

1 **Reconstruction of the murine extrahepatic biliary tree using primary**
2 **human extrahepatic cholangiocyte organoids.**

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7

1 **Abstract**

2 Treatment of common bile duct disorders such as biliary atresia or ischaemic
3 strictures is limited to liver transplantation or hepatojejunostomy due to the
4 lack of suitable tissue for surgical reconstruction. Here, we report a novel
5 method for the isolation and propagation of human cholangiocytes from the
6 extrahepatic biliary tree and we explore the potential of bioengineered biliary
7 tissue consisting of these extrahepatic cholangiocyte organoids (ECOs) and
8 biodegradable scaffolds for transplantation and biliary reconstruction in vivo.
9 ECOs closely correlate with primary cholangiocytes in terms of transcriptomic
10 profile and functional properties (ALP, GGT). Following transplantation in
11 immunocompromised mice ECOs self-organize into tubular structures
12 expressing biliary markers (CK7). When seeded on biodegradable scaffolds,
13 ECOs form tissue-like structures retaining biliary marker expression (CK7)
14 and function (ALP, GGT). This bioengineered tissue can reconstruct the wall
15 of the biliary tree (gallbladder) and rescue and extrahepatic biliary injury
16 mouse model following transplantation. Furthermore, it can be fashioned into
17 bioengineered ducts and replace the native common bile duct of
18 immunocompromised mice, with no evidence of cholestasis or lumen
19 occlusion up to one month after reconstruction. In conclusion, ECOs can
20 successfully reconstruct the biliary tree following transplantation, providing
21 proof-of-principle for organ regeneration using human primary cells expanded
22 in vitro.

23

24

1 Disorders of the extrahepatic bile ducts carry considerable morbidity and
2 mortality. Indeed, 70% of pediatric liver transplantations are performed to treat
3 biliary atresia¹, Primary Sclerosing Cholangitis (PSC) alone accounts for 5%
4 of US liver transplantations² and biliary complications are the leading cause of
5 graft failure following deceased liver transplantation^{3,4}. Treatment options
6 remain limited^{5,6} due to the lack of healthy tissue that can be used to
7 reconstruct and replace diseased bile ducts. In vitro expansion of native
8 cholangiocytes could address this challenge and provide cells suitable for
9 tissue engineering applications such as biliary reconstruction. However, the
10 culture of primary biliary epithelium remains problematic⁷. Here we report a
11 novel method for the isolation and propagation of primary human
12 cholangiocytes from the extrahepatic biliary tree, compatible with regenerative
13 medicine applications. The resulting Extrahepatic Cholangiocyte Organoids
14 (ECOs) express key biliary markers such as Cytokeratin 7 (KRT7 or CK7),
15 Cytokeratin 19 (KRT19 or CK19), Gamma Glutamyl-Transferase (GGT),
16 Cystic fibrosis transmembrane conductance regulator (CFTR) and maintain
17 their functional properties in vitro including Alkaline Phosphatase (ALP), GGT
18 activity and responses to secretin and somatostatin. The potential of ECOs for
19 tissue engineering and clinical applications is further illustrated by their
20 capacity to populate biodegradable scaffolds, organize into a functional biliary
21 epithelium and rescue a murine model of extrahepatic biliary injury (EHBI).

22

23 **Results**

24 **Human extrahepatic cholangiocytes can be propagated as organoids**

1 We first focused on identifying optimal conditions to isolate primary
2 cholangiocytes from the biliary epithelium which forms a monolayer covering
3 the luminal surface of the biliary tree⁸. We tested several approaches for
4 recovering these cells and mechanical dissociation by brushing or scraping
5 the bile duct lumen was associated with improved survival compared to
6 enzymatic digestion (Figure 1a, Supplementary Fig 1a). Furthermore, the
7 majority of the resulting cells co-expressed the biliary markers CK7 and CK19
8 ($94.6 \pm 2.4\%$, SD; n=3); while no contamination from mesenchymal cell types
9 was detected (Supplementary Fig 2). Consequently, mechanical dissociation
10 constitutes the optimal method for harvesting extrahepatic cholangiocytes.

11 To discern appropriate conditions for the maintenance and propagation of
12 these cells, we optimized our recently established system for 3D culture of
13 human induced pluripotent stem cell-derived intrahepatic cholangiocytes^{9,10}.
14 Screening of multiple growth factors known to support expansion of
15 cholangiocytes and epithelial organoids^{11,12} (Supplementary Fig 1b-1c)
16 identified that the combination of Epidermal Growth Factor (EGF), R-spondin
17 and Dickkopf-related protein 1 (DKK-1) promoted the growth of primary
18 cholangiocytes into organoids (Supplementary Fig 3a, 3b). Due to the
19 paradoxical requirement for both a Wnt potentiator (R-spondin) and an
20 inhibitor (DKK-1), we characterized the canonical and non-canonical/PCP Wnt
21 pathway activity in ECOs. Our results demonstrate higher β -catenin
22 phosphorylation in ECOs compared to cells treated with R-spondin but no
23 DKK-1 (Supplementary Fig 1d-1e), signifying lower WNT canonical pathway
24 activity in these cells. Furthermore ECOs exhibit higher Rho Kinase activity
25 compared to cells treated with R-spondin but no DKK-1 (Supplementary Fig

1 1f), which could be consistent with enhanced non-canonical/PCP signaling in
2 ECOs. Thus, it is possible that non-canonical Wnt signaling controls ECO
3 expansion marking a notable difference with previous organoid culture
4 conditions¹².

5 Under these conditions, we derived 8 different ECO lines (Supplementary
6 Table 1) from a variety of deceased donors aged from 33 to 77 years.
7 Notably, we obtained similar results by using cholangiocytes isolated from the
8 gallbladder or by harvesting common bile duct cholangiocytes using an
9 Endoscopic Retrograde Cholangio-Pancreatography (ERCP) brush instead of
10 scrapping the lumen (Supplementary Fig 4). Consequently, ECOs can be
11 derived from different areas of the extra-hepatic biliary tree and harvested
12 using peri-operative (dissection and scrapping) or minimally invasive (ERCP
13 brushings) approaches.

14

15 **ECOs maintain key biliary markers and function in culture**

16 The resulting cells were expanded in vitro for prolonged periods of time
17 (Supplementary Fig 5a) while maintaining their genetic stability
18 (Supplementary Fig 5b-5c). Electron microscopy revealed the presence of
19 characteristic ultrastructural features including cilia, microvilli and tight
20 junctions¹³ (Supplementary Fig 3c), while QPCR and immunofluorescence
21 (IF) analyses established the expression of key biliary markers such as *KRT7*
22 or *CK7*, *KRT19* or *CK19*, Hepatocyte Nuclear Factor 1 beta (*HNF1B*), *GGT*,
23 Secretin Receptor (*SCTR*), sodium-dependent bile acid transporter
24 (*ASBT/SLC10A2*), *CFTR* and SRY-box 9 (*SOX9*)⁹ (Figure 1b-1c,

1 Supplementary Fig 4c-4d, 3d-3e). Of note, stem cell markers such as
2 *POU5F1* or *OCT4*, *NANOG*, prominin 1 (*PROM1*), leucine rich repeat
3 containing G protein-coupled receptor (*LGR*) *LGR-4/5/6*; markers of non-
4 biliary lineages including albumin (*ALB*), α 1-antitrypsin (*SERPINA1* or *A1AT*),
5 keratin 18 (*KRT18*), pancreatic and duodenal homeobox 1 (*PDX1*), insulin
6 (*INS*) and glucagon (*GCG*); and EMT markers (vimentin (*VIM*), snail family
7 transcriptional repressor 1 (*SNAI1*) and S100 calcium binding protein A4
8 (*S100A4*) were not detected (Supplementary Fig 6a-6c). On the other hand,
9 $98.1\% \pm 0.9\%$ (SD; n=3) of the cells co-expressed CK7 and CK19 following
10 20 passages (Supplementary Fig 2) thereby confirming the presence of a
11 near homogeneous population of cholangiocytes.

12 Transcriptomic analyses (Figure 1d, Supplementary Fig 7, Supplementary
13 Table 2) revealed that ECOs maintain a stable gene expression profile over
14 multiple passages (Pearson correlation coefficient for Passage 1 (P1) vs.
15 Passage 20 (P20) $r=0.99$, Supplementary Fig 7a-b), express key biliary
16 markers (Supplementary Fig 7c) and cluster closely to freshly isolated
17 cholangiocytes (Supplementary Fig 7d) (Pearson correlation coefficient for
18 Primary Cholangiocytes (PCs) vs. Passage 20 (P20) $r=0.92$; Supplementary
19 Fig 7b). Gene ontology analyses confirmed enrichment of pathways
20 characteristic for the biliary epithelium (Supplementary Fig 7e). Considered
21 collectively, these results demonstrate that primary cholangiocytes derived
22 from the extrahepatic biliary tree can be expanded in vitro without losing their
23 original characteristics.

24 We then further characterized ECOs by focusing on their function following
25 long term culture (20 passages). The biliary epithelium regulates the

1 homeostasis of bile through the transport of ions, water and bile acids^{8,14}. The
2 secretory capacity of ECOs was interrogated using Rhodamine-123, a
3 fluorescent substrate for the cholangiocyte surface glycoprotein Multidrug
4 Resistance protein-1 (MDR1)^{15,16} (Figure 2a-2c). Rhodamine-123
5 accumulated in the ECO lumen only in the absence of the MDR-1 antagonist
6 verapamil, thereby confirming active secretion through MDR-1 (Figure 2a-2c).
7 Luminal extrusion of bile acids¹⁷ was also demonstrated by showing that the
8 fluorescent bile acid Cholyl-Lysyl-Fluorescein (CLF) was actively exported
9 from ECOs (Figure 2d-2f). Furthermore, ECO ALP and GGT activity was
10 comparable to freshly plated primary cholangiocytes (Figure 2g-2h,
11 Supplementary Fig 4e-4f). The response of ECOs to secretin and
12 somatostatin was also assessed. Secretin promotes water secretion,
13 distending the bile duct lumen, while somatostatin negates the effects of
14 secretin¹⁸⁻²⁰. Accordingly, organoids exposed to secretin increased their
15 diameter compared to untreated controls, while somatostatin inhibited the
16 effect of secretin (Figure 2i-2j). Our data, therefore, demonstrate that ECOs
17 maintain their functional properties after long term culture.

18

19 **ECOs self-organize into tubular structures after transplantation**

20 These results prompted us to investigate the potential of ECOs for in vivo use,
21 especially regenerative medicine applications. We first characterized the
22 potential of ECOs for in vivo engraftment and survival by transplanting cells
23 under the kidney capsule of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} (NSG) mice
24 (Supplementary Fig 8a) for 12 weeks²¹. ECOs successfully engrafted forming

1 tubular structures expressing biliary markers such as CK19 (Supplementary
2 Fig 8b-d).

