin A and 2% FCS at day 3. On days 4 to 7, cells were cultured in gut growth medium (Advanced DMEM/ F12 (Thermo Fisher Scientific) with 15 µM HEPES, 2 mM l-glutamine, penicillin–streptomycin, B27 (Life Technologies) and N2 (Gibco)) supplemented with 200 ng ml⁻¹ noggin (NOG; R&D Systems), 500 ng ml⁻¹ fibroblast growth factor 4 (FGF4; R&D Systems) and 2 µM CHIR99021 (Stemgent) for anterior gut cell induction and supplemented with 500 ng ml⁻¹ FGF 4 and 3 µM CHIR99021 for posterior gut cell induction. Cultures for cell differentiation were maintained at 37 °C in an atmosphere of 5% CO₂, 95% air and the medium was replaced daily.

Anterior–posterior boundary sphere formation. On day 7, anterior or posterior gut cells were dissociated to single cells by incubation with TrypLE Express (Life Technologies) at 37 °C. Cells were centrifuged at 1,000 r.p.m. for 3 min and, after removing supernatant, the pellet was resuspended in gut growth medium containing 10 µM Y 27632 dihydrochloride (Tocris Bioscience). The anterior or posterior gut cell suspensions were plated on 96-well round-bottom ultra-low attachment plates (Corning) at density of 10,000 cells per well and incubated at 37 °C for 24 h to form spheroids. On day 8, the generated single anterior spheroid and posterior spheroid were mixed on 96-well round-bottom ultra-low attachment plate in gut growth medium for 24 h to form fused boundary spheroids (AP spheroids). These AP spheroids were cultured in HBPO culture. On day 9, AP spheroids were embedded in Matrigel drop and were cultured in gut growth medium to generate multi-organ HBPOs. For longer-term culture, HBPOs were dissected and/or transferred to Transwells for air–liquid interface culture at day 13. Cultures for spheroids were maintained at 37 °C in an atmosphere of 5% CO₂, 95% air and the gut growth medium were replaced every 4 days. A step–by–step protocol describing HBPO organoid generation is available at Protocol Exchange.

Transplantation. Single HBPOs at day 13 were transplanted into the subcapsule of the kidney in male immune-deficient NOD.Cg-PrkdcreID22R2tm1Ncl/J (Nscg) mice at 12 weeks of age. All experiments were performed under the approval of the Institutional Animal Care and Use Committee of CCHMC (protocols IACUC2018-0096).

H&E staining and immunohistochemistry. Spheroids and organoids were collected from Matrigel, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Sections were subjected to H&E and immunohistochemical staining. Primary antibodies are listed in Supplementary Table 3. Immunohistochemical staining was performed by using ultraView Universal DAB Detection Kit (Roche Diagnostics). The specimens were observed under a microscope.

For whole-mount immunohistochemical staining, spheroids and organoids were collected from Matrigel and remaining Matrigel was removed by treating with cell-recovery solution at 4 °C for 30 min. The tissues were washed with PBS and fixed in 4% PFA at 4 °C overnight. The fixed samples were treated with 4% PFA with 0.5% Triton X-100 at room temperature for 15 min and permeabilized with 0.1% Tween 20 (Sigma) at room temperature for 15 min. The samples were treated with blocking solution (1% BSA and 0.3% Triton X-100) at room temperature for 1 h and were incubated overnight at 4 °C with primary antibodies diluted in blocking solution. After washing, fluorescent dye-conjugated secondary antibodies were added to the samples at room temperature for 2 h. The primary and secondary antibodies are listed in Supplementary Table 3. After the secondary antibody reaction, the samples were washed three times. Nuclei were stained with DAPI mounting solution.

Stained sections and whole-mount samples were observed under a Nikon A1Rsi inverted confocal microscope.

RNA isolation and RT–qPCR. RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription was carried out using the SuperScript IV First-Strand Synthesis System for RT–qPCR (Invitrogen) according to manufacturer's protocol. qPCR was carried out using the TaqMan gene-expression master mix (Applied Biosystems) on a QuantStudio 3 Real-Time PCR System (ThermoFisher). All primers and probe information for each target gene were obtained from the Universal ProbeLibrary Assay Design Center (https://upl.probefinder.org/organism.jsp) and listed in Supplementary Table 4.

