

ONLINE SUPPLEMENT

ONLINE ANIMATIONS AND VIDEOS

Animations.

- Animations of embryological development of the biliary tree in dogs (representative of most mammals) versus pigs
- Animation of biliary tree in humans

Movies:

- **Video 1.** Scan of porcine biliary tree at low magnification to demonstrate the extensive ramifications of the biliary tree from the intrahepatic region to the duodenum.
- **Video 2.** Scan of porcine biliary tree from liver to duodenum magnified to show branching.
- **Video 3.** Scan of porcine biliary tree in the pancreas

ONLINE SUPPLEMENT FIGURES

Figure S1. Serial sections of common bile duct and the associated pancreatic duct in 4-weeks-old piglets confirmed findings in past studies that there is no hepato-pancreatic common duct in postnatal pigs.

A. A simple graphic was used to show the procedure of tissue grossing, processing, embedding and serial section. A total of 300 sections were collected starting from the inside of the duodenum's mucosa to the surface of the outside of the muscular layer of the duodenum.

B. H&E staining of serial sections with a thickness of 5 μm . The stained section was scanned for every single slide from slides #1 to #300. The opening of the entrance of bile duct appeared by #50. The injected green tissue dye can be distinguished by slides # 118. An enlarged lumen of common bile duct with green tissue dye can be found from #168 to #208. There were few mucosae shown when sections were taken from the layer of #282.

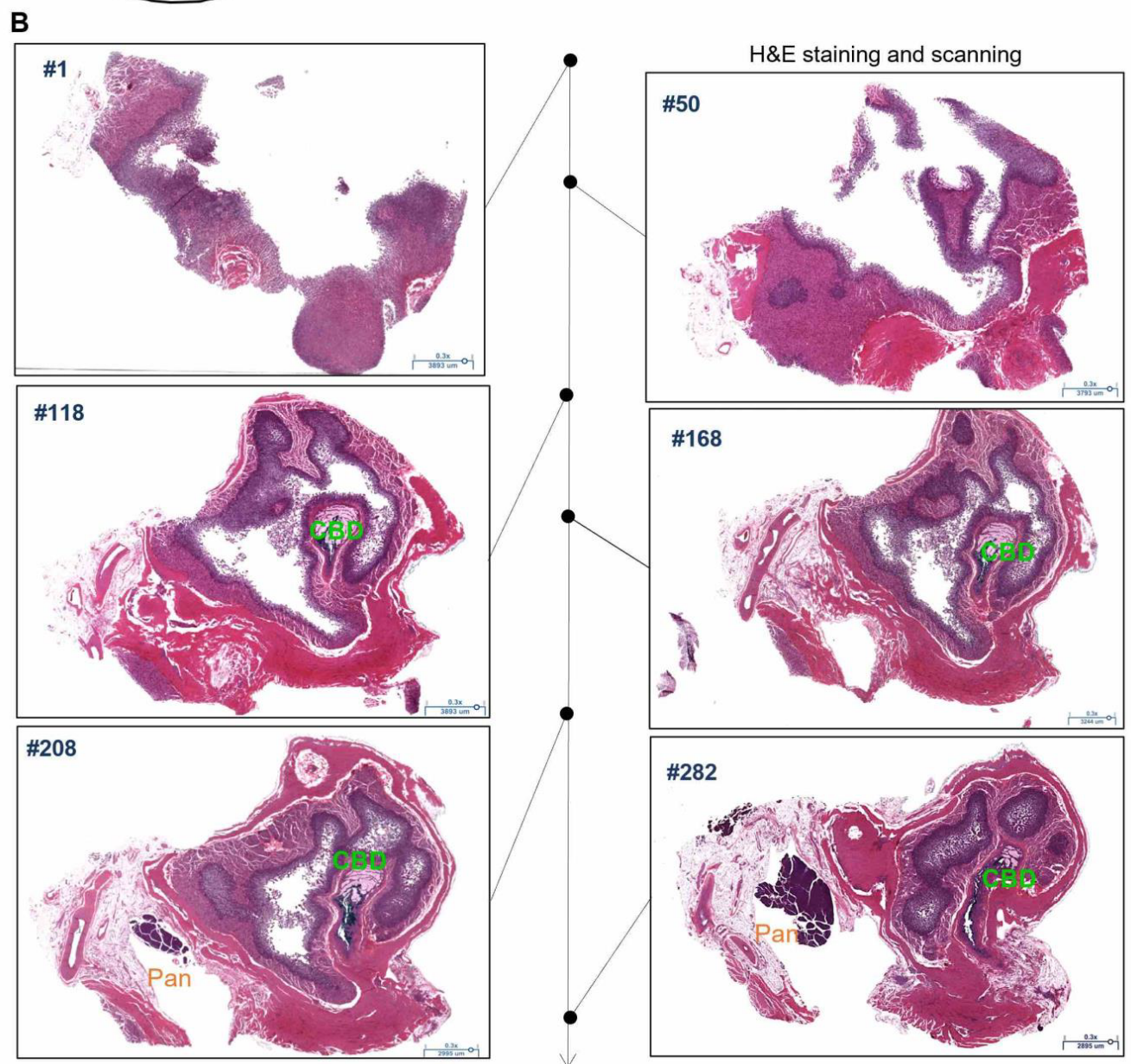
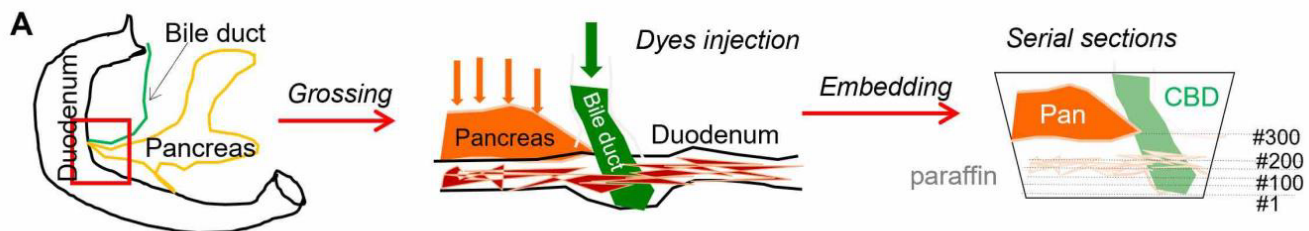


Figure S2. Histology for 7-weeks-old pig liver, biliary tree, and duodenum

Masson's trichrome staining of sections from the 7-weeks-old piglet embedded in the large FFPE paraffin block. Compared to newborn piglets, the pancreas of the 7-weeks-old piglet is dramatically larger. (**A**) Magnification 0.6X. The gallbladder has more villi in the mucosa compared to that in the newborn (**A, a**). Intramural glands in the walls of the pancreatic duct (**A, b**). When the intrahepatic duct (**A, c**), and extrahepatic duct (**A, d**) and the common bile duct are close to the duodenum (**A, e**) there are also sites enriched for PBGs. A simple flow chart along the histology graphs of each duct, shows the hepatic-biliary tree and the pancreatic biliary tree merge into the duodenum separately. **B**, Blue=intra-/extrahepatic duct; Green=gallbladder cystic duct; Yellow=pancreatic duct.