3 Notably, no tumour formation or markers of differentiation to other lineages
4 were detected (Supplementary Fig 8d). Thus, ECOs appear to maintain their
5 basic characteristics even after prolonged engraftment in vivo under the
6 kidney capsule.

7

8 **ECOs populate biodegradable scaffolds**

9 To assess the potential of ECOs for tissue engineering, we first interrogated
10 their capacity for populating Polyglycolic Acid (PGA) biodegradable scaffolds
11 commonly used to provide the structural and mechanical support required for
12 tissue reconstruction²². Indeed, PGA is one of the most widely used synthetic
13 polymers since it does not induce inflammatory responses in the surrounding
14 tissue; it is biodegradable; and it is more flexible and easier to process
15 compared to natural polymers such as collagen²³. To facilitate tracking of the
16 cells, ECOs expressing Green Fluorescent Protein (GFP) were generated
17 through viral transduction (Supplementary Fig. 9a-9b). The resulting cells
18 were seeded on PGA scaffolds, attached to the PGA fibers after 24-48 hours
19 and continued to grow for 4 weeks until the scaffold was confluent (Figure 3a-
20 3d). Primary cholangiocytes plated in 2D conditions demonstrated limited
21 expansion potential and failed to reach confluency when seeded on the
22 scaffolds (Supplementary Fig 10a-b), suggesting that the proliferative capacity
23 of ECOs is crucial for successful scaffold colonization. The populated PGA
24 scaffolds (Figure 3b-3c), could easily be handled with forceps and divided into

1 smaller pieces with a surgical blade. Furthermore, the cells populating the
2 scaffolds retained expression of biliary markers such as CK7 and CK19
3 (Figure 3e-3f), demonstrated no evidence of epithelial–mesenchymal
4 transition (EMT; Figure 3e, 3g) and maintained their functional properties
5 including ALP and GGT activity (Figure 3h-3i). Therefore, ECOs can
6 successfully populate PGA scaffolds, while maintaining their functionality and
7 marker expression.

8

9 **ECO-populated scaffolds reconstruct the gallbladder wall**

10 Following these encouraging results, we decided to define the capacity of
11 ECOs to repair the biliary epithelium. For that, we developed a mouse model
12 of extrahepatic biliary injury (EHBI). More specifically, to simulate biliary tree
13 wall defects requiring biliary reconstruction²⁴, the biliary tree of healthy NSG
14 mice was compromised through a longitudinal incision in the gallbladder wall
15 (Figure 4a). The surgical defect in the gallbladder wall was subsequently
16 repaired by transplanting bioengineered tissue into the injured animals, which
17 was generated using GFP-expressing ECOs (see previous section; Figure 3).
18 Acellular PGA scaffolds and scaffolds populated with GFP-expressing
19 fibroblasts (Supplementary Fig 11a-11d) were used as a negative controls.
20 Animals receiving acellular scaffolds died within 24 hours of the operation
21 (Figure 4b) and post-mortem examination revealed yellow pigmentation of the
22 peritoneal cavity and seminal vesicles consistent with bile leak
23 (Supplementary Fig 12a); while all animals in the fibroblast-scaffold group
24 failed to reconstruct their gallbladder which was replaced by fibrotic tissue

1 incompatible with bile transport or storage (Supplementary Fig 13e-13g). In
2 contrast, animals transplanted with scaffolds containing ECOs survived for up
3 to 104 days without complications and were culled electively (Figure 4b).
4 Notably, the reconstructed gallbladders in the ECO group were fully
5 remodeled resembling the morphology of their native counterparts (Figure 4c,
6 Supplementary Fig 12b). Histology (Figure 3d), IF and QPCR analyses of the
7 ECO-reconstructed gallbladders (Figure 3e, Supplementary Fig 14c-14d)
8 unveiled integration of GFP-positive ECOs expressing biliary markers, such
9 as *KRT19*, *KRT7*, *HNF1B*, *SOX9*, *CFTR* and a human-specific epitope for
10 Ku80 (Figure 4e, Supplementary Fig 12c). Of note, these IF analyses also
11 showed the presence of mouse mesenchymal cells expressing vimentin and
12 endothelial cells expressing CD31 in the reconstructed biliary epithelium
13 suggesting that the scaffold is colonized by endogenous cells after
14 transplantation (Supplementary Fig 12c). Furthermore, we also identified a
15 population of GFP+/vimentin+/CK19- cells, suggesting that ECOs may also
16 contribute to the scaffold stroma in vivo; possibly through EMT
17 (Supplementary Fig 12c, 12e). The integrity of the reconstructed gallbladder
18 lumen and its exposure to bile through continuity with the biliary tree were
19 demonstrated using Magnetic Resonance Cholangio-Pancreatography
20 (MRCP) imaging prior to removal of the organ and was further confirmed with
21 FITC cholangiograms (Figure 4f-4g, Supplementary Fig 12f, Supplementary
22 Video 1). Post mortem surgical examination and full body Magnetic resonance
23 Imaging 104 days post transplantation revealed no evidence of tumor
24 formation (Supplementary Fig 12f, Supplementary Video 2) while IF analyses
25 revealed no GFP+ cells in the adjacent liver tissue (data not shown). On the

1 contrary, gallbladders reconstituted with fibroblasts controls exhibited
2 obliteration of the gallbladder lumen (Supplementary Fig 11h-11i) and
3 replacement of the lumen and biliary epithelium by fibroblasts expressing
4 Fibroblast Specific Antigen S100A4 (Supplementary Fig. 11i-11j). Considered
5 collectively, our findings demonstrate the capacity of ECOs to colonize their
6 physiological niche and regenerate part of the biliary tree without any
7 complications.

8

9 **ECOs on collagen scaffolds generate bioengineered bile ducts**

10 Reconstruction of the gallbladder wall provided proof-of-principle for the
11 capacity of ECOs to regenerate the biliary epithelium after injury; however, the
12 majority of extrahepatic bile duct disorders affect the common bile duct (CBD).
13 Therefore, we focused on the generation of a tubular ECO-populated scaffold,
14 which could be used for bile duct replacement surgery. The internal diameter
15 of the mouse CBD is approximately 100µm with a wall thickness of less than
16 50µm, which precluded the use of a PGA scaffold due to mechanical
17 properties. Instead, we generated densified collagen tubular scaffolds (Figure
18 5a-5b) which were populated with GFP-expressing ECOs (Figure 5c-5e). The
19 use of densified collagen enabled the generation of constructs with an
20 external diameter ranging from 250 to 600µm and adequate strength to
21 maintain a patent lumen (Figure 5d). Notably, the cells populating the collagen
22 scaffolds maintained expression of biliary markers such as *KRT19*, *KRT7*,
23 *HNF1B*, *SOX9* and *CFTR* (Figure 5f-5g) and exhibited GGT and ALP
24 enzymatic activity (Figure 5h-5i). Primary epithelial cells of different origin

1 (human mammary epithelial cells; HMEC) failed to survive and adequately
2 populate densified collagen tubes under the same conditions (Supplementary
3 Fig. 13a). Moreover, plated HMECs failed to survive in a 10% (vol/vol) bile
4 solution compared to ECOs (Supplementary Fig. 13b), further confirming that
5 ECOs constitute the only cell type capable of generating bile resistant bio-
6 engineered bile ducts. Collectively, these results demonstrate the capacity of
7 ECOs for populating tubular densified collagen scaffolds without losing their
8 original characteristics.

9

10 **Bioengineered bile ducts replace the native mouse bile duct**

11 We then decided to explore the possibility to replace the native CBD of NSG
12 mice with a bioengineered duct consisting of an ECO-populated densified
13 collagen tube (Figure 5). A mid-portion of the native CBD was removed and
14 an ECO-populated collagen tube was anastomosed end-to-end to the
15 proximal and distal duct remnants (Figure 6a). Fibroblast populated tubes
16 were used as a negative control. Biliary reconstruction was achieved in all
17 animals transplanted with ECO-populated tubes (Figure 6b-6c,
18 Supplementary Fig 14a-14d), which were followed up for up to a month post
19 transplantation (Supplementary Fig 14d). Histology and IF and QPCR
20 analyses (Figure 6d-6f, Supplementary Fig 14a-14b) revealed a patent lumen,
21 with formation of a biliary epithelium by the transplanted GFP+ cells (Figure
22 6e-6f, Supplementary Fig 14a-14b); confirmed the expression of biliary
23 markers, such as *KRT19*, *KRT7*, *HNF1B*, *CFTR*, *SOX9* (Figure 6d, 6f,
24 Supplementary Fig 14b) by the engrafted cells; but also illustrated the

1 presence of mouse stromal and endothelial cells (Supplementary Fig 14b).
2 Moreover, we observed minimal apoptosis and proliferation in the
3 transplanted tubes 1 month after transplantation, confirming the stability and
4 integrity of the reconstituted biliary epithelium (Supplementary Fig 14b-14c).
5 Lumen patency was further confirmed by Fluorescein Isothiocyanate (FITC)
6 cholangiogram, MRCP and serum cholestasis marker measurements (Figure
7 6g, Supplementary Fig 14e-14f, Supplementary video 3). Accordingly animals
8 receiving ECO-populated tubes exhibited no elevation in serum cholestasis
9 markers (Bilirubin, ALP; Supplementary Fig 14e) and a patent lumen on
10 imaging (Figure 6g, Supplementary Fig 14f); while the bio-artificial common
11 bile ducts retained their ALP activity in vivo (Figure 6h).

12 On the contrary, all fibroblast-populated collagen tubes failed due to lumen
13 occlusion (Figure 6b-6c, 6e-6g, Supplementary Fig 14d), resulting in high
14 biliary pressures and bile leak through the site of anastomosis (Figure 6b). In
15 conclusion, our results demonstrate the capacity of ECO-populated collagen
16 tubes to replace the native CBD in vivo.

17

18 **Discussion**

19 We have demonstrated that epithelial cells from the extrahepatic biliary tree
20 can be expanded and propagated in vitro while maintaining their
21 cholangiocyte transcriptional signature and functional characteristics. In
22 addition, our results show that primary cholangiocytes expanded in vitro as
23 organoids have a unique potential for organ regeneration. Indeed, our system
24 provides the first proof-of-principle for the application of regenerative medicine

1 in the context of common bile duct pathology. The capacity to replace a
2 diseased common bile duct with an in vitro bio-engineered ECO-tube could
3 have a considerable impact for the management of disorders such as biliary
4 atresia, which constitutes the leading cause for pediatric liver transplantation¹;
5 or ischemic strictures which are one of the most common complications
6 following transplantation³. Consequently ECO-populated scaffolds constitute a
7 novel system with high clinical relevance in the field of cholangiopathies.

