Non-canonical Wnt signalling regulates scarring in biliary disease via the planar cell polarity receptors

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The number of patients diagnosed with chronic bile duct disease is increasing and in most cases these diseases result in chronic ductular scarring, necessitating liver transplantation. The formation of ductular scarring affects liver function; however, scar-generating portal fibroblasts also provide important instructive signals to promote the proliferation and differentiation of biliary epithelial cells. Therefore, understanding whether we can reduce scar formation while maintaining a pro-regenerative microenvironment will be essential in developing treatments for biliary disease. Here, we describe how regenerating biliary epithelial cells express Wnt-Planar Cell Polarity signalling components following bile duct injury and promote the formation of ductular scars by upregulating pro-fibrogenic cytokines and positively regulating collagen-deposition. Inhibiting the production of Wnt-ligands reduces the amount of scar formed around the bile duct, without reducing the development of the pro-regenerative microenvironment required for ductular regeneration, demonstrating that scarring and regeneration can be uncoupled in adult biliary disease and regeneration.

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Introduction: Biliary diseases, also known as cholangiopathies, account for approximately one-third of all adult liver disease and 70% of childhood liver disease. Chronic damage to the bile duct typifies biliary disease and results in the proliferation of biliary epithelial cells (BECs) and the formation of a regenerative microenvironment populated by immune cells and fibroblasts. These cells provide both instructive signals to BECs and maintain tissue integrity by depositing scar tissue. This study demonstrates how non-canonical Wnt signalling plays a role in bile duct disease and regeneration and has not been determined.

Here, we demonstrate that Wnt ligands associated with non-canonical Wnt signalling, particularly Wnt5a, are upregulated in biliary injury. In this context, therapeutic inhibition of the Wnt signalling pathway, through the prevention of Wnt-ligand secretion, reduces the level of fibrosis deposited around proliferating BECs, without affecting BEC number. We then go on to demonstrate that Wnt ligands regulate this process through Planar Cell Polarity receptors that activate the JNK/c-JUN signalling pathway specifically in BECs. In turn, this Wnt-PCP signalling plays a role in bile duct disease and regeneration and has not been determined.

Results

Wnt-PCP signalling is activated during duct regeneration. BEC proliferation is required during bile duct regeneration; however, the role that Wnt signalling plays in this process remains controversial, with conflicting reports describing variable roles for Wnt-β-catenin. Using tissue from patients with primary sclerosing cholangitis (PSC), a progressive human biliary disease in which BECs proliferate and also two mouse models of BEC proliferation (thioacetamide, TAA or 3,5-diethoxycarbonyl-1,4-dihydrocollidine, DDC) we sought to determine whether the Wnt-β-catenin pathway is activated in BECs. To do this, we assessed Axin2 mRNA expression and the nuclear translocation of β-catenin in BECs. We failed to see that β-catenin translocates into the nucleus of BECs nor did we see the expected increase in Axin2 expression in any of these contexts (Supplementary Fig. 1).

Despite seeing no changes in these models, we have found that as in many other systems, Axin2 mRNA expression is responsive to changes in canonical Wnt signalling in BECs. In both mouse and human BECs, Axin2 mRNA is increased following β-catenin stabilisation using a GSK3β inhibitor, CHIR99021, and decreases when β-catenin-dependent transcription is inhibited by PR724 (Supplementary Fig. 1a). Therefore, our data suggest that whilst the Wnt-β-catenin pathway can be activated pharmacologically in BECs, activation of this pathway does not increase in BECs during bile duct regeneration. These data are in concordance with recent work showing that BECs do not express LGR proteins necessary for Wnt signalling potentiation, and that LRP-dependent Wnt signalling is dispensable for BEC organoid growth in vitro.

In addition to activating Wnt-β-catenin signalling, Wnt ligands also act via an alternative Wnt pathway known as Wnt-PCP signalling, which, through the activation of Rho-GTPases and JNK/c-JUN, promotes ductular formation in a number of embryonic contexts. In liver tissue from patients with PSC, the number of BECs with phosphorylated JNK (phospho-JNK (Thr183/Y185) is significantly increased, and while c-JUN is expressed broadly within BECs, c-JUN phosphorylation (phospho-c-JUN (S73)) is increased in PSC patients compared with those without disease (Fig. 1a, b), indicating that in ductular regeneration, the Wnt-PCP signalling pathway is likely activated.