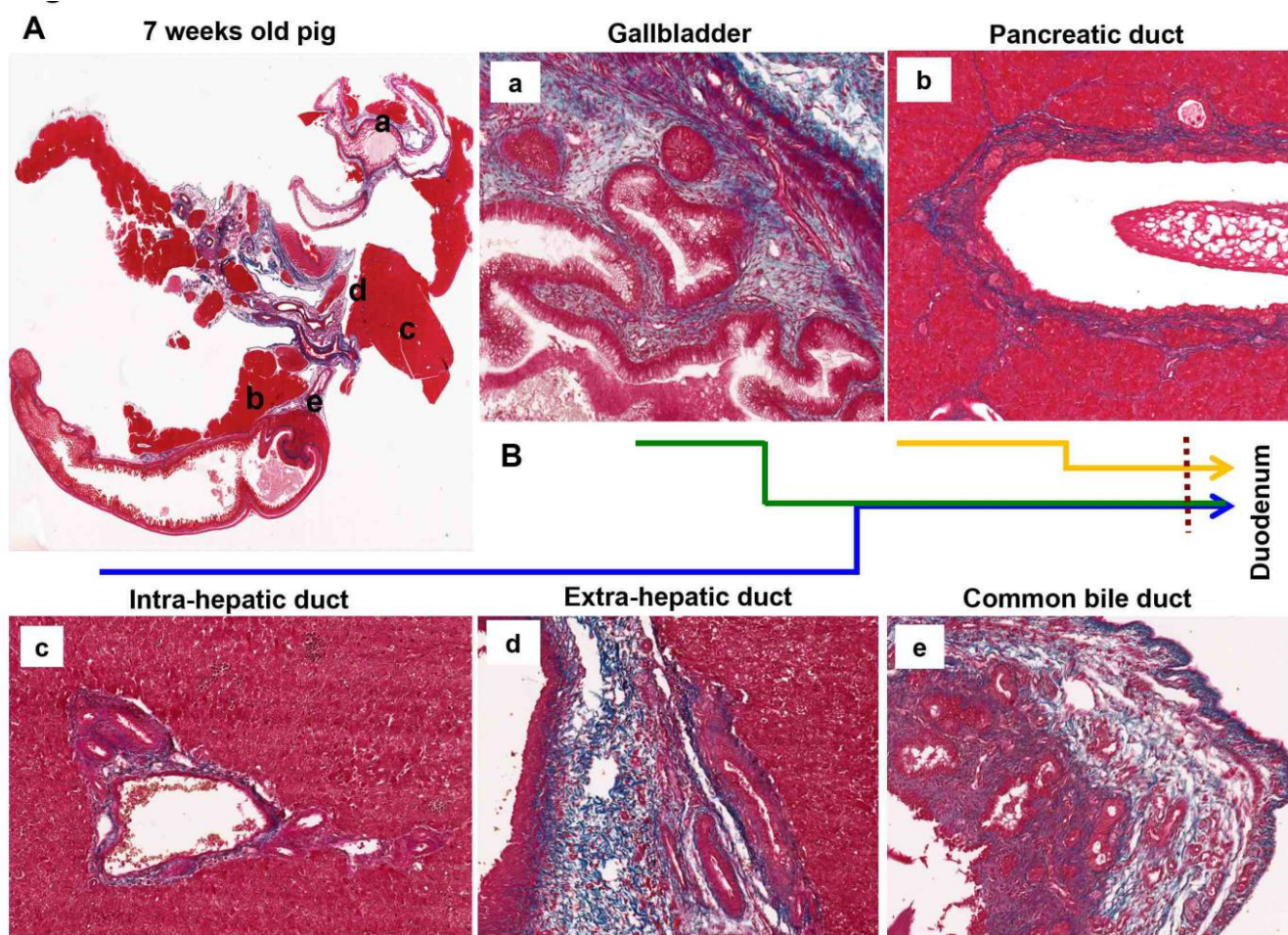


Figure S3. Histology of the biliary tree and its connections with the liver and pancreas

These indicate the ductal structures for 7-weeks-old piglets. A section of the pancreas and the duodenum is used to indicate the structure of the junction and connections between the biliary tree and the pancreas for weanling piglets.

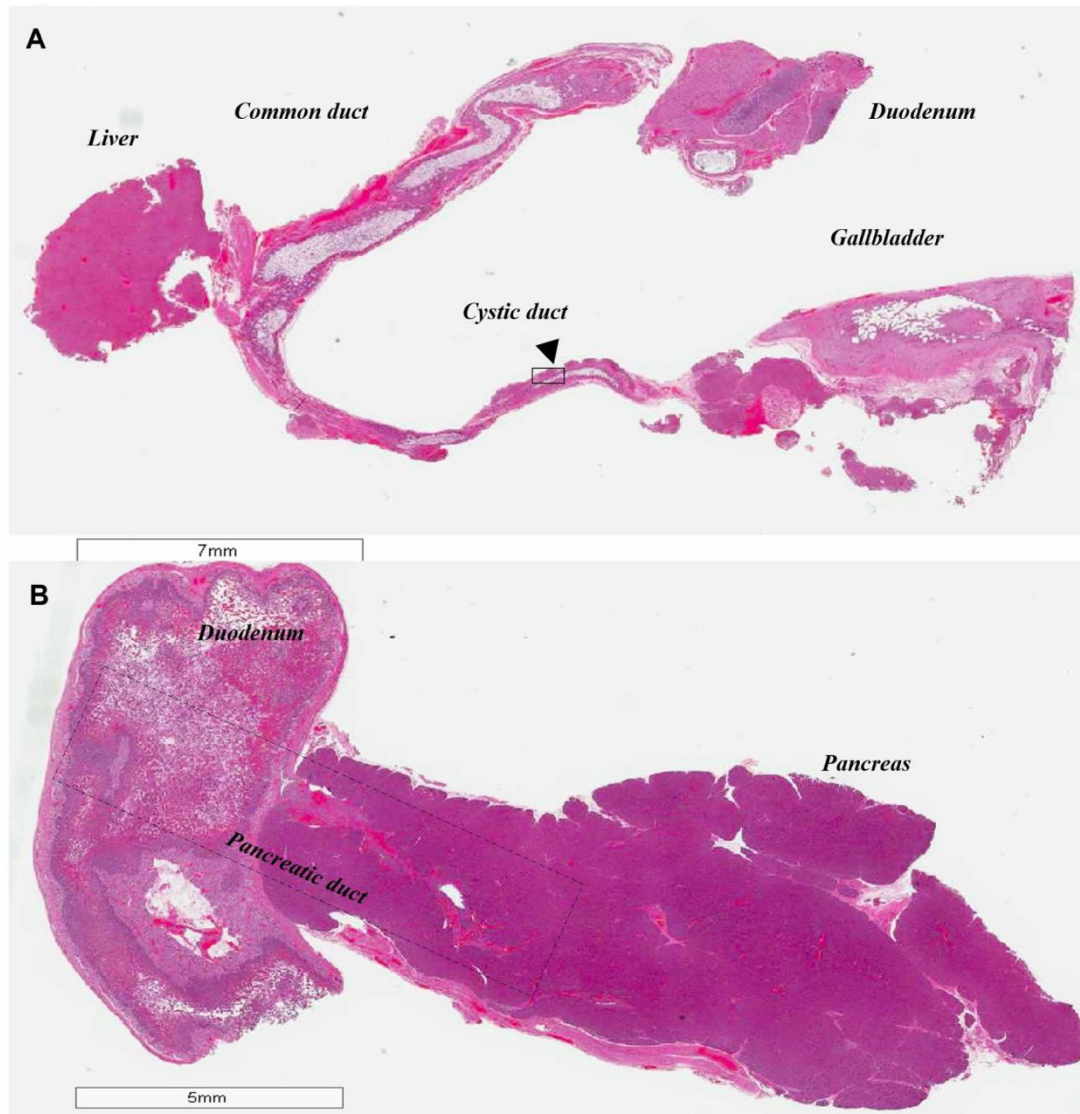


Figure S4. Comparison of niches to those in the human biliary tree.

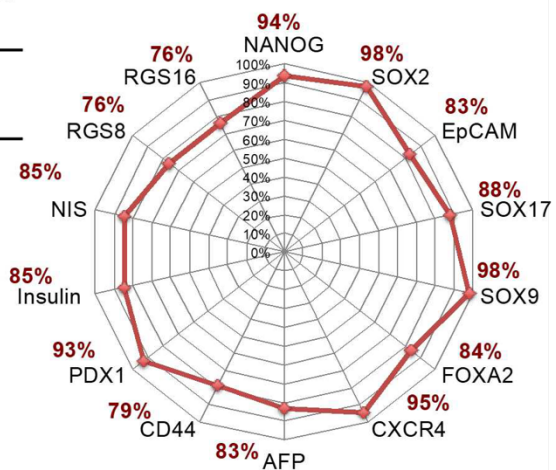
- A.** A table shows the current stem cell versus mature cell markers used for human endodermal stem cells studies.
- B.** A summary of the similarity of the stem cell and lineage cell markers between humans and pigs at both the gene level and protein level.
- C.** IHC staining for human biliary tree tissues of endodermal stem cell markers. Paraffin sections of a 10% neutral-buffered-formalin-fixed and paraffin-embedded biliary tree tissue prepared from a 9-year-old, male human were used as positive controls for candidate antibodies screening and titration. H&E staining showed the preparation of the tissue preserved with the peribiliary glands (PBGs). IHC results with antibodies for porcine tissue study showed the PBGs expressed pluripotency markers (SOX2) and multiple endodermal stem cell markers (EpCAM and SOX9). Scale bar=50 μ m.
- D.** The comparison of the expression patterns showed by RNA seq of three distinct subpopulations of cells: 1) porcine biliary tree stem/progenitors (BTSCs), 2) EpCAM+ hepatic stem cells and hepatoblasts, and 3) diploid, adult parenchymal cells. Missing are any from neonatal pancreas due to the inability to obtain isolated cell populations undamaged by pancreatic digestive enzymes.
- E.** Expression patterns of stem cell markers versus mature lineage markers in the three types of porcine cells.