8 Furthermore, studies of the extrahepatic biliary epithelium have been limited
9 by technical challenges in long-term culture and large-scale expansion of
10 primary cholangiocytes. These challenges have so far precluded large scale
11 experiments such as transcriptomic and genome-wide analyses which are
12 urgently needed to better understand bile duct diseases, such as PSC and
13 cholangiocarcinoma. The capacity of ECOs for large scale expansion, could
14 address this challenge. Indeed, we demonstrate that starting from 10^5
15 extrahepatic cholangiocytes we can generate between 10^{20} – 10^{25} cells after
16 20 passages. Therefore, ECOs not only represent a novel source of cells for
17 cell based therapy but also provide a unique model system for studying the
18 physiology and modeling disorders of the extrahepatic biliary tree in vitro.

19 Access to human tissue constitutes a considerable limitation for systems
20 based on primary cells. However, we show that ECOs can be obtained not
21 only from the common bile duct but also from the gallbladder. Gallbladder
22 tissue is easily accessible and routinely discarded following liver
23 transplantation and cholecystectomy, one of the most common surgical
24 procedures performed. Furthermore, in individuals not having surgery the
25 common bile duct can be accessed using minimally invasive procedures, such

1 as Endoscopic Retrograde Cholangio-Pancreatography (ERCP) and we
2 demonstrate that cholangiocytes can be obtained through brushings, which
3 are routinely performed to acquire histology specimens. Notably, no
4 morphological or functional differences were observed between organoids
5 obtained with these different methods. Moreover, due to the scalability of our
6 system only a small amount of starting material is required. Finally, recent
7 progress in replacing Matrigel by custom made hydrogels to grow gut
8 organoids²⁵ suggest that translating our system from Matrigel to Good
9 Manufacturing Practice (GMP) could be feasible. Considered together, these
10 approaches effectively address challenges of tissue availability and open the
11 possibility of autologous as well as allogeneic cell based therapy.

12 Notably, the derivation of primary hepatic stem cells using an organoid culture
13 system has been reported previously¹². However, the capacity of the resulting
14 cells to differentiate into functional cholangiocytes and populate the biliary tree
15 in vivo remains to be demonstrated. Furthermore, in vivo applications of such
16 platforms could be restricted by contaminating stem cells with a capacity to
17 proliferate inappropriately after transplantation and/or differentiate into non-
18 biliary cell types. Despite the association between organoids and adult stem
19 cells²⁶, we never observed the expression of hepatocyte or pancreatic
20 markers during our experiments either in vitro or after transplantation,
21 suggesting that the differentiation capacity of ECOs is limited to their lineage
22 of origin. Moreover, canonical WNT signaling, which is crucial for the
23 expansion of adult stem cell organoids²⁷ is blocked in our culture conditions
24 through the use of DKK-1 and further studies may be required to fully
25 elucidate the role of R-spondin in our system. Considered together, these

1 observations suggest that our culture system does not include a stem cell
2 population. However, we cannot completely exclude that these cells could
3 represent a biliary progenitor population based on their ability to self-
4 propagate and generate organoids from single cells.

5 Our system provides proof-of-principle for the application of primary cells in
6 regenerative medicine; however, the use of stem cells has been suggested as
7 an alternative for cell based therapy. Although we have recently established a
8 system for the generation of stem cell-derived cholangiocyte-like cells
9 (CLCs)⁹, there are considerable differences between ECOs and CLCs that
10 render ECOs better suited to regenerative therapies for extrahepatic biliary
11 injury. CLCs correspond to intrahepatic cholangiocytes, while ECOs represent
12 extrahepatic biliary epithelium. These two cell types are distinct in terms of
13 embryological origin and disease involvement¹⁴. Furthermore, CLCs still
14 express fetal markers and therefore are more immature compared to ECOs
15 derived from primary cells⁹. Therefore, CLCs may require a period of
16 adjustment and further maturation in vivo, while mature, functional cells, such
17 as ECOs, are required for coping with biliary injury in the acute setting.
18 Finally, although hiPSCs provide a very good source of cells capable of
19 generating almost any tissue, fully differentiated CLCs cannot be expanded;
20 initial derivation/characterization of hiPSC lines remains time consuming;
21 while variability in capacity of differentiation still constitutes a challenge. ECOs
22 can be derived in less than 24 hours with a very high efficiency and can be
23 expanded for multiple passages without losing their original characteristics.
24 Consequently, ECOs are comparable to CLCs in terms of scalability, while

1 their mature phenotype provides a unique advantage for regenerative
2 medicine applications in the context of tissue repair.

3 In conclusion, our results open up novel avenues for the use of extrahepatic
4 primary biliary tissue as a novel platform for in vitro studies, disease modeling
5 and cell based therapy applications.

6

7 **Accession codes**

8 Accession number for microarray data: E-MTAB-4591.

9

10 **Data availability statement**

11 The microarray data are open access and available online on ArrayExpress
12 (<https://www.ebi.ac.uk/arrayexpress/>)

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1

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4 collection and interpretation of data, production of figures, manuscript writing,
5 editing and final approval of manuscript; AWJ: Conception of the technique,
6 scaffold design and generation of densified collagen tubular scaffolds; OCT:
7 animal experiments including kidney capsule injections; cell culture, provision
8 and harvesting of mouse tissue; StS: Magnetic Resonance Imaging (MRI);
9 EMG, SSU: MRI review and reporting; RLG: Animal experiments, IF, tissue
10 histology; MCDB: Cell culture, generation of viral particles, viral transduction,
11 generation of GMP-ECOs; NLB, LV: Animal experiments; MJGV, PM:
12 Bioinformatics analyses; DO: Flow cytometry analyses; LY: Western blot
13 analyses; AR: IF and QPCR analyses and provision of positive controls for IF
14 and QPCR; AB: Flow cytometry analyses, bioinformatics support; JB: Tissue
15 histology, IF; MarZ: Scaffold preparation; MTP: Generation of viral particles,
16 viral transduction, generation of GMP-ECOs; MP: Generation of viral particles;
17 GMS: scaffold generation; PMM,KES: maintenance and provision of fibroblast
18 controls; NP: tissue culture; NG, CAR: Harvesting and preparation of primary
19 tissue; IS: Karyotyping, CGH analyses; SD: Histology review and reporting;
20 WS, JC, KBJ, MatZ, SaS, WTHG, GJA, SEB, TW, THK, EM: critical revision
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1 LV: Design and concept of study, study supervision, interpretation of data,
2 editing and final approval of manuscript.

3

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5

6

1 **Figure Legends**

2

3 **Figure 1**

4 Derivation and characterization of Extrahepatic Cholangiocyte Organoids
5 (ECOs). **(a)** Schematic representation of the method used for the derivation of
6 ECOs. **(b)** Quantitative real time PCR (QPCR) confirming the expression of
7 biliary markers in Passage 1 (P1), Passage 10 (P10) and Passage 20 (P20)
8 ECOs compared to freshly isolated Primary Cholangiocytes (PC) and
9 Embryonic Stem (ES) cells used as a negative control, n=4 ECO lines.
10 Center line, median; box, interquartile range (IQR); whiskers, range (minimum
11 to maximum). Values are relative to the housekeeping gene
12 Hydroxymethylbilane Synthase (*HMBS*) **(c)** Immunofluorescence (IF)
13 analyses confirming the expression of biliary markers in ECO organoids.
14 Scale bars: 100 μ m. Single channel and higher magnification images are
15 provided in Supplementary Fig 3. **(d)** Euclidean hierarchical clustering
16 analysis comparing the transcriptome of primary cholangiocytes (Primary),
17 passage 20 ECOs (ECO), hIPSC-derived intrahepatic cholangiocyte-like-cells
18 (iChoLC), ES cells (ES) and hepatocytes (HEP). For each probe, standard
19 scores (z-scores) indicate the differential expression measured in number of
20 standard deviations from the average level across all the samples. Clusters of
21 genes expressed in ECOs, primary cholangiocytes or both cell types are
22 indicated. GO analyses for each cluster are provided in Supplementary Fig
23 7e. The data corresponds to 3 ECO lines.

24

1 **Figure 2**

2 Functional characterization of ECO organoids. **(a)** Fluorescence images
3 demonstrating secretion of the MDR1 fluorescent substrate rhodamine 123 in
4 the lumen of ECOs, which is inhibited by the MDR1 inhibitor verapamil. Scale
5 bars: 100 μm . **(b)** Fluorescence intensity along the white line in **(a)**. **(c)** Mean
6 intraluminal fluorescence intensity normalized to background in freshly plated
7 Primary Cholangiocytes (Rho PC), Passage 20 ECOs (Rho P20) and P20
8 ECOs treated with verapamil (Ver). Error bars, Standard Deviation (SD);
9 $n=1565$ measurements in total. Asterisks (****) indicate statistical significance
10 ($P<0.001$, Kruskal-Wallis test with Dunn's correction for multiple comparisons)
11 **(d)** Luminal extrusion of the fluorescent bile acid CLF compared to controls
12 loaded with FITC, confirming bile acid transfer. Scale bars: 100 μm . **(e)**
13 Fluorescence intensity along the white line in **(d)**. **(f)** Mean intra-luminal
14 fluorescence intensity normalized over background, $n=1947$ total
15 measurements. Error bars, SD; asterisks as in **(c)**. **(g)** ALP staining of ECOs.
16 Scale bars: Light microscopy: 500 μm , Whole well images: 1 cm. **(h)** Mean
17 GGT activity of P20 ECOs vs. PCs; error bars, SD; $n=3$, asterisks as in **(c)**;
18 one-way ANOVA with Dunnett's correction for multiple comparisons. **(i,j)**
19 Mean diameter measurements **(i)** and live images **(j)** of ECOs treated with
20 secretin or secretin and somatostatin, $n=8$. Error bars, SD; $***P<0.001$;
21 $\#P>0.05$ (Kruskal-Wallis test with Dunn's correction for multiple comparisons).
22 **(a-j)** Data representative of 3 different experiments.