RNA-seq. Sample preparation for RNA-seq was performed using SMART-seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech) according to the manufacturer's user manual.

In brief, first-strand cDNA synthesis was primed by the 3’ SMART-seq cDNA Primer II A and used the SMART-Seq v4 oligonucleotide for template switching at the 5’ end of the transcript. PCR Primer II A amplified cDNA from the SMART sequences introduced by 3’ SMART-Seq cDNA Primer II A and the SMART-Seq v4 Oligonucleotide by PCR. PCR-amplified cDNA was purified by immobilization on AMPure XP beads. The beads were then washed with 80% ethanol and cDNA was eluted with elution buffer. Amplified cDNA was validated using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent) according the kit user manual. The full-length cDNA output of the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing was processed with the Nextera XT DNA Library Preparation Kit (Illumina).

The RNA profiles were compiled with Kallisto software and expressed as transcription per million transcripts (TPM). The datasets filtered by the threshold requiring greater than 0 in at least 1 sample were first subjected to gene functional classification on the basis of gene sets related to FGF, BMP, Hedgehog, Notch and retinoic acid signalling pathways. The lists of gene sets were acquired from Molecular Signatures Database (v6.2). Cluster analysis for filtered datasets performed by using Cluster 3.0 software. Detail of gene sets included in each cluster are listed in Supplementary Table 1. Gene set enrichment analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) analysis was applied to identify significant enrichment of pathway genes across genes between the different regions in fused organoid. GSEA results for comparison of anterior–boundary and boundary–posterior combinations in day 9, 11 and 12 RNA-seq data are listed in Supplementary Table 2. Significant differences were validated by false discovery rate q value <0.25.

Flow cytometry. For flow cytometry, anterior gut cells were differentiated from GFP-labelled iPSCs and posterior gut cells were differentiated from mCherry-labelled iPSCs. At day 13, AP spheroids were dissociated to single cells by treatment with TrypLE Express for 10 min at 37 °C. After washing with PBS, the single cells were incubated with 421-conjugated EpCAM antibody (BioLegend) at room temperature for 30 min. After washing with PBS, cell sorting was performed in a BD FACS AriaII (BD Biosciences). Analysis was performed by BD FACSDIVA software and FlowJo (FlowJo).

CRISPR editing. The plasmid encoding Cas9–2A–GFP was acquired from Addgene (no. 44719). Guide RNA targeting the N terminus of PROX1 or HES1 was synthesized by Integrated DNA Technologies, cloned into the pGL3-U6-sgRNA-PGK-purovycin vector (Addgene no. 51133) and sequenced using the RV3 universal primer. To construct the homology-directed repair template, homology arms flanking the PROX1 start codon were independently amplified from genomic DNA and then fused to tdTomato by overlap-extension PCR using the high-fidelity Taq polymerase ipProof (Bio-Rad). The resulting PCR product was then cloned into the pCR-Blunt II-TOPO cloning vector (Invitrogen) and confirmed by Sanger sequencing.

Human iPSCs were transfected with 2 µg of each plasmid using Lipofectamine 3000 following the manufacturer's instructions. Twenty-four hours after transfection, cells were sorted by GFP expression to select for transfected cells. Clonal cells were expanded for two weeks and screened for inserted PROX1-ttdTomato or deleted HES1 exon 1 sequence and karyotyped.

Amlyase ELISA. To measure amylase secretion level of organoids, 200 µl of culture supernatant was collected from organoid embedded in Matrigel. The culture supernatants were collected at 72 h after the culture and stored at −80 °C until use. The supernatant was centrifuged at 1,500 r.p.m. for 3 min to pellet debris and the resulting supernatant was assayed with Human Amylase ELISA Kit (Invitrogen) according to the manufacturer’s instructions.

Mouse whole-embryo culture. The Rotatory-type Bottle Culture System (Ikemoto Scientific Technology) was used for whole-embryo culture. E9.0 PROX1–GFP mouse embryo was dissected and transferred to a culture bottle with Advanced DMEM/F12 supplemented with B27 and N2 supplements. The temperature inside the embryo-culture system was maintained at 37 °C.