Native liver explants from patients with PSC are severely fibrotic/cirrhotic and so represent end-stage disease. Therefore, it is not possible to determine whether Jnk/c-Jun signalling is dynamically regulated during disease progression. To characterise this, we evaluated the expression of phospho-JNK (Thr183/Y185) and phospho-c-JUN (S73) during regeneration in two models of biliary injury and BEC proliferation and found that the number of phospho-JNK (Thr183/Y185) and phospho-c-JUN (S73) positive biliary cells is significantly increased compared with uninjured bile ducts (Fig. 1c, d). Furthermore, in isolated bile ducts from mice with biliary disease, matrix metalloproteinase-7 (Mmp7) and connective tissue growth factor (Ctgf), known c-JUN target genes, which are associated with biliary scarring, are also transcriptionally upregulated (Fig. 1e) and localise to BECs specifically (Fig. 1f, positive and negative RNAscope controls, supplementary Fig. 1h).

Wnt-Jnk/c-Jun regulates biliary scar formation. Previous studies have found that loss of Wnt-ligand secretion following Wntless (Wls) deletion alters biliary regeneration and fibrosis. However, recent data suggesting that these ligands are not functioning through a Wnt-β-catenin pathway in BECs, led us to hypothesise that Wnt ligands activate Wnt-PCP signalling in bile ducts. To validate that Wnt ligands were upregulated in response to injury, we evaluated the mRNA expression of all Wnt ligands in isolated bile ducts from both the DDC and TAA models (Supplementary Fig. 2), thereby identifying a number of ligands that were transcriptionally overexpressed compared with healthy ducts. To resolve whether Wnt ligands are activating a Wnt-PCP signalling pathway during biliary repair, wild-type mice with biliary injury were given LGK974, a highly selective inhibitor of the MBOAT acyltransferase, Purporcin, which is required to add lipid modifications to Wnt ligands (LGK974 from hereon is referred to as Purporcin-i). Inhibition of Purporcin results in the global suppression of Wnt-ligand secretion and renders Wnt ligands unable to bind to the CRD domain of Frizzled. Our experimental strategy is summarised in Fig. 2a.

Following administration of Purporcin-i during biliary regeneration, isolated bile ducts had a reduced transcriptional expression of Ctgf and Mmp7 (Fig. 2b). However, Wnt-β-catenin target genes
including Axin2 were not changed following Porcupine inhibition (Supplementary Fig. 3a). There was an obvious reduction in glutamine synthetase (GS) positivity, a known target of Wnt-β-catenin signalling in hepatocytes (Supplementary Fig. 3b), confirming that Wnt-ligand inhibition across the liver had been successful. In proliferating BECs (in both the TAA and DDC models) Porcupine-i results in a significant reduction in phospho-JNK$^{183/185}$ and phospho-c-JUN$^{573}$ positivity, indicating that in biliary epithelial cells the activation of Jnk/c-Jun signalling is, in part, regulated by Wnt ligands (Fig. 2c).

Having defined that Porcupine-i alters Jnk/c-Jun signalling in BECs we sought to then determine whether the inhibition of Wnt-ligand secretion affects the regeneration of the biliary tree. Whilst the number of BECs (stained for Keratin-19) and fibroblasts (stained for Desmin) remained the same between Porcupine-i and control mice (Fig. 2d), the amount of total fibrillar collagen (determined through staining for Picrosirius Red) and specifically Collagen-1 that is deposited during bile duct regeneration is significantly reduced following Porcupine inhibition (Fig. 2d).