23

24 **Figure 3**

1 ECOs dissociated to single cells (ECO-SCs) can populate biodegradable PGA
2 scaffolds. **(a,b)** Photographs of a PGA scaffold before **(a)** and after **(b)**
3 treatment with ECOs. Scale bars: 1 cm. **(c)** Light microscopy images of a
4 PGA scaffold populated with ECO-SCs. Red arrowheads: Fully populated
5 scaffold; black arrowheads: cells recruiting new PGA fibers; white
6 arrowheads: PGA fibers. Scale bars: 100 μ m. **(d)** Confocal microscopy images
7 demonstrating cell expansion at different time-points after seeding of GFP-
8 positive ECO-SCs on a PGA scaffold. White lines indicate the position of PGA
9 fibers. Scale bars: 100 μ m. **(e)** IF demonstrating the expression of biliary
10 markers and lack of EMT markers in ECO-SCs seeded on PGA scaffolds.
11 Scale bars: 100 μ m **(f)** QPCR analyses demonstrating the expression of
12 biliary markers in ECOs before (ECOs) and after (scaffold) seeding on PGA
13 scaffolds, n=4 ECO lines. Center line, median; box, interquartile range (IQR);
14 whiskers, range (minimum to maximum). Values are relative to the
15 housekeeping gene *HMBS*. **(g)** Mean ratio of CK7+/CK19+ and
16 CK19+/Vimentin (VIM)+ cells in IF analyses similar to the image shown in **(e)**.
17 Error bars represent SD; n=6. ** P <0.01 (Mann-Whitney test). **(h)** Mean GGT
18 activity of ECO-SCs populating a PGA scaffold, n=4. Error bars represent SD.
19 **** P <0.001 (two-tailed t-test). **(i)** ALP staining of PGA scaffolds populated by
20 ECO-SCs. Scale bars: 500 μ m.

21

22 **Figure 4**

23 Biliary reconstruction in an extrahepatic biliary injury (EHBI) mouse model
24 using ECOs. **(a)** Schematic representation of the method used for biliary

1 reconstruction. **(b)** Kaplan–Meier survival analysis, demonstrating rescue of
2 EHBI mice following biliary reconstruction with ECO-populated scaffolds.
3 **** $P < 0.01$** (log-rank test). **(c)** Images of gallbladders reconstructed with
4 acellular PGA scaffolds (scaffold only), PGA scaffolds populated with ECOs
5 (transplanted) and native un-reconstructed gallbladder controls (not
6 transplanted), demonstrating full reconstruction with ECO populated scaffolds.
7 CD: cystic duct, CBD: common bile duct, CHD: common hepatic duct, F:
8 fundus, A: anterior surface, P: posterior surface. Scale bars: 500 μm . **(d)** H&E
9 staining of the reconstructed gallbladders. L: lumen. Scale bars: 100 μm **(e)** IF
10 analyses demonstrating the presence of GFP-positive ECOs expressing
11 biliary markers in the reconstructed gallbladders. L: lumen Scale bars: 100
12 μm . Higher magnification images are provided in Supplementary Fig 12 **(f,g)**
13 FITC cholangiogram (n=1) **(f)** and MRCP images (n=2) **(g)** of reconstructed
14 (transplanted) vs. native control (not transplanted) gallbladders (GB)
15 demonstrating a patent lumen and unobstructed communication with the rest
16 of the biliary tree. Scale bars: 1 mm.

17

18 **Figure 5**

19 ECOs can populate densified collagen tubular scaffolds. **(a)** Schematic
20 representation of the method used for the generation of densified collagen
21 tubular scaffolds. **(b)** Image of a densified collagen construct prior to tube
22 excision. Scale bar, 500 μm . **(c)** Maximum intensity projection image
23 demonstrating a GFP+ ECO-populated tube after its generation. Scale bar; 30
24 μm **(d)** Confocal microscopy image demonstrating lumen patency of an ECO-

1 populated collagen tube. Scale bar; 30 μm . **(e)** Images of a near confluent
2 GFP+ ECO-tube. Scale bar; 100 μm . **(f)** IF analyses demonstrating the
3 expression of biliary markers by ECOs following the generation of ECO-tubes.
4 Scale bar; 100 μm . **(g)** QPCR analyses demonstrating the expression of
5 biliary markers before (ECOs) and after (Scaffold) the generation of ECO-
6 populated collagen tubes. ES cells are used as a negative control, n=4 ECO
7 lines. Center line, median; box, interquartile range (IQR); whiskers, range
8 (minimum to maximum). Values are relative to *HMBS* expression. **(h, i)** ECO-
9 tubes exhibit ALP **(h)** and GGT **(i)** activity. Scale bars, 500 μm ; MEFs, Mouse
10 Embryonic feeders used as negative control; Scaffold, ECO-populated,
11 densified collagen tubes; error bars, SD; n=3.

12

13 **Figure 6**

14 Bile duct replacement using ECO-populated densified collagen tubes. **(a)**
15 Schematic representation of the method used. **(b)** Postmortem images of
16 mice receiving ECO-populated collagen tubes (ECOs) vs. mice receiving
17 fibroblast-populated tubes (fibroblasts). Bile flow results in yellow
18 pigmentation of ECO-tubes. The white color of the fibroblast conduit
19 combined with a dilated bile-filled (yellow color) Proximal Bile Duct (PBD)
20 suggests luminal occlusion, resulting in bile leak (yellow peritoneal
21 pigmentation; white dashed line). SC: Collagen tubes/scaffolds; DBD: Distal
22 Bile Duct; scale bars 500 μm . **(c)** Images of a thin walled construct resembling
23 the native bile duct in animals receiving ECO-populated tubes vs. a thickened
24 construct with no distinguishable lumen in animals receiving fibroblast tubes.

1 Scale bars 500 μm . **(d)** QPCR using human-specific primers confirming the
2 expression of biliary markers by transplanted ECO-populated tubes (ECOs in
3 vivo) compared to cultured ECOs (ECOs in vitro) and mouse biliary tissue
4 used as a negative control, n=4 **technical replicates**. Center line, median; box,
5 interquartile range (IQR); whiskers, range (minimum to maximum). Values are
6 relative to *HMBS* expression. **(e)** H&E staining demonstrating the presence of
7 a biliary epithelium and a patent lumen in ECO-tubes but not fibroblast
8 constructs. Scale bars 100 μm . **(f)** IF analyses demonstrating a GFP+/ CK19+
9 epithelium lining the lumen of ECO-constructs, vs. obliteration of the lumen by
10 fibroblasts in fibroblast constructs. Scale bars 100 μm . **(g)** FITC
11 cholangiogram, demonstrating lumen patency in ECO-tubes vs. lumen
12 occlusion in fibro-constructs. Scale bars: ECO, 100 μm ; Fibroblasts, 500 μm
13 **(h)** ALP activity is observed only in ECO-tubes, but not in fibroblast
14 constructs. Scale bars: ECO, 100 μm ; Fibroblasts, 500 μm .

15

1 **Online Methods**

2 **Primary biliary tissue**

3 Primary biliary tissue (bile duct or gallbladder) was obtained from deceased
4 organ donors from whom organs were being retrieved for transplantation. The
5 gallbladder or a section of the bile duct was excised during the organ retrieval
6 operation after obtaining informed consent from the donor's family (REC
7 reference numbers: 12/EE/0253, NRES Committee East of England -
8 Cambridge Central and 15/EE/0152 NRES Committee East of England -
9 Cambridge South).

10 **Isolation of primary cholangiocytes**

11 Excised bile duct segments were placed in a 10 cm plate and washed once
12 with William's E medium (Gibco, Life Technologies). A longitudinal incision
13 was made along the wall of the excised bile duct segment exposing the lumen
14 and 10-15 ml of William's E medium were added to cover the tissue. The
15 luminal epithelium was subsequently scraped off using a surgical blade, while
16 submerged in medium. The supernatant was collected and the tissue and
17 plate were washed 2-3 times with William's E medium to harvest any
18 remaining cells. The supernatant and washes were centrifuged at 444g for 4
19 minutes. The pellet was washed with William's E, re-centrifuged and the
20 supernatant was discarded (Figure 1a).

21 Excised gallbladders were placed in a 15 cm plate, a longitudinal incision was
22 made along the wall of the excised gallbladder and the lumen was washed
23 once with William's E medium (Gibco, Life Technologies). Cholangiocytes

1 were isolated and harvested following the method described above
2 (Supplementary Fig 3a).

3 For isolation through brushings, an excised bile duct segment was placed in a
4 10 cm plate and cannulated using an ERCP brush. The lumen was brushed
5 10-20 times and the cells were harvested by washing the brush several times
6 in a falcon tube containing 40-50 ml of William's E medium (Supplementary
7 Fig 3b).

8 **Generation and culture of ECOs**

9 Isolated primary cholangiocytes were centrifuged at 444g for 4 minutes and
10 re-suspended in a mixture of 66% matrigel (BD Biosciences, catalogue
11 number: 356237) and 33% William's E medium (Gibco, Life Technologies)
12 supplemented with 10mM nicotinamide (Sigma-Aldrich), 17mM sodium
13 bicarbonate (Sigma Aldrich), 0.2mM 2-Phospho-L-ascorbic acid trisodium salt
14 (Sigma-Aldrich), 6.3mM sodium pyruvate (Invitrogen), 14mM glucose (Sigma-
15 Aldrich), 20mM HEPES (Invitrogen), ITS+ premix (BD Biosciences), 0.1µM
16 dexamethasone (R&D Systems), 2mM Glutamax (Invitrogen), 100U/ml
17 penicillin per 100µg/ml streptomycin, 20ng/ml EGF (R&D Systems), 500ng/ml
18 R-Spondin (R&D Systems) and 100ng/ml DKK-1 (R&D Systems). The cell
19 suspension was plated in 24-well plate format, at 50µl/well, so that a small
20 dome of matrigel was formed in the centre of each well and then incubated at
21 37°C for 10-30 minutes until it solidified. Subsequently, 1ml of William's E
22 medium with supplements was added. The culture medium was changed
23 every 48 hours.

1 To split the cells, the matrigel was digested by adding Cell Recovery Solution
2 (Corning) for 30 minutes at 4°C. The resulting cell suspension was harvested,
3 centrifuged at 444g for 4 minutes, washed once with William's E medium and
4 re-suspended in 66% matrigel and 33% William's E medium with
5 supplements, as described above.

6 All experiments were performed using passage 20 ECOs unless otherwise
7 stated.

8 **Cell line identity**

9 Demographic data for donor corresponding to the each ECO lines is provided
10 in supplementary table 1. Following derivation ECO lines were authenticated
11 by matching their karyotype (Supplementary Fig. 4b) to the sex of the donor of
12 origin. The lines were tested on a regular basis and found to be negative for
13 mycoplasma contamination.

14 **Immunofluorescence, RNA extraction and Quantitative Real Time PCR**

15 IF, RNA extraction and QPCR were performed as previously described⁹. A
16 complete list of the primary and secondary antibodies used is provided in
17 supplementary table 3. A complete list of the primers used is provided in
18 supplementary table 4.