Fig S4

A Table 1. Lineage markers for human biliary tree stem cells and hepatic-/pancreatic- mature cells

HUMAN CELL TYPES/LINEAGE	LINEAGE/STAGES SPECIFIC MARKERS
Pluripotent Stem Cells	SOX2 NANOG SALL4
Biliary Tree Stem Cells	EpCAM NIS FOXA2 CD44 SOX17 PDX1 SOX9 CXCR4
Hepatic lineage	HNF4A AFP ALBUMIN TF
Pancreatic Lineage	RGS8/16 INSULIN TRYPSIN
Cholangiocytes	CFTR Aquaporin-1 Aquaporin-9
Duodenal Cell Types	PDX1 CK8 CK9 MUC5

B



C

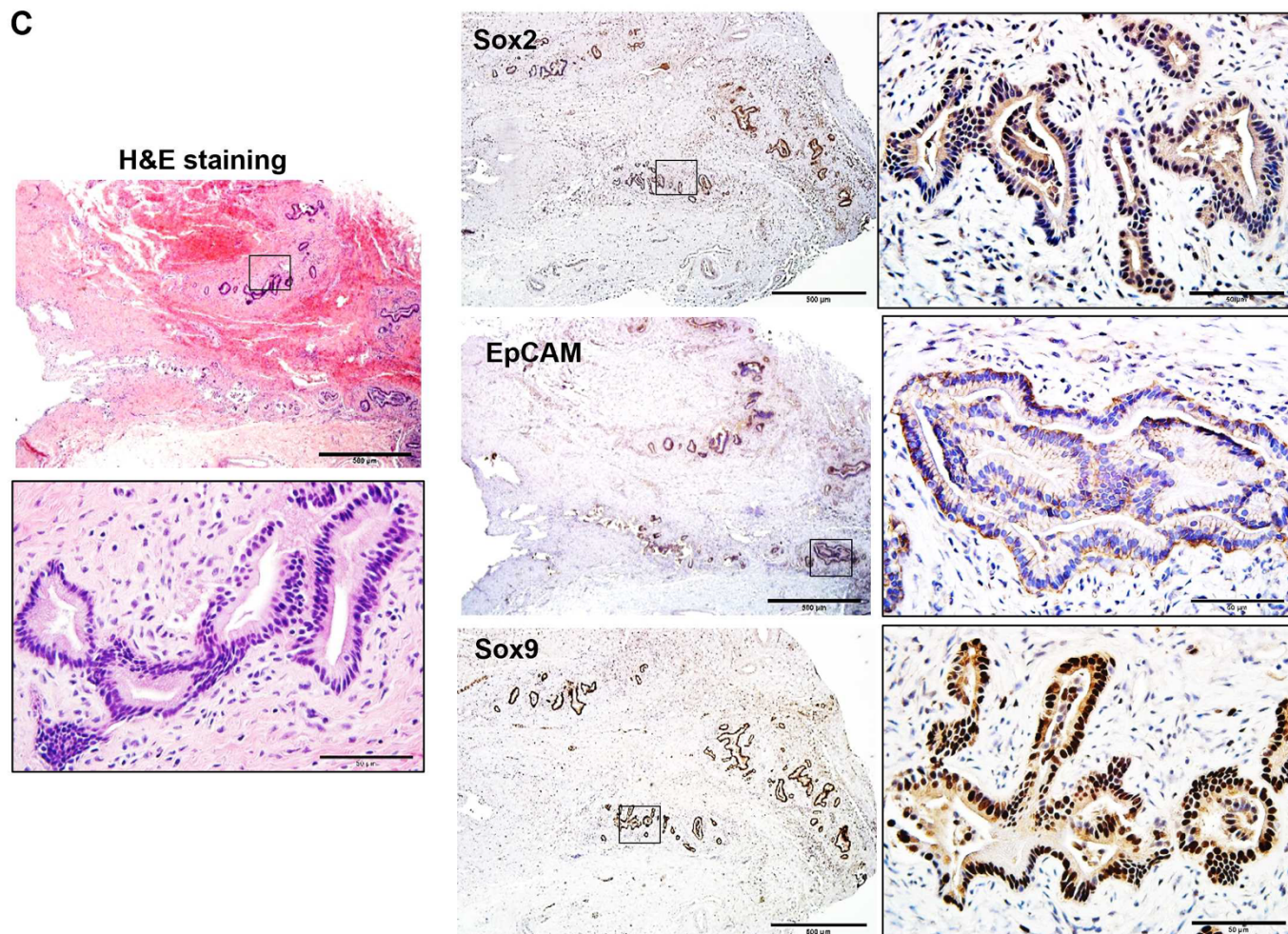


Fig S5. *Ex Vivo* Differentiation potential of pBTSC/ELSMC organoids

Wholly defined, serum-free culture conditions (**Table S3**) were used to establish BTSC organoids from isolated porcine biliary tree cells by strategies that remove mature stromal cells by panning procedures and so enriched for epithelial stem/progenitors (pBTSCs) and early lineage stage mesenchymal cells (ELSMCs), angioblasts and precursors to endothelia and to stellate cells¹. The organoids that resulted were subjected to distinct serum-free conditions for stemness versus for lineage restriction towards an hepatic versus pancreatic fates. The details of the different conditions are provided in **Table S3**. The porcine BTSC organoids were maintained either in serum-free Kubota's Medium supplemented with factors as described below:

A. For maintenance as organoids of stem/progenitors, the cells were maintained in Kubota's Medium and 0.1% hyaluronan, conditions devoid of growth factors and of hormones other than insulin and transferrin/Fe. Under these conditions the organoids remained as stem/progenitors of pBTSCs partnered with ELSMCs (angioblasts and their descendants, precursors to endothelia and to stellate cells) and expressing SOX2, SOX9, NCAM, SOX17 and PDX1. They did not express mature hepatic or pancreatic markers (e.g. P450s, insulin), nor early or intermediate lineage stage hepatic or pancreatic traits (e.g. alpha-fetoprotein, ICAM-1, MUC 6).

B. For lineage restriction towards hepatic fates, Kubota's Medium was supplemented with higher calcium levels (0.6 mM), copper (10^{-10} M), and the hormones and growth factors (glucocorticoids, HGF, EGF, Oncostatin M) and at concentrations as noted in **Table S3**. For rapid and optimal differentiation, the organoids were plated onto substrata of type IV collagen and laminin, mixed at a 1:1 ratio. Within 2-3 days, the organoids converted to monolayer cultures showing classic parenchymal morphology. Shown after one week under the differentiation conditions is their expression of lipids (oil red O staining). They also expressed other mature hepatic traits (not shown) that included albumin, P450s, and CFTR.

C. For lineage restriction towards pancreatic islet fates, Kubota's Medium was prepared without glucocorticoids and further supplemented with 0.6 mM calcium, 1% B27, 0.1 mM ascorbic acid, 0.25 μ M cyclopamine, 1 μ M retinoic acid, 20 ng/ml of FGF-7 for 4 days, and then changed to one supplemented with 50 ng/ml exendin-4 and 20 ng/ml of HGF for 6 more days of induction. Though the pancreatic islet fates were achieved without addition of matrix components, more rapid and complete differentiation occurred with supplementation of the medium with type IV collagen and laminin (ratio of 1:1). Within less than a week, the cells transitioned to islets and acquired expression of insulin, glucagon, and other islet hormones.