**Fig. 1 Activation of the Jnk-Jun signal in biliary disease.** a Immunohistochemistry on serial sections of either non-diseased (upper panels) or primary sclerosing cholangitis tissue (bottom panels) stained for phosphorylated JNK$^{183/185}$, total c-JUN and phosphorylated c-JUN$^{573}$. Dotted lines demarcate the boundary of bile ducts. Red arrows identify biliary epithelial cells with positive phosphorylated c-JUN$^{573}$ expression. b Quantification of phosphorylated JNK$^{183/185}$, total c-JUN or phosphorylated c-JUN$^{573}$ and the quantification of c-JUN:phospho-c-JUN$^{573}$ in normal human and primary sclerosing cholangitis tissue. c Immunohistochemistry of phosphorylated JNK$^{183/185}$, total c-JUN and phosphorylated c-JUN$^{573}$ in a time course of DDC injury to model fibrotic initiation or in established fibrosis induced by treatment with TAA. Red arrows denote positive nuclei. Dotted lines demarcate biliary epithelial cells and bile ducts. d Quantification of phosphorylated JNK$^{183/185}$, total c-JUN, phosphorylated c-JUN$^{573}$ and the ratio of total c-JUN:phosphorylated c-JUN$^{573}$ in the DDC and TAA models. e mRNA expression of Wnt-PCP target genes in mice undergoing DDC and TAA-induced biliary injury, normalised to the housekeeping gene, Ppia. f RNAscope of mRNA for Ctgf and Mmp7 in healthy bile ducts and following bile duct injury. Red arrows denote RNAscope positivity. Scale bar = 50 μm. p.v.—portal vein, b.d.—bile duct. Porphyrin (labelled) accumulation in the duct is a consequence of the DDC model. Source data are provided as a Source Data file. In graphs where two groups are included, a Student’s t test is used. When comparing multiple groups, a one-way ANOVA with post hoc correction for multiple testing is used. In dot plots, data are presented as mean ± S.E.M. Each data point (N) represents an individual animal or patient.
Fig. 2 Therapeutic inhibition of Wnt reduces scarring. a Schematic of Porcupine inhibition with Porcupine-i during bile duct regeneration induced by DDC or TAA. b mRNA expression of Wnt-PCP pathway target genes Ctgf and Mmp7 in isolated bile ducts following Porcupine-i treatment, normalised to the housekeeping gene Ppia. c Immunofluorescent staining of biliary epithelial cells (Keratin-19-positive, red) with phosphorylated JNKT183/Y185 (upper panels) or phosphorylated c-JUNS73 (lower panels), green. White arrows denote positivity in biliary epithelial cells, yellow arrows show positivity in other, non-Keratin-19-positive cells. Positivity of phosphorylated JNKT183/Y185 and phosphorylated c-JUNS73 quantified in dot plots and represented as a proportion of total biliary epithelial cells counted. d Histological quantification of Keratin-19 (biliary epithelial cells), Desmin (fibroblasts), Picrosirius Red, PSR (fibrillar collagens) and Collagen-1 in livers of mice treated with either DDC or TAA, following Porcupine inhibition. Scale bar = 50 µm. p.v. — portal vein, b.d. — bile duct. Source data are provided as a Source Data file. In all comparisons, a Student’s t test is used. Box-whisker plots represent min–max range of the data. In dot plots, data are presented as mean ± S.E.M. Each data point (N) represents an individual animal.

Given that the inhibition of Wnt-ligand secretion is sufficient to alter Jnk/c-Jun signalling and regulate biliary fibrosis, we then sought to define the origin of the Wnt ligands involved in this process. Previous work from our lab and others has shown that myeloid cells (including macrophages) are a source of multiple Wnt ligands3,9,45, including the archetypal Wnt-PCP ligand, Wnt5a46, and indeed, in CD45+/CD11b+ macrophages isolated from the livers of mice with biliary injury, we found that macrophages expressed WNT5A. Moreover, in macrophages from mice treated with porcupine-i, 50% of cells retained WNT5A in their ER compared with 32% in vehicle-treated animals (Supplementary Fig. 3c–f). WNT5A is transcriptionally upregulated in liver patients with PSC compared with livers from patients without underlying disease (Supplementary Fig. 4a), and similarly, in mouse models of biliary regeneration Wnt5a transcript expression is induced following injury (Fig. 3a). In these murine models of bile duct regeneration and BEC proliferation, CD68-positive macrophages, but not Desmin-positive myofibroblasts, in close proximity to BECs express Wnt5a mRNA (Supplementary Fig. 4c) and WNT5A protein47 (Fig. 3b). Moreover, in PSC patient tissues WNT5A-positive cells localise to the scars surrounding the bile duct (Supplementary Fig. 4b), suggesting that the provision of Wnt ligand by macrophages could promote biliary fibrosis.