19 All QPCR data are presented as the median, interquartile range (IQR) and
20 range (minimum to maximum) of four independent ECO lines unless
21 otherwise stated. Values are relative to the housekeeping gene
22 Hydroxymethylbilane Synthase (*HMBS*).

23 All IF images were acquired using a Zeiss Axiovert 200M inverted microscope
24 or a Zeiss LSM 700 confocal microscope. Imagej 1.48k software (Wayne

1 Rasband, NIHR, USA, <http://imagej.nih.gov/ij>) was used for image processing.

2 IF images are representative of 3 different experiments. IF images of

3 reconstructed gallbladder sections are representative of 5 different animals.

4 **Microarrays**

65 RNA for microarray analysis was collected from 3 different ECO lines (n=3).

66 The RNA was assessed for concentration and quality using a SpectroStar

67 (BMG Labtech, Aylesbury, UK) and a Bioanalyser (Agilent Technologies,

68 Cheadle, UK). Microarray experiments were performed at Cambridge

69 Genomic Services, University of Cambridge, using the HumanHT-12 v4

70 Expression BeadChip (Illumina, Chesterford, UK) according to the

71 manufacturer's instructions. Briefly, 200ng of Total RNA underwent linear

72 amplification using the Illumina TotalPrep RNA Amplification Kit (Life

73 Technologies, Paisley, UK) following the manufacturer's instructions. The

74 concentration, purity and integrity of the resulting cRNA were measured by

75 SpectroStar and Bioanalyser. Finally cRNA was hybridised to the HumanHT-

76 12 v4 BeadChip overnight followed by washing, staining and scanning using

77 the Bead Array Reader (Illumina). The microarray data are available on

78 ArrayExpress (Accession number: E-MTAB-4591). For reviewer access,

79 please use the following login details Username: Reviewer_E-MTAB-4591

80 Password: rtlImbi0

81 **Microarrays analysis**

82 Raw data was loaded into R using the lumi package from bioconductor²⁸ and

83 divided into subsets according to the groups being compared; only the

84 samples involved in a given comparison are used. Subsets were then filtered

85 to remove any non-expressed probes using the detection p-value from

1 Illumina. Across all samples probes for which the intensity values were not
2 statistically significantly different ($P>0.01$) from the negative controls were
3 removed from the analysis. Following filtering the data was transformed using
4 the Variance Stabilization Transformation²⁹ from lumi and then normalised to
5 remove technical variation between arrays using quantile normalisation.
6 Comparisons were performed using the limma package³⁰ with results
7 corrected for multiple testing using False Discovery Rate (FDR) correction.
8 Finally the quality of the data was assessed along with the correlations
9 between samples within groups.

10 Probes differentially expressed between HEP and ECOs representing the
11 aggregate transcriptional “signature” of ECOs were selected for Euclidean
12 hierarchical clustering using Perseus software (MaxQuant). Standard scores
13 (z-scores) of the log₂ normalized probe expression values across the different
14 conditions were calculated and used for this analysis. Heatmaps and Primary
15 Component Analysis (PCA) plots were generated using the MaxQuant
16 Perseus software (<http://www.perseus-framework.org/>)³¹. Functional
17 annotation and gene ontology analyses were performed on the genes
18 differentially expressed between PCs and ECOs (Figure 1d) using the
19 NIAID/NIH Database for Annotation, Visualization and Integrated Discovery
20 (DAVID) v6.8 (<https://david.ncifcrf.gov/>)^{32,33}.

21 **Western Analysis**

22 Total protein was extracted with lysis buffer (50mM Tris pH 8, 150mM NaCl,
23 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and protease and
24 phosphatase inhibitors). Protein concentrations were determined by BCA
25 Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s

1 instructions. Samples were prepared for Western blot by adding 1x NuPAGE
2 LDS Sample Buffer with 1% β -mercaptoethanol and incubated for 5 minutes
3 at 95°C. Protein (25 μ g) was separated by 4-12% NuPAGE Bis-Tris protein
4 gels (Invitrogen) and transferred onto PVDF membranes (Bio-Rad). Proteins
5 were detected by probing with antibodies specific to Phospho- β -catenin
6 (Ser33/37/Thr41) (Cell Signalling Technology), Active- β -catenin (Millipore),
7 Total- β -catenin (R&D), α -tubulin (Sigma) followed by incubation with
8 horseradish peroxidase anti-mouse, anti-goat or anti-rabbit secondary
9 antibodies. Membranes were developed using Pierce ECL Western blotting
10 substrate (Thermo Scientific) according to the manufacturer's instructions.

11 **Rho Kinase activity analyses**

12 Rho Kinase activity was measured using a commercially available kit (Cell
13 Biolabs, STA-416) according to the manufacturer's instructions

14 **Flow cytometry analyses**

15 ECO organoids were harvested using Cell Recovery Solution (Corning) for 30
16 minutes at 4°C, centrifuged at 444g for 4 minutes and dissociated to single
17 cells using TrypLE™ Express (Gibco). The cells were subsequently fixed
18 using 4% PFA for 20 minutes at 4°C. Cell staining and flow cytometry
19 analyses were performed as previously described^{9,34}.

20 **Karyotyping**

21 ECO organoids were harvested using Cell Recovery Solution (Corning),
22 dissociated to single cells as described above, plated in gelatin coated plates
23 and cultured using William's E medium with supplements. When the cells
24 were sub-confluent, usually after 72hrs, the cultures were incubated for 3-4
25 hours with William's E medium with supplements containing 0.1 μ g/ml

1 colcemid (Karyomax®, Gibco). The cells were then harvested using Trypsin-
2 EDTA (0.05%) (Gibco) for 4-5 minutes at 37°C, centrifuged at 344g for 5
3 minutes and re-suspended in 5mls of KCl hypotonic solution (0.055M). The
4 suspension was re-centrifuged at 344g for 5 minutes, 2 mls of a 3:1 100%
5 methanol:glacial acetic acid solution were added and slides were prepared as
6 previously described³⁵

7 **Comparative Genomic Hybridization analyses**

8 Genomic DNA was labeled using the BioPrime DNA Labeling Kit (Invitrogen),
9 according to the manufacturer's instructions and samples were hybridised to
10 Agilent Sureprint G3 unrestricted CGH ISCA 8x60K human genome arrays
11 following the manufacturer's protocol, as previously described³⁶. The data
12 was analysed using the Agilent CytoGenomics Software.

13 **Rhodamine123 transport assay**

14 The Rhodamine 123 transport assay was performed as previously described⁹
15 and images were acquired using a Zeiss LSM 700 confocal microscope.
16 Fluorescence intensity was measured between the organoid interior and
17 exterior and luminal fluorescence was normalized over the background of the
18 extraluminal space. Each experiment was repeated in triplicate. Error bars
19 represent SD.

20 **Cholyl-Lysyl-Fluorescein transport assay**

21 To achieve loading with Cholyl-Lysyl-Fluorescein (CLF, Corning
22 Incorporated), ECO organoids were split in 5µM of CLF and incubated at 37°C
23 for 30 minutes. Images were acquired using a Zeiss LSM 700 confocal
24 microscope and fluorescence intensity was measured between the organoid
25 interior and exterior as described for the Rhodamine 123 transport assay. To

1 demonstrate that the changes in CLF fluorescence intensity observed were
2 secondary to active export of CLF from the organoid lumen, the experiment
3 was repeated with 5 μ M of unconjugated Fluorescein Isothiocyanate (FITC)
4 (Sigma-Aldrich) as a control. Fluorescence intensity measurements were
5 performed as described for the Rhodamine 123 transport assay. Each
6 experiment was repeated in triplicate. Error bars represent SD.

7 **GGT activity**

8 GGT activity was measured in triplicate using the MaxDiscovery™ gamma-
9 Glutamyl Transferase (GGT) Enzymatic Assay Kit (Bioo scientific) based on
10 the manufacturer's instructions. Error bars represent SD.

11 **Alkaline Phosphatase staining**

12 Alkaline phosphatase was carried out using the BCIP/NBT Color
13 Development Substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue
14 tetrazolium) (Promega) according to the manufacturer's instructions.

15 **Response to Secretin and Somatostatin**

16 Responses to secretin and somatostatin were assessed as previously
17 described⁹.

18 **Generation of ECOs expressing Green Fluorescent Protein**

19 EGFP expressing VSV-G pseudotyped, recombinant HIV-1 lentiviral particles
20 were produced with an optimized second generation packaging system by
21 transient co-transfection of three plasmids into HEK 293T cells (ATCC CRL-
22 11268). EGFP expression is under control of a core EF1 α -promoter. All
23 plasmids were a gift from Didier Trono and obtained from addgene (pWPT-
24 GFP #12255, psPAX2 #12260, pMD2.G, #12259). Viral infection of organoids
25 was performed as previously described³⁷. Infected ECOs were expanded for 2

1 passages, harvested as described above for flow cytometry analyses and cell
2 sorting by flow cytometry for GFP positive cells was performed. GFP
3 expressing single cells were plated using our standard plating method and
4 cultured in William's E medium with supplements for 1-2 weeks until fully
5 grown ECO organoids developed.

6 **Generation of ECO populated PGA scaffolds**

7 1mm thick PolyGlycolic Acid (PGA) scaffolds with a density of 50mg/cc were
8 used for all experiments. Prior to seeding cells, the PGA scaffolds were pre-
9 treated with a 1M NaOH for 10-30 seconds washed 3 times, decontaminated
10 in a 70% ethanol solution for 30 minutes and then air-dried for another 30
11 minutes until all the ethanol had fully evaporated. All scaffolds were a gift from
12 Dr Sanjay Sinha and obtained from Biomedical Structures (Biofelt).

13 ECOs were harvested and dissociated to single cells as previously described
14 for flow cytometry analyses. $5-10 \times 10^6$ cells were re-suspended in 100 μ l of
15 William's E medium with supplements, seeded on a scaffold surface area of
16 1cm^2 and incubated at 37°C for 30-60 minutes to allow the cells to attach to
17 the scaffold. The scaffolds were placed in wells of a 24-well plate and
18 checked at regular intervals during this period to ensure the medium did not
19 evaporate. If necessary, 10-20 μ l of William's E medium with supplements
20 were added. After 1 hour, 2-3 mls of William's E medium with supplements
21 were added to the wells and the medium was changed twice weekly.