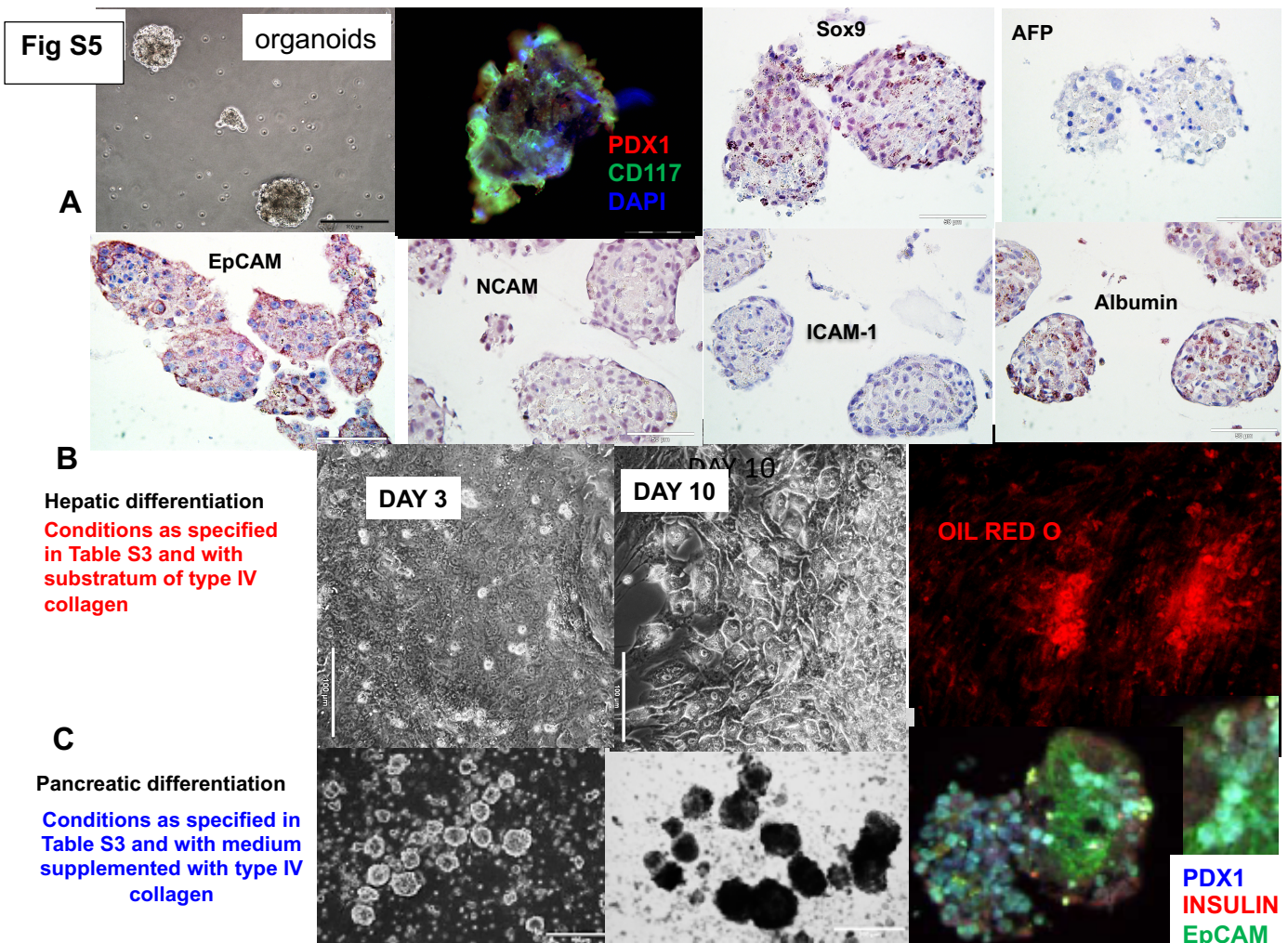


Figure S6. Summary of the anatomy and stem/progenitor niches in porcine biliary tree

A. Illustration of the lobule patterns for porcine liver, biliary tree and pancreas. Abbreviation for enlarged liver lobes: CL, caudate lobe; **RLL**, right lateral lobe; **RML**, right medial lobe; **LML**, left medial lobe; **LLL**, left lateral lobe ;**G**, gallbladder; Abbreviation for enlarged pancreas: **S**, stomach; **D**, duodenum; **H**, head of pancreas; **T**, tail of pancreas; **Pr**, Descending process; **UB**, upper bridge; **LB**, lower bridge.

B. Locations of the porcine hepato/pancreatic co-stem/progenitor cell niches

The highest numbers of the hepato/pancreatic co-stem/progenitor niches are in the submucosal duodenal glands, the Brunner's Glands, in peribiliary glands (PBGs) throughout the biliary tree and in the accessory duct to the dorsal pancreas. The major distinctions between pigs and humans are that humans, but not pigs, have the hepatopancreatic common duct, the location of the highest number of niches in the biliary tree In all other mammals other than pigs or oxes; pigs, but not humans, have stem/progenitor niches in the pancreatic duct glands (PDGs) within the pancreas proper and that are in regions near to the duodenum. Both human and pigs have committed bipotent and unipotent progenitors, but not the stem cells, elsewhere in the pancreas and in the gallbladder though the ones in human gallbladder retain some traits of stemness albeit muted relative to those in the PBGs.

Fig 6A

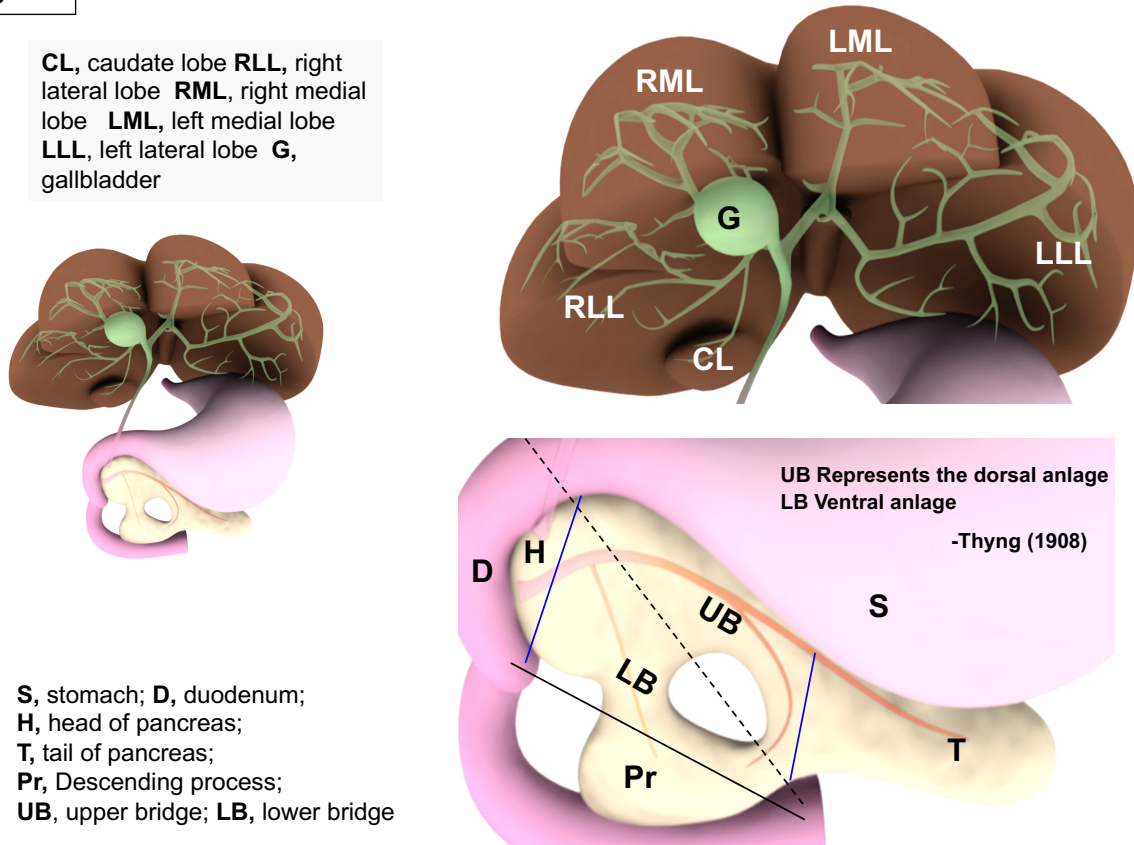
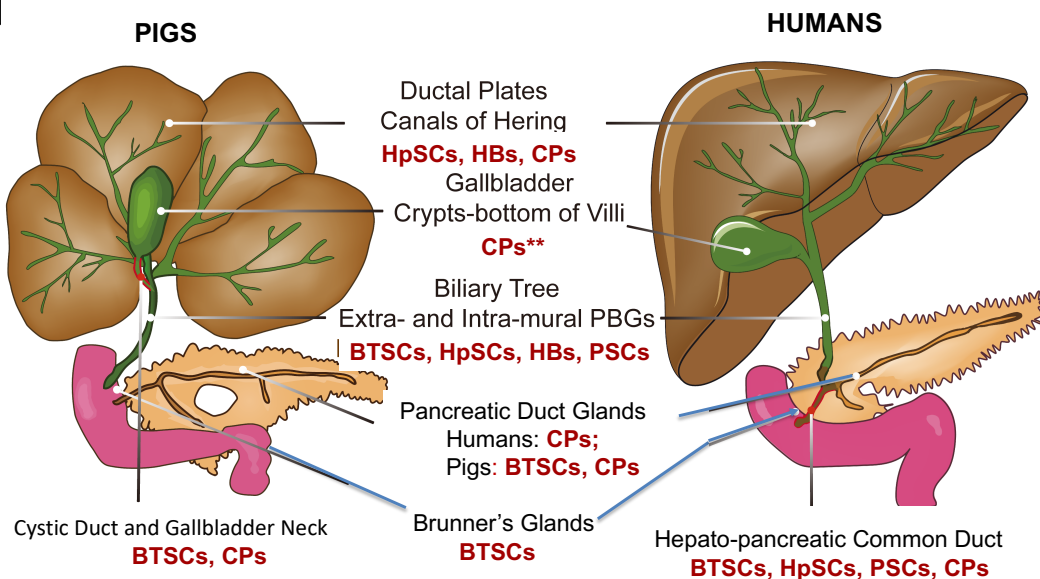