To understand whether Wnt5a-expressing myeloid cells regulate biliary scarring, we made use of a mouse line in which Cre recombinase is expressed by myeloid lineages to delete Wnt5a specifically in these cells (LysMCre::Wnt5a ΔBox,
Fig. 3 Myeloid Wnt5a promotes biliary scar deposition. a mRNA expression of Wnt5a in whole liver from mice with either DDC (left panel) or TAA (right panel) induced biliary disease. b Immunofluorescence showing the localisation of WNT5A protein (red) to CD68-positive cells (green, left panel), not αSMA-positive cells (green, right panel), yellow arrows show dual-positive cells. c mRNA from DDC-treated mice showing loss of Wnt-PCP target genes, Ctgf and Mmp7, normalised to Ppia in isolated bile ducts following deletion of Wnt5a from monocyte lineages. d Histological quantification of Keratin-19 (biliary epithelial cells), Desmin (fibroblasts), Picrosirius Red, PSR (fibrillar collagens) and Collagen-1 in livers of mice treated with DDC following myeloid-specific deletion of Wnt5a. e mRNA from TAA-treated mice showing loss of Wnt-PCP target genes, Ctgf and Mmp7 normalised to Ppia in isolated bile ducts following deletion of Wnt5a from monocyte lineages. f Histological quantification of Keratin-19 (biliary epithelial cells), Desmin (fibroblasts), Picrosirius Red, PSR (fibrillar collagens) and Collagen-1 in livers of mice treated with TAA following myeloid-specific deletion of Wnt5a. Scale bar = 50 μm. Source data are provided as a Source Data file. In graphs where two groups are included, a Student’s t test is used. Box-whisker plots represent min-max range of the data. In dot plots, data are presented as mean ± S.E.M. Each data point (N) represents an individual animal.

Supplementary Fig. 4d–g). Following biliary injury with either DDC or TAA, isolated bile ducts showed a reduced mRNA expression of Ctgf and Mmp7 when myeloid Wnt5a was deleted compared with ducts isolated from mice in which Wnt5a was intact (Fig. 3c, e), but did not show changes in Wnt-β-catenin pathway target expression (Supplementary Fig. 4h). Moreover, in both models of bile duct regeneration, the amount of PSR staining and Collagen-1 immunostaining was significantly reduced following Wnt5a deletion in myeloid cells, without affecting the number of keratin-19-positive BECs or Desmin-positive myofibroblasts (Fig. 3d, f). Interestingly, in our acute (DDC) model of biliary regeneration, high Wnt5a expression does not persist throughout injury. When Wnt5a is deleted from myeloid cells specifically at later time points in this model, the changes in Collagen-1 and PSR are lost, suggesting that either Wnt5a is only required for the initiation of ductular fibrosis in this context or that other Wnt ligands or signalling pathways compensate for Wnt5a loss in myeloid cells.

Wnt-PCP receptors regulate ductular scarring. There is a high level of redundancy within the Wnt-ligand family and conflicting data as to whether Wnt ligands are faithful activators of particular downstream receptors. Therefore, we sought to validate our findings that Wnt ligands and particularly Wnt5a regulate biliary scarring through Wnt-PCP activation. In Wnt-PCP signalling a number of receptors, including Frizzled receptors and orphan receptor tyrosine kinases such as ROR1/2 and PTK7 are
known to bind Wnt ligands. These then converge on the scaffolding proteins VANGL1 and VANGL2, which become phosphorylated, a process that is necessary for Wnt-PCP activity. VANGL1 and VANGL2 are transcriptionally upregulated in PSC patients. Moreover, in PSC patients, VANGL2 and PTK7 proteins localise to the plasma membranes of proliferating BECs, but not in healthy bile ducts suggesting that upregulation of Wnt-PCP pathway components occurs in biliary disease.