22 **Generation of densified collagen tubes**

23 Densified collagen tubes were prepared using a novel approach. A 3D printed
24 chamber was fabricated, consisting of a funnel piece and a base plate. A
25 250 μ m thick metallic wire was mounted into the base plate and fed through

1 the centre of the funnel. Absorbent paper towels were compacted between
2 the two 3D printed parts, which were then screwed together. 5 mg mL⁻¹
3 collagen gel solution, loaded with cells, was poured into the funnel and gelled
4 at 37°C for 30 min. After that time, the screws were loosened and, by placing
5 the 3D printed chambers at 37°C for 2-4h, water was drawn out of the
6 collagen gel. A cell-loaded densified collagen tube was thus formed with a
7 250µm lumen and a wall thickness of 30-100 µm, determined by the duration
8 of the drying phase. Upon removal from the chamber, the tube was trimmed
9 for excess collagen and cut to the required length.

10 **Culture of Human Mammary Epithelial Cells (HMECs)**

11 HMECs and the required tissue culture consumables were purchased as a kit
12 from Lonza (cat no. cat no. CC-2551B) and the cells were cultured according
13 to the supplier's instructions

14 **Animal experiments**

15 All animal experiments were performed in accordance with UK Home Office
16 regulations (UK Home Office Project License numbers PPL 80/2638 and PPL
17 70/8702). Immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice which
18 lack B, T and NK lymphocytes³⁸ were bred in-house with food and water
19 available ad libitum pre- and post-procedures. A mix of male and female
20 animals were used, aged approximately 6-8 weeks. All the ECO-constructs
21 used were populated with ECOs derived from the common bile duct.

22 **Generation of Extra-Hepatic Biliary Injury (EHBI) mouse model**

23 To generate a model of extrahepatic biliary injury, midline laparotomy was
24 performed and the gallbladder was first mobilized by dividing the ligamentous
25 attachment connecting its fundus to the anterior abdominal wall under

1 isoflurane general anesthesia. A longitudinal incision was then made along
2 2/3 of the length of the gallbladder, from the fundus towards Hartmann's
3 pouch (neck of gallbladder).

4 **Biliary reconstruction in EHBI mice**

5 To reconstruct the gallbladder, a scaffold section measuring approximately 1 x
6 1 mm (seeded with ECOs or without ECOs in controls) was sutured as a
7 'patch' to close the defect using 4 – 6 interrupted 10'0 non-absorbable nylon
8 sutures under 40x magnification. The laparotomy was closed in two layers
9 with continuous 5'0 absorbable Vicryl sutures. The animals were given
10 buprenorphine (temgesic 0.1 mg/kg) analgesia as a bolus and observed every
11 15 minutes in individual cages until fully recovered.

12 8 animals underwent biliary reconstruction using an ECO-populated scaffold.
13 All animals survived up to 104 days without complications and were culled
14 electively for further analyses. Two control experiments were performed,
15 where the animals underwent biliary reconstruction using acellular scaffolds.
16 Both animals died within 24 hours from bile leak, therefore no further control
17 experiments were performed to minimize animal discomfort.

18 **Bile duct replacement**

19 The native common bile duct was divided and a short segment excised. The
20 populated densified collagen tube was anastomosed end-to-end, using
21 interrupted 10'0 nylon sutures, between the divided proximal and distal
22 common bile duct. A length of 5'0 nylon suture material (diameter 100 µm)
23 was inserted into the collagen tube and fed into the proximal and distal
24 common bile duct to ensure patency of the lumen during the anastomosis.
25 After the anastomosis was complete, the 5'0 suture was pushed into the

1 duodenum through the distal bile duct and was removed through an incision in
2 the duodenum, which was then closed with interrupted 10'0 nylon sutures.
3 Lumen patency was assessed at the time of transplantation through light
4 microscopy and cannulation of the lumen with a 5'0 non-absorbable suture.
5 Transplantation was abandoned as futile in case of fully occluded tubes due
6 to cell infiltration. These events were considered construct/tube failure rather
7 than surgical complications and therefore were not censored in the survival
8 analysis.

9 **Bile duct ligation**

10 C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor,
11 ME). The mice were housed and bred in a Minimal Disease Unit at the animal
12 facility at Oslo University Hospital, Rikshospitalet, Oslo. All experiments were
13 performed on male mice between 8 and 12 weeks of age. A median
14 laparotomy was performed, the common bile duct exposed and ligated close
15 to the junction of the hepatic bile ducts. Sham operated mice underwent the
16 same procedure without ligation. Serum was harvested after 5 days. Alanine
17 transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase
18 (ALP) were measured in serum using an ADVIA 1800 (Siemens) at The
19 Central Laboratory, Norwegian School of Veterinary Science. All animal
20 experiments were approved by the Norwegian Food Safety Authority (project
21 license no FOTS 8210/15) and all animals received human care in line with
22 "Guide for the Care and Use of Laboratory Animals" (National Institutes of
23 Health Publication, 8th Edition, 2011).

24 **Blood sample collection**

1 Blood was taken using a 23g needles directly from the inferior vena cava
2 under terminal anaesthesia at the time the animals were electively culled and
3 transferred into 1.5ml Eppendorf tubes for further processing.

4 **Blood sample processing**

5 The blood samples were routinely processed by the University of Cambridge
6 Core biochemical assay laboratory (CBAL). All of the sample analysis was
7 performed on a Siemens Dimension EXL analyzer using reagents and assay
8 protocols supplied by Siemens.

9 **Light microscopy imaging**

10 Light microscopy images of excised reconstructed gallbladders were acquired
11 using a Leica MZFLIII fluorescence dissecting microscope. The images are
12 representative of 5 animals.

13 **Cryosectioning and Histology**

14 Excised gallbladders were fixed in 4% PFA, immersed in sucrose solution
15 overnight, mounted in optimal cutting temperature (OCT) compound and
16 stored at -80°C until sectioning. Sections were cut to a thickness of 10µm
17 using a cryostat microtome and mounted on microscopy slides for further
18 analysis

19 **Haematoxylin and Eosin (H&E) Staining**

20 H&E staining was performed using Sigma-Aldrich reagents according to the
21 manufacturer's instructions. Briefly, tissue sections were hydrated, treated
22 with Meyer's Haematoxylin solution for 5 minutes (Sigma-Aldrich), washed
23 with warm tap water for 15 minutes, placed in distilled water for 30-60
24 seconds and treated with eosin solution (Sigma-Aldrich) for 30-60 seconds.

1 The sections were subsequently dehydrated and mounted using the Eukitt®
2 quick-hardening mounting medium (Sigma-Aldrich). Histology sections were
3 reviewed by an independent histopathologist with a special interest in
4 hepatobiliary histology (SD).

5 **TUNEL assay**

6 The TUNEL assay was performed using a commercially available kit (abcam,
7 ab66110) according to the manufacturer's instructions.

8 **Fluorescein Isothiocyanate (FITC) cholangiography**

9 In situ FITC cholangiography was performed in sacrificed animals after
10 dissection of the gallbladder free from the adherent liver lobes, but before
11 surgical interruption of the extrahepatic biliary tree. The distal bile duct was
12 cannulated with a 23½ gauge needle and FITC injected retrogradely into the
13 gallbladder and images taken under a fluorescent microscope.

14 **Magnetic Resonance Cholangio-Pancreatography (MRCP)**

15 Magnetic resonance cholangio-pancreatography was performed after sacrifice
16 of the animals. MRCP was performed at 4.7T using a Bruker BioSpec 47/40
17 system. A rapid acquisition with relaxation enhancement sequence was used
18 with an echo train length of 40 echoes at 9.5ms intervals, a repetition time of
19 1000ms, field of view $5.84 \times 4.18 \times 4.18 \text{cm}^3$ with a matrix of $256 \times 180 \times 180$
20 yielding an isotropic resolution of 230 μm . The actively-decoupled four-
21 channel mouse cardiac array provided by Bruker was used for imaging.

22 For the second mouse imaged, for higher signal to noise ratio to give
23 improved visualisation of the biliary ducts a two-dimensional sequence was
24 used with slightly varied parameters (24 spaced echoes at 11ms intervals to
25 give an effective echo time of 110ms; repetition time 5741ms; matrix size of

1 256×256; field of view of 4.33×5.35cm² yielding a planar resolution of
2 170×200µm²). Fifteen slices were acquired coronally through the liver and gall
3 bladder with a thickness of 0.6mm. For this acquisition, a volume coil was
4 used to reduce the impact of radiofrequency inhomogeneity.

5 To examine the biliary ducts and gall bladder, images were prepared by
6 maximum intensity projections. Structural imaging to rule out neoplastic
7 growths was performed using a T1-weighted 3D FLASH (fast low-angle shot)
8 sequence with a flip angle of 25°, repetition time of 14ms and an echo time of
9 7ms. The matrix was 512×256×256 with a field of view of 5.12×2.56×2.56cm³
10 for a final isotropic resolution of 100 µm.

11 The MRCP images were reviewed by 2 independent radiologists with a
12 special interest in hepatobiliary radiology (EMG, SU).

13 **Statistical analyses**

14 All statistical analyses were performed using GraphPad Prism 6. For small
15 sample sizes where descriptive statistics are not appropriate, individual data
16 points were plotted. For comparison between 2 mean values a 2-sided
17 student's t-test was used to calculate statistical significance. The normal
18 distribution of our values was confirmed using the D'Agostino & Pearson
19 omnibus normality test where appropriate. Variance between samples was
20 tested using the Brown-Forsythe test. For comparing multiple groups to a
21 reference group one-way ANOVA with Dunnett correction for multiple
22 comparisons was used between groups with equal variance, while the
23 Kruskal-Wallis test with Dunn's correction for multiple comparisons was
24 applied for groups with unequal variance. Survival was compared using log-
25 rank (Mantel-Cox) tests. Where the number of replicates (n) is given this

1 refers to ECO lines or number of different animals unless otherwise stated.
2 Further details of the statistical analyses performed are provided in
3 Supplementary table 5.

4 For animal experiments, group sizes were estimated based on previous study
5 variance. Final animal group sizes were chosen to allow elective culling at
6 different time point while maintaining $n > 4$ animals surviving past 30 days to
7 ensure reproducibility. No statistical methods were used to calculate sample
8 size. No formal randomization method was used to assign animals to study
9 groups. However; littermate animals from a cage were randomly assigned to
10 experimental or control groups by a technician not involved in the study. No
11 animals were excluded from the analysis. No blinding was used when only
12 one group of animals survived for radiology imaging. In cases, such as
13 gallbladders reconstructed with fibroblasts vs. ECOs where more than one
14 groups survived to be imaged, both radiologists reviewing the images (EG
15 and SU) where blinded to the method of reconstruction.