Fig 6B



Highest numbers of niches= Brunner's glands and PBGs near duodenum (both pigs and humans); **Humans**: PBGs in hepato-pancreatic common duct; **Pigs**: PBGs in cystic duct, gallbladder neck and PDGs in ventral and dorsal pancreas near duodenum. ** in humans, but not pigs, gallbladder niches contain mostly CPs but with some cells with muted and late-stage stem cell traits

METHODS

Companies providing equipment, reagents and/or supplies: Abcam, Cambridge, MA; **ACD Labs**, Toronto, CA; **Acris Antibodies**, Inc), San Diego, CA; **Advanced Bioscience Resources, Inc (ABR)**, Rockville, MD; **Agilent Technologies**, Santa Clara, CA; **Alpco Diagnostics**, Salem, NH; **Applied Biosystems**, Foster City, CA; **BD Pharmingen**, San Jose, CA; **Becton Dickinson**, Franklin Lakes, NJ; **Bethyl Laboratories**, Montgomery, TX; **BioAssay Systems**, Hayward, CA; **Cambridge Isotope Laboratories**, Tewksbury, MA; **Biotime, Inc**, Alameda, CA; **Carl Zeiss Microscopy**, Thornwood, NY; **Carolina Liquid Chemistries, Corp.**, Winston-Salem, NC; **Charles River Laboratories International, Inc**, Wilmington, MA; **Chenomx, Inc**, Alberta, Canada; **Cole-Parmer**, Court Vernon Hills, IL; **DiaPharma**, West Chester Township, OH; **Fisher Scientific**, Pittsburgh, PA; **Gatan, Inc**, Pleasanton, CA; **Illumina**, San Diego, CA; **Ingenuity**, Redwood City, CA; **Life Technologies Corp.**, Grand Island, NY; **Leica**, Washington, DC; **LifeSpan Biosciences, Inc**, Seattle, A; **Molecular Devices**, Sunnyvale, CA; **Olympus Scientific Solutions Americas Corp.**, Waltham, MA; **PhoenixSongs Biologicals (PSB)**, Branford, CT; **Polysciences, Inc**, Warrington, PA; **Qiagen**, Germantown, MD; **R&D Systems**, Minneapolis, MN; **RayBiotech**, Norcross, GA; **Roche Diagnostics**, Mannheim, Germany; **Santa Cruz Biotechnology, Inc**, Dallas, TX; **Sigma-Aldrich**, St. Louis, MO; **Sofregen**, Medford, MA; **Takara**, Otsu, Japan; **Tousimis Research Corp.**, Rockville, MD; **Triangle Research Labs (TRL)**, Research Triangle Park, NC; **Umetrics**, Umea, Sweden; **Varian Medical Systems, Inc**, Palo Alto, CA; **Vector Laboratories**, Burlingame, CA; **VWR Scientific**, Radnor, PA)

EXPERIMENTAL MODELS

ETHICAL CONDUCT OF RESEARCH

All animal experiments were performed in strict accordance with the approved Institutional Animal Care and Use Committees of North Carolina State University (NCSU) in Raleigh and at the University of North Carolina (UNC) in Chapel Hill and so have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. All procedures used in animal studies and those involving use of human tissues were approved by IACUC and IRB committees at both institutions: the UNC and at the College of Veterinary Medicine at NCSU. The IACUC approval numbers are 16-316.0 and 17-225.0. Studies for human cells in this project were evaluated by the IRB committee and were provided an IRB approval number of 97-1063.

PORCINE MODELS

Location of Facilities for Pigs. Animals used as hosts or as donors for cells were maintained in facilities at the College of Veterinary Medicine at North Carolina State University (NCSU, Raleigh, NC). Surgeries, necropsies, and the collection of all biological fluids and tissues were performed at these facilities.

Pig Hosts Used for the Grafts. The pigs used as recipients were a mixture of six different breeds: a six-way cross consisting of Yorkshires, Large Whites, Landraces (from the sows), Durocs, Spots, and Pietrans (from

the boars). This highly heterogeneous genetic background is desirable in that it parallels the heterogeneous genetic constitutions of human populations². The host animals were all females, approximately six weeks of age and ~15 kg.

GFP+ Transgenic Pig Donors were established carrying a H2B histone-eGFP transgene. The GFP+ donor animals were obtained by breeding a transgenic H2B-GFP boar with a wild type gilt by standard artificial insemination³. The model was developed via CRISPR-Cas9 mediated homology-directed repair (HDR) of IRES-pH2B-eGFP into the endogenous β -actin (ACTB) locus. Transgenic animals show ubiquitous expression of pH2B-eGFP in all tissues. Fusion of the GFP to H2B results in localization of the GFP marker to the nucleosome and allows clear nuclear visualization as well as the study of chromosome dynamics. The founder line has been analyzed extensively and ubiquitous and nuclear localized expression has been confirmed. In addition, breeding has demonstrated transmission of the H2B-GFP to the next generation. All animals were healthy, and multiple pregnancies have been established with progeny showing the expected Mendelian ratio for the transmission of the pH2B-eGFP. The male offspring were genotyped at birth, and those that were positive for the transgene were humanely euthanized for tissue collection and isolation of donor cells.

Genotyping of Animals. For each donor and recipient animal, the swine leucocyte antigen class I (SLA-I) and class II (SLA-II) loci have been PCR amplified using primers designed to amplify known alleles in these regions based on the PCR-sequence-specific-primer strategy.⁴ The system consists of 47 discriminatory SLA-I primer sets amplifying the SLA-1, SLA-2, and SLA-3 loci, and 47 discriminatory SLA-II primer sets amplifying the DRB1, DQB1, and DQA loci⁵. These primer sets have been developed to differentiate alleles by groups that share similar sequence motifs and have been shown easily and unambiguously to detect known SLA-I and SLA-II alleles. When used together, these primer sets effectively provided a haplotype for each animal that was tested, thus providing an assay to confirm easily a matched or mismatched haplotype in donor and recipient animals.

Surgeries on Pigs

Anesthesia was induced by administering a combination of ketamine/xylazine (2-3 mg/kg weight each) injected IV or 20 mg/kg ketamine plus 2gm/kg xylazine IM and was maintained by isoflurane in oxygen administered via a closed-circuit gas anesthetic unit.

Grafts on the Liver and general surgical procedures – The pigs were positioned in dorsal recumbency, and the ventral abdomen was clipped from xyphoid to pubis. The skin was aseptically prepared with alternating iodinated scrub and alcohol solutions. After entry into the surgery suite, preparation of the skin was repeated using sterile technique, and the area was covered with a topical iodine solution before application of sterile surgical drapes. The surgeons used appropriate aseptic technique. A mid-ventral incision was made through the skin, through subcutaneous tissues and *linea alba*, starting at the xiphoid process and extending caudally 8-12 cm. The left hepatic division was exposed, and a 3 x 4.5 cm patch graft was applied to the ventral surface of the liver and containing 1X HA (~60 Pa) with embedded organoids placed onto the

backing containing 10X HA (~760 Pa), and the patch was placed in direct contact onto the surface of the liver capsule. The patch graft was sutured to the liver using 4-6 simple, interrupted sutures of 4-0 polypropylene. The exposed surface of the graft was then treated with 2 mls of 2X HA hydrogel (~200-300 Pa), a level of rigidity that was fluid enough to permit it to be painted or coated onto the outside of the graft; it served further to minimize adhesions from neighboring tissues. Following placement of the surgical graft, the *linea alba* was closed with a simple continuous suture using 0-PDS. The *linea* was blocked with 2 mg/kg 0.5% bupivacaine, IM. The subcutaneous tissues and skin were closed with continuous 2-0 PDS and 3-0 Monocryl sutures, respectively. Tissue adhesive was placed on the skin surface.