As Vangl2 is functionally dominant over its homologue Vangl1, we elected to study whether Wnt signalling via Vangl2 regulates the formation of biliary scars. Similar to human, healthy mouse bile ducts express low levels of Vangl1 and Vangl2 at the transcriptional level, and positive staining for VANGL1/2

Fig. 4 Wnt-PCP receptors are expressed by regenerating bile ducts. a mRNA expression of VANGL2 in healthy liver tissue and tissue from patients with primary sclerosing cholangitis. b Immunofluorescence of VANGL2 (red) and PTK7 (green) in tissue from healthy liver and patients with primary sclerosing cholangitis. c mRNA expression of Vangl2 and Ptk7 normalised to Ppia in isolated bile ducts and whole liver from DDC-induced ductular regeneration. d mRNA expression of Vangl2 and Ptk7 in animals with TAA-induced biliary injury normalised to Ppia. e Upper panels: RNAScope of Vangl2 mRNA in DDC and TAA-induced bile duct regeneration (yellow arrows denote positivity and dotted lines denote bile ducts). Lower panels: immunohistochemistry for VANGL2 (green) and PTK7 (red) in healthy bile ducts, or bile ducts injured with either DDC or TAA. White arrows denote PTK7 single positive stromal cells. Yellow arrows denote biliary epithelial cells that express both VANGL2 and PTK7 in biliary epithelial cells. Scale bar = 50 µm. Source data are provided as a Source Data file. In graphs where two groups are included, a Student’s t test is used. When comparing multiple groups, a one-way ANOVA with post hoc correction for multiple testing is used. Box-whisker plots represent min–max range of the data. In dot plots, data are presented as mean ± S.E.M. Each data point (N) represents an individual animal or patient.
proteins in the plasma membranes of BECs is infrequent (Fig. 4c–e and Supplementary Fig. 5b). Similarly, in healthy BECs, Ptk7 expression is low, although PTK7-positive portal fibroblasts can be seen surrounding healthy ducts (Fig. 4e). Following bile duct injury with either DDC or TAA, Vangl2 and Ptk7 mRNA levels increase in isolated bile ducts (Fig. 4e and Supplementary Fig. 5b). Similarly, in healthy Vangl2WT mutant mice compared with controls.

Wnt-PCP signalling can be effectively inhibited through modulating Vangl2 activity using a semi-dominant hypomorphic Vangl2 mutant, Vangl2S464N, also known as Looptail22. Mice homozygous for this mutation die shortly after birth; however, heterozygous mice (Vangl2S464N/+, hereafter denoted as Vangl2S464N) are viable, reach adulthood and have been used to determine the role of Vangl2 in a number of adult contexts53. In isolated bile ducts from Vangl2S464N mice given either DDC or TAA injury (Fig. 5a), we found a significant reduction in the mRNA expression of Mmp7 and Ctgf (Fig. 5b, c), indicating that in both short-term and long-term fibrotic injury, signalling downstream of BEC-expressed VANGL2 regulates Ctgf and Mmp7 levels. Having shown that mutations in Vangl2 phenocopy the transcriptional changes seen with Porcupine inhibition (Fig. 2) we sought to define whether mice carrying the Vangl2S464N mutation had a reduced ability to form bile duct scars following injury. Following DDC or TAA injury, mice with wild-type Vangl2 developed progressive ductular fibrosis. In littermates that carried a heterozygous mutation, however, the level of scarring was significantly reduced (Fig. 5d). Interestingly, mutations in Vangl2 did not affect the number of BECs (Fig. 5e), nor did reduced VANGL2 function result in changes in the number of Desmin-positive fibroblasts (Fig. 5f) found within the liver. In concordance with the mRNA expression (Fig. 5b, c), the levels of CTGF protein were significantly reduced in Vangl2S464N mice following bile duct injury compared with control littermates (Fig. 5g). Given that the Vangl2S464N mutant mouse phenocopies Wnt-ligand loss following Porcupine inhibition, we suggest that Wnt-PCP signalling is required for the establishment of biliary scars in the mouse.