16

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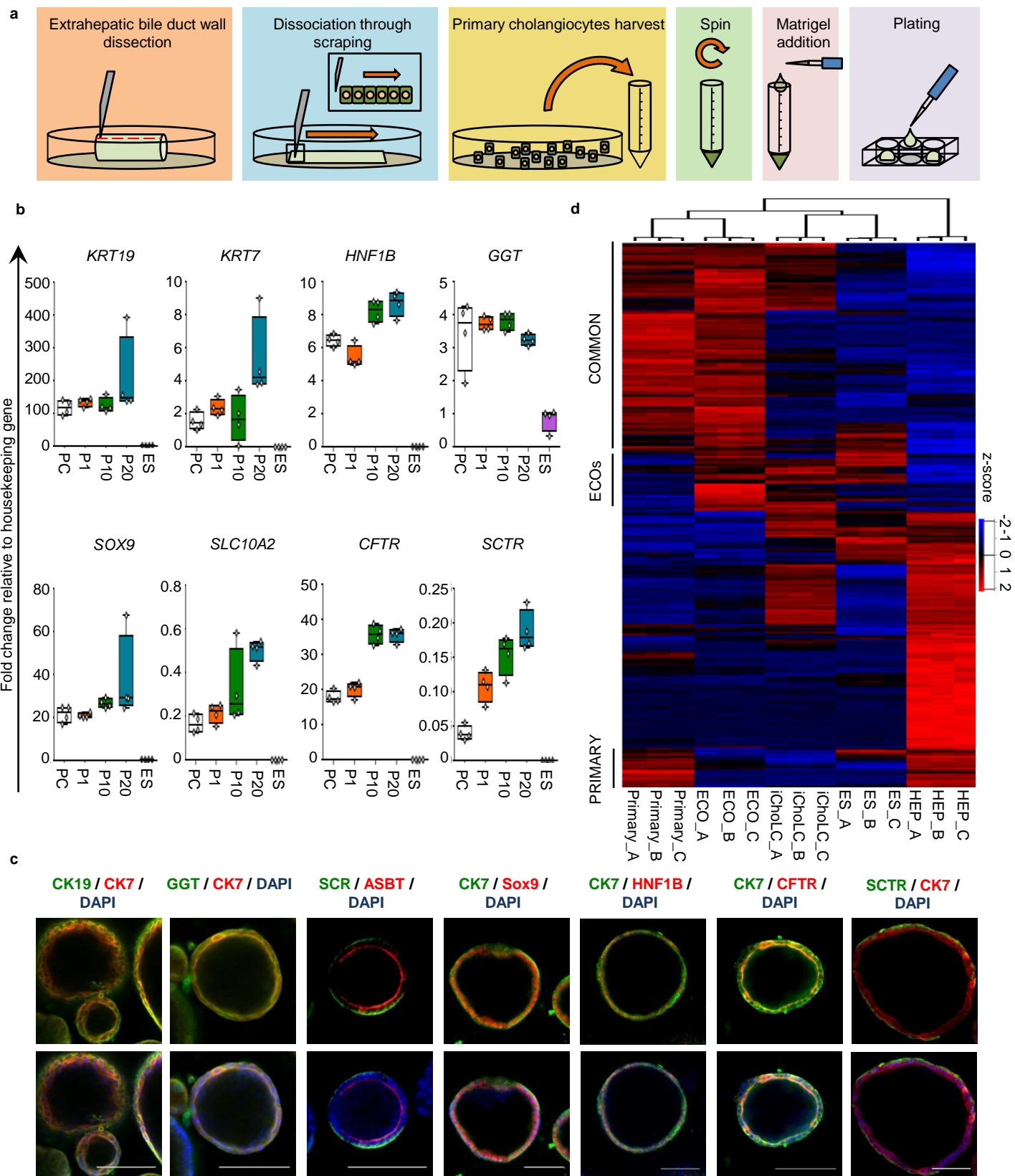
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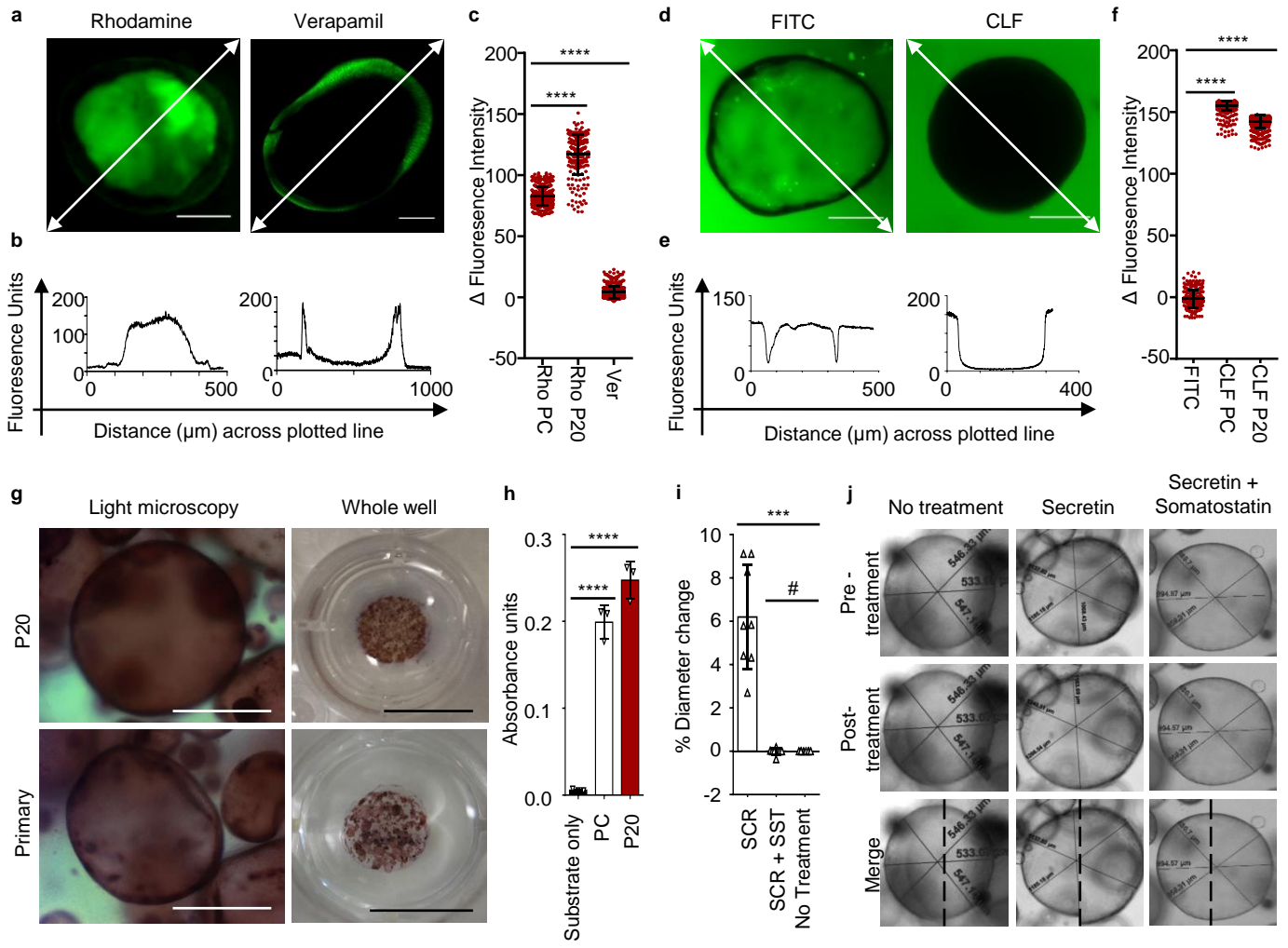
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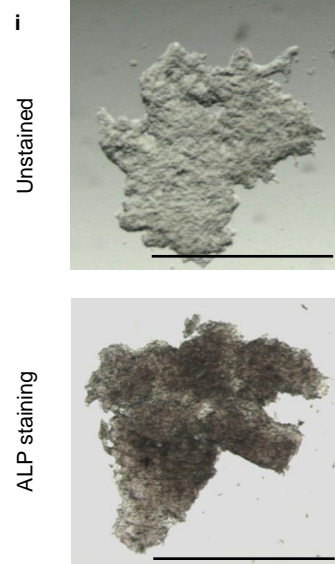
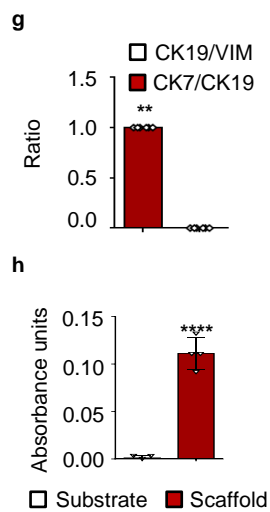
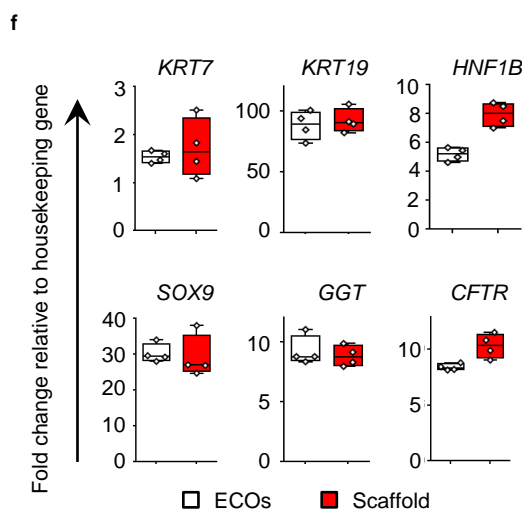
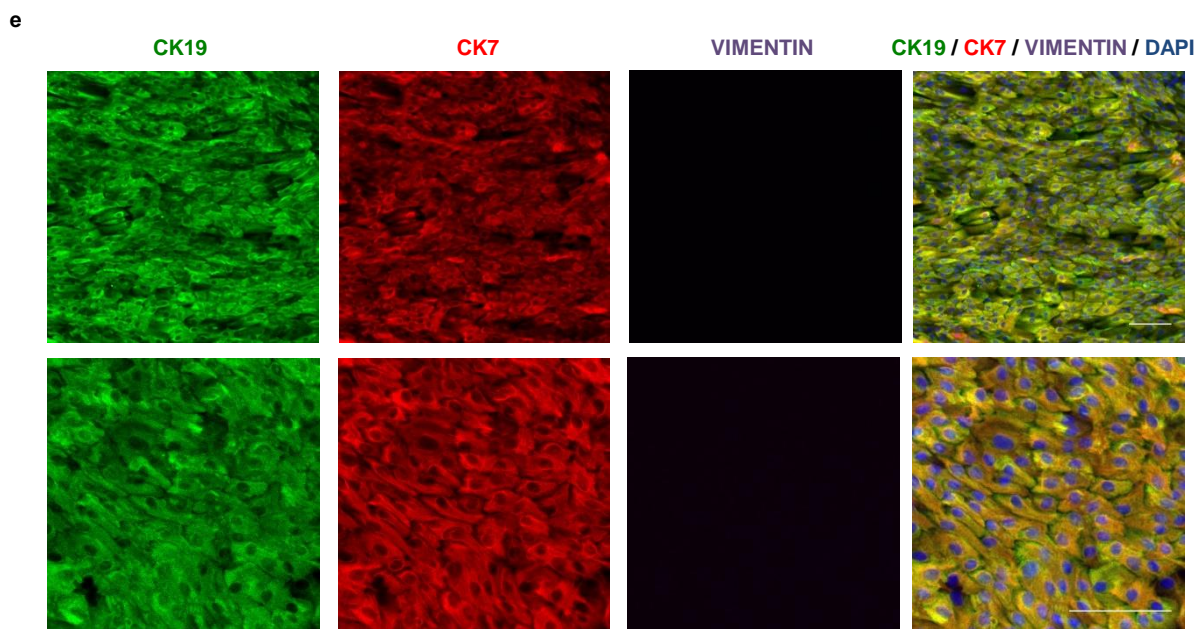
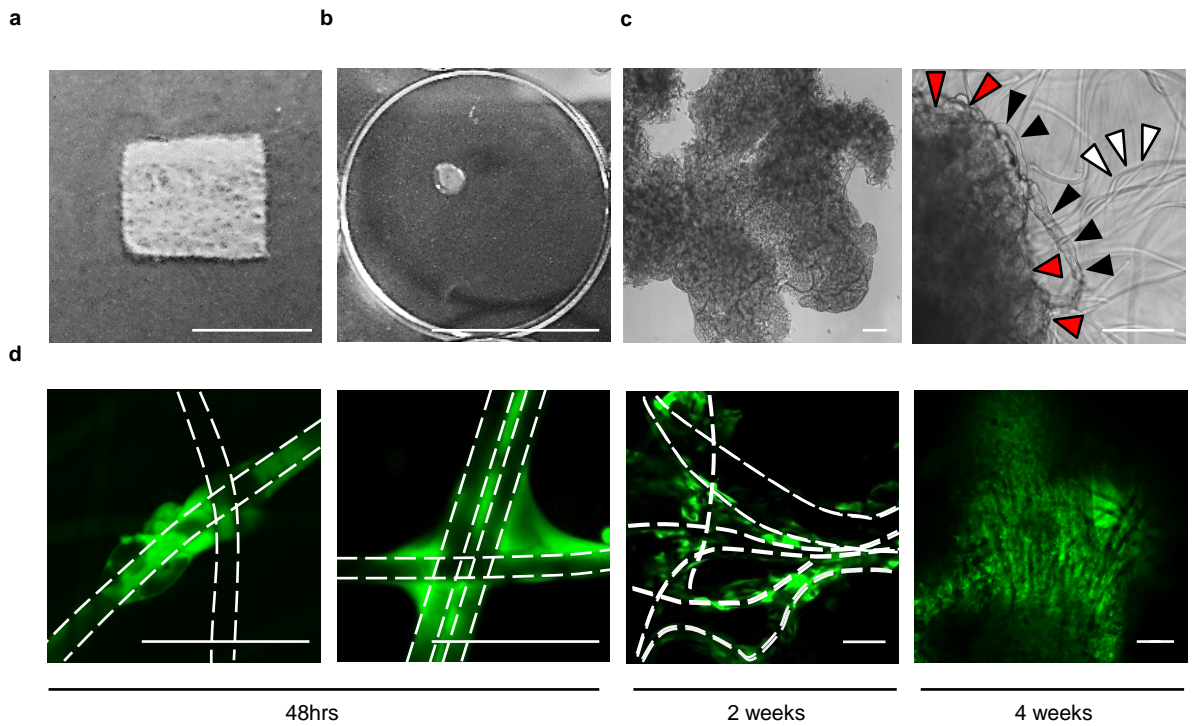
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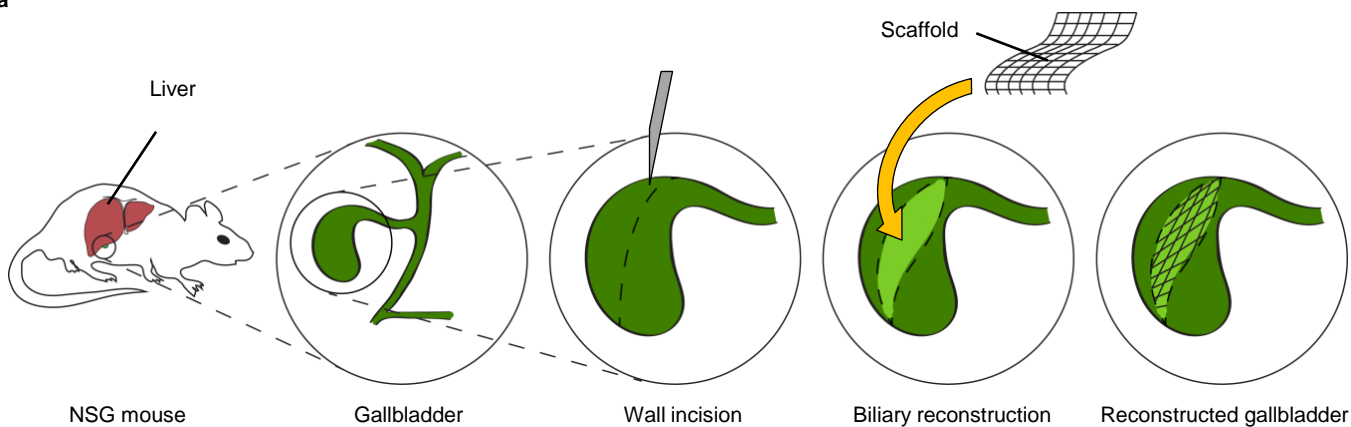
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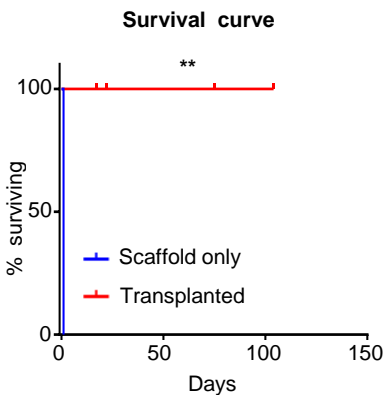