Statistical analysis. Analysis was performed using unpaired two-tailed Student’s t-test, Dunn–Hollander–Wolfe test or Welch’s t-test. Results are presented as mean ± s.d. or s.e.m.; P < 0.05 was considered statistically significant.
Unless noted otherwise, n numbers refer to biologically independent replicates. For comparisons between more than two samples, we performed one-way ANOVA followed by Tukey’s test, Dunnett’s test, non-parametric Kruskal–Wallis or post hoc Dunn–Holland–Wolf test.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

Sequence data used in this study have been deposited in the Gene Expression Omnibus with accession number GSE121830. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Author contributions** H.K., K.I. and R.O. carried out the experiment and analysed data. M.M. and A.F. performed the organoid experiment. K.G. and N.S. performed computational analysis. H.K. and T.T. wrote the manuscript with support from K.I., R.O. and W.L.T. M.K., J.M.W. and A.M.Z. helped to supervise the project. H.K. and T.T. conceived the original idea. T.T. supervised the project.

**Competing interests** The authors declare no competing interests related to this manuscript.

**Additional information**

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Extended Data Fig. 1 | Anterior- and posterior-gut cell specification and boundary organoid formation. a, Flow cytometry of EpCAM in day 7 anterior and posterior gut cells using TkDA3 human iPSCs and 72_3 human iPSCs. The gating strategy was forward scatter (FSC) area (A)/side scatter (SSC) A, FSC height (H)/FSC width (W), SSC H/SSC W, propidium iodide (PI)/FSC A and EpCAM–BV421/SSC A. Representative image of three independent experiments showing similar results. b, Whole-mount immunostaining, flow cytometry showing percentage of the population in each quadrant, qPCR for SOX2 and CDX2, and organoid images at each time point. Data are mean ± s.d.; n = 3 independent experiments. Unpaired, two-tailed r-test. Scale bars, 50 µm. c, Image of day-11 boundary organoids. Anterior and posterior gut spheroids were differentiated from H1 ESCs or 1383D6 iPSCs, mixed and transferred into Matrigel. Independent experiments were repeated twice for each line with similar results. Scale bar, 200 µm. d, Whole-mount Immunofluorescent staining of PDX1, CDX2, FOXF1 and HHEX in boundary organoids derived from 72_3 iPSCs at day 12. Representative image of n = 30 independent organoids showing similar results. The arrowhead indicates the boundary of the organoid. Scale bar, 100 µm. e, Whole-mount immunofluorescent staining of CDX2, E-cadherin and HHEX in the boundary region of organoids derived from H1 ESCs at day 12. Representative image of n = 6 independent organoids showing similar results. Scale bar, 50 µm.
Extended Data Fig. 2 | Cell–cell contact of anterior–posterior gut spheroids induced HBP marker expression. a, Anterior and posterior spheroids were mixed on day 8, fused on day 9, cultured, and collected on day 12 for quantitative RT-qPCR. The spheroids that did not fuse were also collected on day 12 for comparison. Independent experiments were repeated twice with similar results. b, Gene expression of PDX1 and HHEX in fused, non-fused, posterior spheroids (day 8) and iPSCs. Data are mean ± s.d. from two independent experiments. c, Comparison of different combinations of anterior and posterior gut spheroids. Immunofluorescent staining of CDX2, HHEX, and PDX1 in AP, AA and PP spheroids at day 12. Images are representative of n = 4 (AA and PP) and n = 6 (AP) independent organoids showing similar results. Scale bar, 200 µm.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | HBP progenitors developed from posterior gut cells. **a**, Starting on day (d)0, unlabelled iPSCs were differentiated into anterior spheroids and GFP-labelled (GFP sequence inserted into AAVS1 (Adeno-associated virus integration site 1) locus) iPSCs were differentiated into posterior spheroids. Top, bright-field and GFP fluorescence image during boundary organoid formation. Middle and bottom, whole-mount immunostaining for HHEX and PDX1 at day 13. HHEX expression overlapped with GFP expression. Images are representative of \( n = 3 \) independent organoids showing similar results. Scale bar, 200 \( \mu \text{m} \). **b**, H2B–GFP labelled and unlabelled PROX1::tdTomato reporter iPSCs were differentiated into anterior and posterior spheroids, respectively. tdTomato expression was detected only in unlabelled original posterior gut spheroids. Independent experiments were repeated twice with similar results. Scale bar, 200 \( \mu \text{m} \). **c**, Anterior and posterior gut spheroids were differentiated from unlabelled iPSCs and PROX1::tdTomato reporter iPSCs. Reporter cell-derived anterior and unlabelled cell posterior spheroid (left), or unlabelled cell-derived anterior and reporter cell-derived posterior spheroid (right) were examined for tdTomato expression. Top, bright-field image; bottom, tdTomato fluorescence. Images are representative of \( n = 3 \) independent organoids showing similar results. Scale bar, 200 \( \mu \text{m} \).
Extended Data Fig. 4 | Characterization of HBP progenitors from boundary organoids. a, Schematic of PROX1-tdTomato reporter generation using the CRISPR-Cas9 system. b, PROX1 reporter activity in boundary organoids. All images are boundary organoids derived from the PROX1::tdTomato reporter line at day 12. Images are oriented with anterior at the top and posterior at the bottom. Arrowheads indicate PROX1::tdTomato expression at the boundary of each spheroid. Independent experiments were repeated three times with similar results. Scale bar, 100 µm. c, Transcriptomic characterization of dissected anterior, boundary and posterior domains by RNA-seq. Heat map shows downstream gene expression related to FGF, BMP, hedgehog, Notch and retinoic acid signalling pathways selected by gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway category. The heat map was separated into eight groups (C1–C8) by hierarchical clustering. d, Default developmental potential of transplanted boundary organoids. Middle, H&E staining and immunohistochemistry; right, immunofluorescence. The experiment was repeated with three independent samples with similar results. Scale bar, 100 µm.
Extended Data Fig. 5 | Depletion of HHEX, PDX1 and PROX1 by exposure of human boundary organoids and mouse embryos to retinoic acid receptor. 