The Vangl2S464N mutation is constitutive, and therefore we could not preclude that there were developmental deficiencies in bile duct development and patterning, which are simply
exacerbated during ductular regeneration. To overcome this, we utilised a transgenic mouse line in which Vangl2 can be specifically deleted in cells (Vangl2\textsuperscript{lox/lox})\textsuperscript{54}.

To confirm that Vangl2 can be deleted in the BEC lineage, we isolated bile ducts from Vangl2\textsuperscript{lox/lox} mice or wild-type littermates and grew these as organoids (Fig. 6a). Following infection with lentiviral-Cre (Lv-Cre), BECs in these organoids recombine floxed alleles in a mosaic way (Supplementary Fig. 6a). Furthermore, Vangl2\textsuperscript{lox/lox} mice grow normally following expression of Cre, however they have significantly reduced Vangl2 mRNA expression (Supplementary Fig. 6b). Additionally, Vangl2 protein levels are reduced and Vangl2 no longer localises to the plasma membrane of BECs in these organoids (Fig. 6a, b, from herein these organoids are known as Vangl2\textsuperscript{ATM}). Following Vangl2 loss, Vangl2\textsuperscript{ATM} organoids show reduced levels of nuclear phospho-c-JUN\textsuperscript{573} (Fig. 6a, lower panels), and whilst the levels of total JNK and c-JUN remain unchanged in Vangl2\textsuperscript{WT} versus Vangl2\textsuperscript{ATM} organoids, BECs in organoids lacking Vangl2 have notably reduced levels of phosphorylated JNK\textsuperscript{T183/Y185} and have a 40% reduction of phosphorylated c-JUN\textsuperscript{573} (Fig. 6b). Furthermore, VANGL2 loss significantly reduces the mRNA levels of Mmp7 and Ctgf (Fig. 6c), confirming that the levels of these transcripts relate to signalling through Vangl2.

Having confirmed that Vangl2-specific deletion in BECs alters Wnt-PCP signalling in vitro, we generated a mouse in which Vangl2 could be specifically deleted in BECs following administration of tamoxifen (K19CreERT\textsuperscript{+}/Vangl2\textsuperscript{lox/lox}). In K19CreERT\textsuperscript{+}/Vangl2\textsuperscript{lox/lox} mice, following injury, we failed to detect phospho-JNK\textsuperscript{T183/Y185} or phospho-c-JUN\textsuperscript{573} in Vangl2\textsuperscript{ATM} BECs (Fig. 6d). Importantly, Vangl2\textsuperscript{ATM} cells (lineage traced with RFP) are retained in vivo in the bile duct following injury (Supplementary Fig. 6c and d), indicating that Vangl2 is not an essential gene for BEC survival. Yet, following Vangl2 deletion, the amount of Mmp7 and Ctgf mRNA in isolated bile ducts and CTGF protein was significantly reduced (Fig. 6e, f). Moreover, the levels of total fibrillar collagen assessed through Picrosirius Red staining and Collagen-1 specifically, surrounding these Vangl2\textsuperscript{ATM} BECs, were also reduced (Fig. 6g) without affecting the number of portal

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fibroblasts (Fig. 6h), confirming that lineage-specific deletion of Vangl2 in BECs is sufficient to reduce biliary scarring and validating the phenotype found using Vangl2S464N mutant mice.

Discussion
Canonical Wnt-β-catenin signalling is widely regarded as a principal regulator of the cell cycle in adult tissue homeostasis and regeneration, where the stabilisation of β-catenin and its translocation into the nucleus promotes the transcription of a number of pro-proliferative and pro-survival pathways in a plethora of tissues. In the context of cancers too, the role of Wnt-β-catenin has been widely and extensively studied as mutations in β-catenin and in its hallmark regulators APC and Axin promote deregulated cell proliferation and are now considered oncogenes in a wide array of contexts.