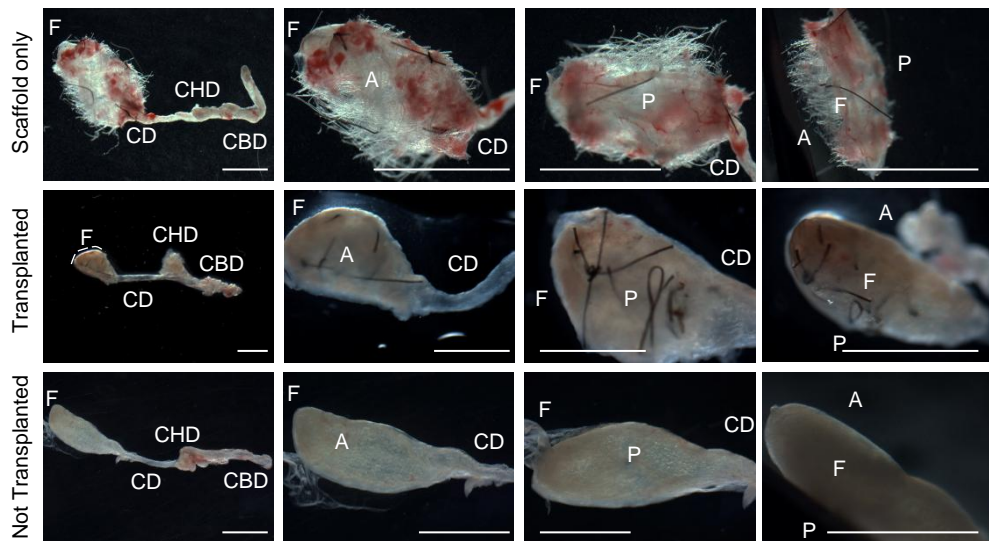
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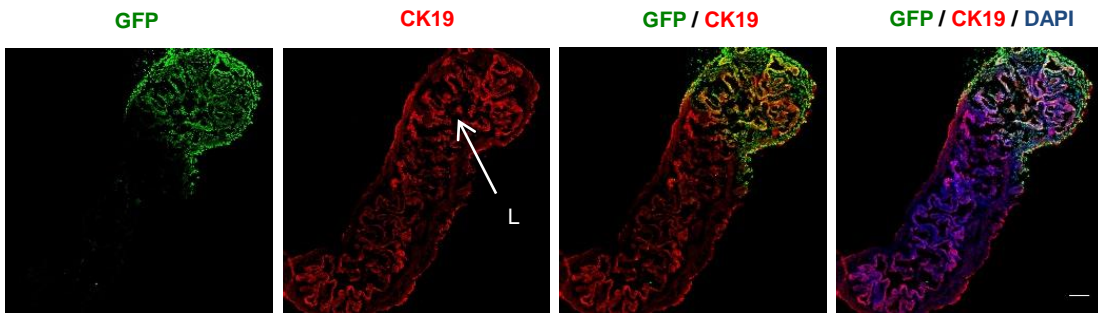
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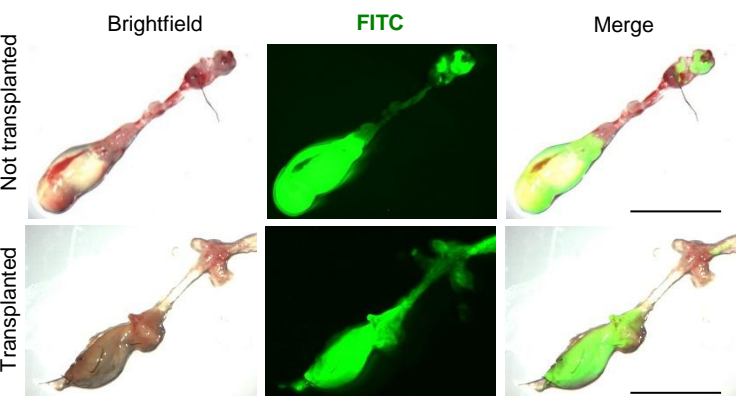
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