Grafts on the Pancreas. A mid-ventral incision was made, followed by blunting the dissection to the linea alba and use of Balfour retractors. For disclosure of the pancreas, stay sutures were placed in the descending duodenum. The graft was placed onto the head of the pancreas and duodenum with the hydrogel containing the organoids in direct contact with the pancreas. We used sutures at the four corners (4-0 Prolene); the first two corner sutures were placed in the descending duodenum; the other 2 were placed in the mesentery and avoiding the pancreatic parenchyma. The serosal surface of the graft was covered with 2X HA hydrogel to minimize adhesions. The abdomen was closed with continuous absorbable (PDS) sutures in the linea, subcutaneous and subcuticular layers. Tissue adhesive was used on the skin to avoid skin sutures.

Immunosuppression. Transplants from transgenic pigs to the wild type recipients were allogeneic and so required immunosuppression. Immune-suppression protocols used were ones established by others.^{6, 7} All pigs received oral dosages of the immunosuppressive drugs Tacrolimus (0.5 mg/kg) and Mycophenolate (500 mg) twice daily, beginning 24 hours prior to surgery. The drugs were given continuously for the entire experimental period. These could be given to the animals easily if mixed with their favorite foods.

Isolation of Normal Tissues. Newborn piglets weighed ~10 lbs; older pigs, 12 weeks of age, were ~50-60 lbs. Pigs were sacrificed by penetrating captive bolt euthanasia followed by jugular exsanguination. The method meets the recommended guidelines of the American Veterinary Medical Association for euthanasia in pigs⁸. A listing of the donors from piglet or adult pigs used is given in **Table S4**.

MEDIA AND SOLUTIONS

All media were sterile filtered (0.22 μ m filter) and kept in the dark at 4°C before use. Basal medium and fetal bovine serum (FBS) were purchased from GIBCO/Invitrogen. All growth factors were purchased from R&D Systems. All other reagents, except those noted, were obtained from Sigma.

Cell Wash. 500 mls of basal medium (RPMI 1640; Gibco # 11875-093) was supplemented with 0.5 grams of serum albumin (Sigma, # A8896-5G, fatty-acid-free), 10^{-9} M selenium, and 5 mls of antibiotics (Gibco #35240-062, AAS). It was used for washing tissues and cells during processing.

Collagenase buffer. Consists of 100 mls of cell wash supplemented with collagenase (Sigma # C5138) with a final concentration of 600 U/ml (R1451 25mg) for biliary tree (ducts) tissue and 300 U/ml (12.5 mg) for organs.

Kubota's Medium, a wholly defined medium designed originally for hepatoblasts⁹, and then found successful for maintenance of any endodermal stem/progenitors, was used to prepare cell suspensions, organoids and hyaluronan hydrogels. It consists of any basal medium (here being RPMI 1640) with no copper, low calcium (0.3 mM), 1 nM selenium, 0.1% serum albumin (purified, fatty-acid -free; fraction V), 4.5 mM nicotinamide, 0.1 nM zinc sulfate heptahydrate, 5 µg/ml transferrin/Fe, 5 µg/ml insulin, 10 µg/ml high density lipoprotein, and a mixture of purified free fatty acids presented complexed with fatty acid-free, highly purified albumin. It is available commercially from *PhoenixSongs Biologicals* (Branford, CT).

Hyaluronans (HAs). Soluble, long chain forms of HA (Sigma Catalog # 52747) were used in stabilization of organoid cultures¹⁰ and in cryopreservation of cell suspensions^{11, 12}. Those used to make the hydrogels, thiol-modified HAs, were obtained from Glycosan Biosciences, a subsidiary of Lineage Cell Therapeutics (Alameda, CA). The components for these thiol-modified HAs were made by a proprietary bacterial-fermentation process using bacillus subtilis as the host in an ISO 9001:2000 process (www.biopolymer.novozymes.com/). The components were produced by Novozymes under the trade name, HyaCare®, and are 100% free of animal-derived materials and residual organic solvent. No animal-derived ingredients are used in the production, and there are low protein levels and no endotoxins. The production follows the standards set by the European Pharmacopoeia. The HA hydrogels were prepared using Glycosil (HyStem® HAs, ESI BIO-CG313), the thiol-modified HAs, that can be triggered to form disulfide bridges in the presence of oxygen, or by forming thio-ether linkages using polyethylene glycol diacrylate (PEGDA). Glycosil® is reconstituted as a 1% solution of thiolated HA in 1% phosphate buffered saline (PBS) using degassed water, or, in our case, in serum-free Kubota's Medium. Upon reconstitution, it remains liquid for several hours but can undergo some gelation if exposed to oxygen. More precise gelation occurs with no temperature or pH changes if Glycosil is treated with a cross-linker such as PEGDA causing gelation to occur within a few minutes^{13, 14, 15}.

The level of cross-linking is the primary contributor to the level of stiffness (viscoelasticity) and can be controlled by adjusting the ratio of the thiol-modified hyaluronans to PEGDA. In prior studies, hepatic stem cell populations were tested in HA hydrogels of varying level of rigidity and were found to remain as stem cells, both antigenically and functionally, only if the level of rigidity was less than ~100 Pa¹⁶. We used this finding to design grafts with a soft layer (~100 Pa) in which to embed organoids of BTSCs and with more rigid layers (~700 Pa) of hyaluronan hydrogels in the backing to form a barrier to migration in directions other than the target tissue as well as to minimize adheions¹⁷.

Macro-scale rheological properties of hydrogels were determined using a stress-controlled cone-and-plate rheometer (TA Instruments, AR-G2, 40 mm cone diameter, 1° angle). Gels actively polymerized on the rheometer while oscillating at 1 rad/s frequency and 0.6 Pa stress amplitude with the modulus monitored continuously to query for sufficient completion of the cross-linking reactions. Once equilibrated, the hydrogels were subjected to an oscillatory frequency sweep (stress amplitude: 0.6 Pa, frequency range: 0.01 - 100 Hz).

Preparation of Cells. Extrahepatic biliary tree tissue (gall bladder, common duct, hepatic ducts) was obtained from pigs [or from human tissue]. For the porcine tissues used to generate organoids, tissues were pounded with a sterilized, stainless-steel mallet to eliminate the parenchymal cells, carefully keeping the linkage of the intra-hepatic and extrahepatic bile ducts. The biliary tree was then washed with the “cell wash” buffer comprised of a sterile, serum-free basal medium supplemented with antibiotics, 0.1% serum albumin, and 1 nM selenium (10^{-9} M). It was then mechanically dissociated with crossed scalpels, and the aggregates enzymatically dispersed into a cell suspension in RPMI-1640 supplemented with 0.1% bovine serum albumin (BSA) [or for human tissues, human serum albumin, HAS], 1 nM selenium, 300 U/ml type IV collagenase, 0.3 mg/ml deoxyribonuclease (DNase) and antibiotics. Digestion was done at 32°C with frequent agitation for 30-60 minutes. Most tissues required two rounds of digestions followed by centrifugation at 1100 rpm at 4°C. Cell pellets were combined and re-suspended in cell wash. Cell suspensions were centrifuged at 30G for 5 minutes at 4°C to remove red blood cells. The cell pellets were again re-suspended in cell wash and filtered through a 40 µm nylon cell strainer (Becton Dickinson Falcon #352340) with fresh cell wash. Cell numbers were determined, and viability was assessed using Trypan Blue. Cell viability above 90-95% was observed routinely.