Wnt ligands do however, interact through a number of highly conserved developmental pathways, which likely evolved to regulate the formation of the anterior–posterior axis in the earliest multicellular organisms, and establish polarity in groups of cells. This pathway, the Wnt-Planar Cell Polarity (Wnt-PCP) pathway, is necessary for establishing any number of axes within the embryo, and through its control of cell shape, migration and planar cell movement is absolutely required for normal organogenesis. The role of Wnt ligands in establishing these body plans likely predates their role in activating β-catenin-dependent transcription, yet whether Wnt-PCP signalling plays a role in embryonic or postnatal contexts is in agreement; however, whereas the role of Wnt ligands, through Wnt-PCP receptor activation, including Ptk7 and Vangl2, results in the phosphorylation of Jnk, which promotes c-Jun-dependent transcription. These observations are concordant with scRNA data in which the AP-1 transcription factor network is enriched in BECs. Inhibition of Wnt ligands, either genetically or using a therapeutic Wnt inhibitor, significantly decreases the amount of biliary scarring that occurs following injury, leading us to demonstrate that Wnt-PCP signalling is upstream of fibrogenic cytokine production. Surprisingly, heterozygous loss of Vangl2, through the Vangl2S464N mutation, is sufficient to reduce biliary scarring, suggesting that this system requires tight regulation. Emerging data from other chronic fibrotic diseases indicate that Wnt-PCP signalling can play a role in the establishment and maintenance of scarring and deposition of the extracellular matrix; here we show that Wnt-PCP is also involved in the formation of biliary scars in the adult liver.

Adult tissue repair, by its very nature, requires fine balance between epithelial regeneration and scarring. While fibroblasts in the regenerative microenvironment are the primary cells that make scar tissue, we and others have previously shown that the fibroblast microenvironment in biliary regeneration provides a number of signals that BECs require to maintain both their proliferation and lineage specification. Deleting these profibrogenic cells or inhibiting their activation/proliferation would therefore have significant consequences for the regenerating biliary epithelium and could ultimately prevent ductular regrowth following injury. Inhibition of Wnt signalling or loss of Wnt-PCP receptors on proliferating BECs does not affect the formation of the cellular regenerative microenvironment, with pro-regenerative fibroblast numbers and the numbers of BECs remaining constant following inhibition. Our data demonstrate that it is possible to pharmacologically uncouple regeneration from scar formation in the diseased bile duct and suggest that inhibition of Wnt-ligand production should be considered as a therapy in chronic biliary diseases.

Methods
Mouse models. All mice were maintained in 12-h light/dark cycles and had access to food and water ad libitum in accordance with UK Home Office Regulations. All experiments were performed under UK Home Office licence PPL 70/8150 held by Dr Luke Boulet; mice were euthanised by an escalating dose of CO2. Animal experiments were given ethical approval by the University of Edinburgh Animal Welfare Ethical Review Board (AWERB).

For the DDC mouse model, 6–8-week-old, male CD1 mice were fed 0.1% 5-diethoxy carbonyl-1,4-dihydrocollidine (DDC) in their diet for up to 14 days and provided with normal drinking water. Mice that drop >20% of their bodyweight were given DDC food softened with water. For the TAA model, mice were given 400 mg/kg thiocetamide in sweetened water for up to 12 weeks. Thiocetamide water was present throughout the model and mice would access this ad libitum. In studies where mice received the Porcupine inhibitor, LGK974 experimental mice were dosed with 5 mg/kg LGK974, twice daily via oral gavage (the vehicle for this was 0.5% methylcellulose and 0.5% Tween-80 in D/H2O). Mice were dosed for 48 h prior to the administration of DDC to ensure inhibited Wnt-ligand production prior to injury. Effectiveness of LGK974 was confirmed through reduction of GS
staining adjacent to central veins and is known to be Wnt-ligand dependent. Finally, to generate livers that overexpress β-catenin

We used a hydrodynamic model in which 20 µg of plasmid 990 beta-catenin (a gift from Xin Chen (Addgene plasmid # 86499; http://n2t.net/addgene/86499; RRID: Addgene_86499) was co-injected with 6 µg of PT2/C-Luci/PGK-SB13 (a gift from John Ohlﬁest (Addgene plasmid # 20207; http://n2t.net/addgene/20207; RRID: Addgene_20207)). These plasmids were delivered in 10% v/v physiological saline in a 5 µl volume.