**Note:** The figures illustrate the depletion of HHEX, PDX1, and PROX1 upon exposure to retinoic acid receptor. The images show whole-mount staining of HHEX, PDX1, and CDX2 in organoids treated with and without BMS493. Expression of these proteins is inhibited at the boundary, suggesting a role for retinoic acid receptor function in establishing the boundary organoid. Images are representative of n = 4 independent organoids showing similar results. Scale bar, 200 µm.

**b, c, PROX1 inhibition by BMS493 exposure with embryonic day (E) 9.0 Prox1::eGFP reporter mouse embryo explant culture.** The whole embryo was cultured in the rotator-type bottle culture system for 24 h. The group treated with retinoic acid receptor antagonist BMS493 was compared with the control (DMSO only) group. **b**, Bright-field image and GFP fluorescence of the embryo after culture. Images are representative of n = 3 independent embryos showing similar results. **c**, The area of GFP-expressing regions was quantified from images represented in b. Data were mean ± s.d. (n = 3). P = 0.0035 by unpaired, two-tailed Student’s t-test. Scale bar, 1 mm.
Extended Data Fig. 6 | Transplantation of dissected PROX1-expressing domain from human organoid and mouse embryo. a, Dissected PROX1-positive boundary domain at day 13 was transplanted into an immunodeficient mouse, and formed a duct-like structure in the tissue, expressing PROX1 and duct marker SOX9 after one month. Images are representative of \( n = 2 \) independent transplants showing similar results. b, E9.0 PROX1–GFP mouse embryonic HBP domain was transplanted and formed limited tissue expressing PROX1 (GFP) or PDX1 seven days after transplantation. Images are representative of \( n = 2 \) independent transplants showing similar results.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Optimization of the in vitro culture system.  