Formation of Organoids Cell suspensions were added to Multiwell Flat Bottom Cell Culture Plates (Corning #353043) in serum-free Kubota's Medium and incubated for ~an hour at 37°C to facilitate attachment of mature mesenchymal cells, occurring typically within 10-15 minutes, even though the medium was serum-free. The cells remaining in suspension were transferred to another dish and again incubated for up to an hour. Repeats of this resulted in depletion of a significant fraction of the mature mesenchymal cells. Afterwards, the remaining floating cells were seeded at $\sim 2 \times 10^5$ cells per wells in serum-free Kubota's Medium in Corning's ultralow attachment dishes (Corning #3471) and were incubated overnight at 37°C in a CO₂ incubator. Organoids comprised of the biliary tree stem cells (BTSCs) partnered with ELMSCs (early lineage stage mesenchymal cells) formed overnight. These organoid cultures survived for weeks in Kubota's Medium, especially if the medium was supplemented (0.1%) with soluble forms of hyaluronans (Sigma). The cells could also be cryopreserved as described below. From each gram of pig biliary tree tissue, we obtained $\sim 1.5 \times 10^7$ cells. We used $\sim 3-6 \times 10^5$ cells per well of 6-well, ultra-low attachment plates and incubated in serum-free Kubota's Medium. The cells produced, on average, 6000 to 20,000 small organoids ($\sim 50-100$ cells/organoid/well). For the grafts, we used at least 100,000 organoids ($>10^7$ cells). Depending on the size of the backing, we were able to increase the number of organoids in the grafts up to 10^8 organoids ($\sim 10^9$ cells) embedded in ~ 1 ml of the soft hyaluronan hydrogel on a 3 cm X 4.5 cm backing.

Cryopreservation of Stem/Progenitors. The most successful approach was cryopreservation of isolated cells and that had been subjected to the panning procedures to minimize the numbers of mature mesenchymal cells. One can cryopreserve the stem/progenitor populations in Cryostor10 (Bioliife, Seattle, Washington). The viability of cells was improved further with supplementation with 0.1% HAs (Sigma #52747)^{12, 18}. The highest viability of the organoids prepared from freshly thawed, cryopreserved cell suspension; lower viability was observed with cryopreserved, fully formed organoids. Cryopreservation was done using CryoMed™ Controlled-Rate Freezers. The viability on thawing was greater than 90% for those cryopreserved as a cell suspension and then, upon thawing, used to form organoids^{11, 12}.

Patch Grafts. Further details of the logic, strategies and methods for patch grafting of organoids into solid organs are given in our prior publication¹⁷. Grafts were formed using a backing, Contour Seri-silk (Sofregen, Medford, MA), onto which were placed the stem/progenitor organoids embedded in soft hyaluronan hydrogels (~100 Pa). These were readily prepared ahead of time and maintained in a culture dish in an incubator overnight. The lack of success with attempts to cryopreserve organoids within soft hydrogels meant that embedding the organoids in the soft hydrogel had to be done just prior to surgery.

Necropsy Procedures. All animals were humanely euthanized at the designated time point by sedation with Ketamine/Xylazine, and isoflurane anesthesia, followed by an intravenous injection of a lethal dose of sodium pentobarbital. Upon confirmation of death, the carcass was carefully dissected, and the target organs were removed, and placed in chilled Kubota's Medium for transportation to the lab. In addition to the liver, the lungs, heart, kidney, and spleen were collected and fixed in 10% neutral formalin.

Histology. Portions of freshly dissected porcine tissues or the grafts on liver or pancreas were fixed with 4% paraformaldehyde (PFA) and then paraffin embedded. Five-micron sections were cut and stained with hematoxylin-eosin for routine histological analyses. The tissues obtained from neonatal pigs were prepared (liver, pancreas, biliary tree and duodenum) in large paraffin blocks to facilitate analyses of the connections throughout the biliary tree and pancreatic duct system.

Immunohistochemistry (IHC). For immunofluorescent staining, 5 µm frozen sections or cultured cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, rinsed with PBS, blocking with 10% goat serum in PBS for 2 hours, and rinsed. Fixed cells were incubated with primary antibodies at 4°C for 14 hours, washed, incubated for 1 hour with labeled isotype-specific secondary antibodies, washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei and viewed using Leica DMIRB inverted microscope (Leica, Houston, TX) or a Zeiss ApoTome Axiovert 200M (Carl Zeiss Inc, Thornwood, NY).

For IHC, the tissues were fixed in 4% PFA overnight and stored in 70% ethanol. They were embedded in paraffin and cut into 5 µm sections. After deparaffinization, antigen retrieval was performed with sodium citrate buffer (pH 6.0) or ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) in a steamer for 20 min. Endogenous

peroxidases were blocked by incubation for 15 min in 3% H₂O₂. Sections were incubated for 30 min at room temperature with ImmPRESS peroxidase staining kits and 3,3'-diaminobenzidine substrates (Vector Laboratories). Sections were counterstained with hematoxylin. Antibodies used are listed in **Table S1**.

RNA-sequencing and Gene Expression Analysis. RNA was purified using Qiagen RNeasy Kit from porcine adult liver, pancreas, gallbladder, and biliary tree tissue and from isolated cell suspensions of hepatocytes (AHEP), hepatic stem cells (HpSCs), biliary tree stem cells (BTSCs), each from three different donors. RNA integrity (RIN) analysis was performed using an Agilent 2000 Bioanalyzer. The cDNA libraries were generated using the Illumina TruSeq Stranded mRNA preparation kit and sequenced on the Illumina HiSeq 2500 platform. Two samples were sequenced per lane, occupying a total of 8 lanes for all the samples (one flow cell). Quality control analysis was completed using FastQC, mapping of sequence reads to the human genome (hg19) was performed with MapSplice2 using default parameters, transcript quantification was carried out by RSEM analysis, and DESeq was used to normalize gene expression and identify differentially expressed genes. MapSplice2 was also used to detect candidate fusion transcripts. Fusion calls were based on the depth and complexity of reads spanning candidate fusion junctions. Gene expression profiles were compared using Pearson's correlation analysis and hierarchical clustering was performed in R. Pathway enrichment analysis was performed with the Ingenuity Pathway Analysis (IPA) software.

Quantitative Reverse Transcription and Polymerase Chain Reaction. Total RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, CA). First strand cDNA synthesized using the Primescript 1st strand cDNA synthesis kit (Takara, Otsu, Japan) was used as a template for PCR amplification. Quantitative analyses of mRNA levels were performed using Faststart Universal Probe Master (Roche Diagnostics, Mannheim, Germany) with ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers were designed with the Universal Probe Library Assay Design Center (Roche Applied Science). Primer sequences are listed in **Table S2**. The primers were annealed at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C (15 s) and 60°C (1 min). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used generally as a standard.

Statistical Analysis Statistically significant differences between samples are calculated by using Student's 2-tailed t test and results are presented as the mean $\pm$ SD. P values of less than 0.05 were considered statistically significant

ONLINE SUPPLEMENT TABLES

Table S1. Key Resources: Antibodies

Antibody	Species Type		Manufacturer	Clone	Catalog#	Dilution for testing
NANOG	Rb	IgG	Peprotech	D73G4	500-P236	1/200(IHC-P, ICC-IF),
SOX2	Rb	IgG	Cell Signaling	D6D9	#3579	1/50(IHC)-400(ICC-IF)
hOCT4	Rb	IgG	Cell Signaling		#2750	1/200(IHC), 1/400(ICC-IF)
EpCAM (C-terminal)	Rb	IgG	Abcam Origene	E144	ab32392	1/200 1/100(ICC-IF)
SOX17	Ms	IgG1	Abcam	3B10	ab84990	1/100(IHC-P) 1/20(ICC-IF)
SOX9	Rb	IgG	Chemicon		AB5535	1/500-2000, IHC-P 1/1000
Ki67	Rb	IgG1	Abcam	SP6	ab16667	1/100(ICC-IF)- 100(IHC)
FOXA2	Rb	IgG	Abcam		ab40874	1/500
CXCR4	Rb	IgG	Abcam		ab2074	1/100(IHC-P)-500(ICC-IF)
RGS8	Rb	IgG	SantaCruz		sc-134552	1/50(IHC-ICC-IF) 1/100(IHC-P)
RGS16	Rb	IgG	Applied Biological Materials		Y054685	1/50(IHC-ICC-IF) 1/75(IHC-P)
AFP	Ms	IgG2a	SIGMA	C3	A-8452	1/100(ICC-IF) 1/500(IHC-P)
ASBT (SLC10A2)	Rb	IgG	Gift-Dawson P	SLC10 AC	7789	1/1000 1/400(IF)
Fibronectin	Rb	IgG	Abcam		ab23751	1/5000(ICC-F, RIA)
Connexin32	Ms	IgG1K	life technologies	CX-2C2	138200	1/250-500(IHC), 1/50-100(ICC-IF)
NIS (sodium iodide symporter)	Ms	IgG1	Abcam	SPM186	ab17795	1/50(IHC-P-IF)
CD44	Ms	IgG2a	Abcam	F10-44-2	ab6124	1/200(IHC-P) 1/100(ICC-IF)
PDX1	Gt	IgG	R&D		AF2419	1/50
Insulin	Rb	IgG	NOVUS		NBP1-50246	1/100
LGR5	Rb	IgG	Sigma		HPA012530	1/500IHC 1/350 ICC
Pig Albumin	Rb	IgG	Abcam		ab79960	IF;1/100
Insulin	Gp		Abcam		ab195956	IHC-P, IHC-Fr

Table S2. Key Resources: Primers (qPCR)

Category	Name	Accession Number	Sequence (5' to 3')	Length (bp)	Tm (°C)
<i>Housekeeping gene</i>	GAPDH	NM_001206359.1	Sense: ATCCTGGGCTACACTGAGGAC	473	60 °C
			Anti-sense: AAGTGGTCGTTGAGGGCAATG		
<i>Pluripotency genes</i>	Nanog	NM_001129971.1	Sense: TTCCTTCCTCCATGGATCTG	214	62 °C
			Anti-sense: ATCTGCTGGAGGCTGAGGTA		
	Sox2	NM_001123197	Sense: GCCCTGCAGTACAACTCCAT	216	60 °C
			Anti-sense: GCTGATCATGTCCCGTAGGT		
	Oct4	JN633978.1	Sense: CGAAGCTGGACAAGGAGAAG	176	60 °C
			Anti-sense: GCTGAACACCTTCCCAAAGA		
<i>Endodermal stem cell genes</i>	EpCAM	NM_214419.1	Sense: ACCAGAGAATGCTATCCAGAA C	314	53 °C
			Anti-sense: CTCACTCGCTCCAAACAGG		
	Lgr5	NM_001315762.1	Sense: CCTTGCCCTGAACAAAATA	110	60 °C
			Anti-sense: ATTTCTTTCCCAGGGAGTGG		
	Sox9	NM_213843.2	Sense: CGGTTCGAGCAAGAATAAGC	229	60 °C
			Anti-sense: GTAATCCGGGTGGTCCTTCT		

	Bmi1	NM_001285971.1	Sense: TCATTGATGCCACAACCATT	189	60 °C
			Anti-sense: TGAAAAGCCCCGGAACCTAAT		
GI tract-related genes	Muc2	NC_010444.3	Sense: GGCTGCTCATTGAGAGGAGT	249	60 °C
			Anti-sense: ATGTTCCCGAACTCCAAGG		
	CDX2	NC_010453.4	Sense: AGAACCCCCAGGTCTCTGTCT T	115	56°C
			Anti-sense: CAGTCCGAAACACTCCCTCAC A		
Hepatic parenchymal cells-related genes	AFP	NM_214317.1	Sense: CGCGTTTCTGGTTGCTTACAC	609	60
			Anti-sense: ACTTCTTGCTCTTGGCCTTGG		
	Albumin	AY663543.1	Sense: AGTCTGCCAAGCTGCTGATA	115	56
			Anti-sense: AGCCTTGGGAAATCTCTGGC		
			Anti-sense: TCTCGCTCTCCAGAATGTGC		

Table S3. Serum-Free, wholly defined conditions used for organoids			
	Endodermal Stem/progenitor Organoids*	Lineage restriction to hepatocytes and cholangiocytes	Lineage restriction to pancreatic islets
Basal Medium	Any rich basal medium (e.g. RPMI 1640 or DME/F12) plus nicotinamide		
Trace elements	---	copper (10 ^{-10th} M)	
		selenium (10 ^{-9th} M), zinc (10 ^{-12th} M)	
Calcium	0.3 mM	0.6 mM	
Insulin	5 µg/ml		
Transferrin/Fe	5 µg/ml		
Growth factors	None	T3 (10 ⁻⁹ M), bFGF (20 ng/ml), HGF (20 ng/ml),	
		Oncostatin M (10 ng/ml) Galactose (2 g/L), EGF (10 ng/ml); VEGF (20 ng/ml)	Cyclopamine (0.25 µM), retinoic acid (1 µM) FGF-7 (20 ng/ml)
Other Hormones	None	Glucocorticoids (10 ^{-8th} M) Glucagon (7 µg/ml)	Exendin (50 ng/ml)
Lipids**	10 µg/ml high density lipoprotein plus a mixture of purified free fatty acids bound to purified albumin		
Matrix components (***elicited rapid differentiation)	0.1% hyaluronans	A substratum of type IV collagen and laminin (ratio 1:1)	Supplementation of medium with type IV collagen and laminin (ratio 1:1)
Oxygen levels	2%	5%	
*The organoids were comprised of biliary tree stem cells partnered with angioblasts and their immediate descendants, precursors to endothelia and to stellate cells. Under the conditions of serum-free Kubota’s Medium ⁹ supplemented with 0.1% soluble hyaluronans, the cells in the organoids remained stably as stem/progenitors for weeks.			
**Details of the preparation of Kubota’s Medium are given in prior publications ^{19, 20, 21, 22, 23,} differentiation occurred with the serum-free, hormonally defined medium alone, but it happened more quickly (within 2-3 days) and more completely if the purified matrix components were used.			

Table S4. Pigs used in the study			
	date	age	Use of the Tissue and the Fixation buffer
PL-01	5/12/15	9 weeks	Anatomical study: pancreas-liver-gallbladder-cystic duct-bile duct-duodenum
PL-02	5/20/15	1 week	Anatomical study: pancreas-liver-gallbladder-cystic duct-bile duct-duodenum
			4% PFA -70% ethanol before processing
PL-03	6/15/15	1 week	Anatomical study: pancreas-liver-gallbladder-cystic duct-bile duct-duodenum
			4% PFA -70% ethanol before processing
PL-04	6/15/15	7 weeks	Anatomical study: pancreas-liver-gallbladder-cystic duct-bile duct-duodenum
			4%PFA -70% ethanol before processing
PL-05	6/26/15	4 weeks	Samples were used for cell isolation
PL-06	7/8/15	3 weeks	Samples were used for cell isolation
PL-07	7/8/15	3 weeks	Samples were used for cell isolation
PL-08	7/30/15	1 week	Samples were used for Indian Blue Ink study
PL-09	8/26/15	12 weeks	Samples were used for cell isolation
PL-10	9/4/15	12 weeks	Anatomical study: pancreas-liver-gallbladder-cystic duct-bile duct-duodenum
			4% PFA 1 week-70% ethanol before processing
PL-11	11/21/15	1 week	Samples were used for cell isolation and cryopreservation
PL-12	2/19/16	9 weeks	Anatomical study and samples were used for cell isolation
PL-13	2/24/16	1 week	Samples were used for cell isolation
PL-14	3/21/16	1 week	Samples were used for cell isolation
PL-15	4/12/16	1 week	Samples were used for cell isolation and cryopreservation
PL-16	4/12/16	1 week	Samples were used for cell isolation and cryopreservation
All animals, the wild type breed, are a mixture of six different breeds: a six-way cross consisting of Yorkshires, Large Whites, Landraces (from the sows), Durocs, Spots, and Pietrans (from the boars). This highly heterogeneous genetic background is desirable in that it parallels the heterogeneous genetic constitutions of human populations. PL-09 was female; all the rest were males.			

Table S5. Information on Pigs and Cell Numbers

Storage#	Pigs	Cell types**	Cell number	Notes
S-2	H2B-GFP-Piglet #1 (61105) (1-day-postnatal)	Biliary tree	3,000,000	BTSCs
S-7	H2B-GFP-Piglet #2 (61102) (1-day-postnatal)	Biliary tree	1,000,000	BTSCs
S-11	H2B-GFP-Piglet #3 (61104) (2-day-postnatal)	Biliary tree	1,000,000	BTSCs
S-15	Wild Type-Piglet #1 (1-day-postnatal)	Biliary Tree	570,000	BTSCs
S-20	Wild Type-Piglet #2 (1-day-postnatal)	Biliary Tree	570,000	BTSCs
** gallbladder and extrahepatic biliary tree				

Table S6. Quality-control Information for RNA Samples for the sequencing Assays

Storage#	Cell types**	RNA Concentration (ng/uL)	260/280	260/230	RIN
S-2	Biliary tree	102.8	1.95	1.83	5.1
S-7	Biliary Tree	80.3	1.93	1.76	3.8
S-11	Biliary Tree	136.8	1.98	2.09	3.9
S-15	Biliary Tree	340.4	1.97	2.46	n/a
S-20	Biliary Tree	350.7	1.98	2.35	6.4
** Biliary tree cells prepared from gallbladder and extrahepatic biliary tree					

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