a, Illustration of the dissection strategy of the PROX1-positive region from organoids with representative images. The imaging experiments were repeated with 12 independent samples with similar results. Scale bar, 100 μm.  
b, Optimization of the organoid culture system, comparing: (1) floating, (2) embedding in Matrigel, (3) embedding in Matrigel and culture in Transwells from day 13 and (4) dissection followed by embedding in Matrigel, and culture in Transwell from day 13. Left, the typical morphology of invaginating or branching organoid. The imaging experiments were repeated with 12 independent samples with similar results. Scale bar, 100 μm.  
c, Illustration of optimization of in vitro culture system. We compared various culture formats to enhance morphological change, such as invagination and branching morphogenesis, of PROX1-positive HBP domains. At day 7, anterior and posterior gut spheroids were mixed after 24 h of culture. Connected spheroids were transferred into a Matrigel drop or a low-binding culture plate to compare between non-floating and floating conditions during emergence of the HBP domain. Organoids in the Matrigel-embedded group started to express tdTomato at day 11. The tdTomato-positive region was manually dissected under the microscope according to the fluorescent signal and transferred into a Matrigel again or a Transwell to compare the effect of various agonists and antagonists in the medium.  
d, Morphogenesis of boundary organoids during 2 days from day 13. Imaging experiments were repeated independently three times with similar results. Scale bar, 100 μm.
Extended Data Fig. 8 | Comparison of organoid size, PROX1-positive area, branching and invagination in various conditions. a, Comparison of PROX1-tdTomato expression in AP, AA and PP spheroids at day 50 of culture. Images are representative image of n = 6 independent organoids showing similar results. Scale bar, 500 μm. b, c, Quantification of entire spheroid surface area (b) and of PROX1-positive region (c). n = 11 (AP), 6 (AA) and 7 (PP). Box plots show the mean, box edges show the 25th and 75th percentiles and whiskers show the range of values. In b, P = 0.0278 (AP versus AA), 0.0052 (AP versus PP) and 0.8566 (AA versus PP); in c, P = 0.0011 (AP versus AA), 0.0022 (AP versus PP) and 0.9063 (AA versus PP). One-way ANOVA, followed by Tukey’s test. d, Percentage of branching and invaginating PROX1-positive organoids, defined as in Extended Data Fig. 7b. The AP combination produced spheroids with branching and invagination, whereas other two combinations did not. e, Failure to branch and invaginate from the posterior region of HBPOs. Dissected posterior region from day 11 organoid cultured until day 30. Whereas HBPO formed a PROX1-expressing branching structure, the posterior dissected region of HBPO that shows PDX1 expression did not form this structure. Images are representative of two independent experiments with similar results. Scale bar, 200 μm.
Extended Data Fig. 9 | Expression of organ domain-specific markers in HBPOs. a, Immunofluorescent staining for AFP, albumin and HHEX at day 30. AFP and albumin were expressed in the same region but HHEX was not expressed. Expression of HHEX, a hepatocyte progenitor marker, was lost at this late stage. The experiment was repeated twice independently with similar results. b, Immunofluorescent staining for NKX6.1, NKX6.3 and PDX1. NKX6.3 was expressed in the vicinity of pancreatic markers PDX1 and NKX6.1. The experiment was repeated three times independently with similar results. c, Immunofluorescent staining for EpCAM, PROX1, SOX9 and CLF. Representative image of $n = 3$ independent organoids showing similar results. Scale bar, 100 µm.
Extended Data Fig. 10 | Upregulation of pancreatic marker genes and depletion of bile duct markers in HES−/− organoids. a, Gene-targeting strategy for HES1-knockout (KO) line using the CRISPR–Cas9 system. b, Confirmation of modified gene sequence of control and HES1 knockout (Del #11). c, Representative image of n = 3 HES1−/− iPSC culture showing similar results. Scale bar, 500 μm. d, HES1 expression in an organoid at day 20. Data are mean ± s.d.; n = 6 independent organoids. Unpaired two-tailed t-test. e, Heat map shows gene-expression profile of pancreas-associated markers at day 22 of HES1+/+ and HES1−/− HBPOs. These data correspond to Fig. 4c. f, Connected structure is disrupted in long-term cultured HES1-knockout organoids. Whole-mount staining of DBA and SOX9 in HES1−/− and HES1+/+ organoids. DBA and SOX9 signal was lost in HES1−/− organoids. The experiment was repeated three times independently with similar results. Scale bar, 200 μm.