Vang2/eGFP mice were provided by Dr Ping Chen, Emory University, USA, and oxed allele. All genetically engineered mice were genotyped by Transnetyx Inc. Mouse RNAScope. RNAScope was performed on formalin-fixed liver tissue. Four-micrometre sections were used throughout, and all RNAScope performed in this study was done by Aqualia Histoplex, Edinburgh, with the exception of Axi2, which was performed at the core histology facility, The Beatson Institute, Glasgow.

Bile duct isolation. To isolate bile ducts from both uninjured and injured livers, diversions were made, the liver was chopped into 0.5 mm³ pieces and digested in 0.3 mg/ml of Collagenase and DNASe-I (Roche) containing Collagenase-IV (Roche) and DNASe-I (Roche). Following digestion and dissociation, bile ducts become obvious as parenchyma is digested away. Bile ducts are strained through a 70-µm filter and extensively washed in PBS to remove any residual cells. Bile ducts are then used for downstream applications.

Organoid culture. Liver organoids were derived from bile ducts of injured mice. Briefly, bile ducts were dispersed in 100% Matrigel. Within 24 h, bile ducts form closed structures, and within 48 h budding can be seen from the duct. Following expansion, these ducts were removed from Matrigel by incubating with ice-cold Versene. Organoids were dissociated with pipetting and then re-plated in fresh 100% Matrigel. This process was repeated to expand organoids. The growth medium used in this study consisted of a base media of DMEM/F-12 supplemented with Glutamax, Penicillin/Streptomycin, and 50 ng/ml recombinant HGF, EGFR, FGFR1, Gastrin, Nicotinamide, N-Acetylcycteine, B-27, Forskolin, N-Acetylcysteine, and Nicotinamide, N-Acetylcycteine. For assays in which signalling was monitored in vivo, organoids were cultured in base media containing Collagenase-IV (Roche) and DNAse-I (Roche). To evaluate the effects of Vangl2 knockout on bile duct organoids (Vangl2fl/fl) versus control organoids (Vangl2WT/WT) Cre was expressed from the CMV promoter, in vitro, by injection of organoid structures with LV-CMV-Cre (University of Edinburgh, SRF facility) at an MOI of 5. For infection media was supplemented with 1/100 of Transdux reagent with Glutamax, Penicillin/Streptomycin, 25 μg/ml Tryptoptic Soybean Inhibitor, 1x Insulin/Transferrin/Selenium, 13.4 μg/ml Bovine Pituitary Extract, 3.93 μg/ml Dexamethasone, 3.4 μg/ml T3 (3,3,5-triiodo-l-thyronine), 0.025 μg/ml IGF and 4 μg/ml Forskolin, and were pH adjusted with 3.4 N NaOH.

Preparation of bone marrow-derived macrophages. Total bone marrow was isolated from adult mouse femurs and grown in DMEM/Ham’s F-12 media containing glutamine, 10% FCS, Penicillin/Streptomycin and 50 mg/ml recombinant MCSF in low-attachment ﬂasks. After 48 h, the concentration of MCSF was reduced to 25 ng/ml, and cells were maintained in this media for five further days. Following differentiation, bone marrow-derived macrophages (BMDM) were plated at 1 x 10⁶ cells/cm² and left to activate for 48 h, at which point they were used experimentally.

FACS isolation of hepatic macrophages. Dissected liver lobes were minced and then digested for 2 h in DMEM/F-12 nutrient media containing 1% of FCS, 0.5 mg/ml Collagelase, 0.5 mg/ml Dispase and 0.1 mg/ml DNase1. Following digestion, liver tissue was washed through a 70-µm filter. Remaining cells and large cell clumps were removed from the cell mixture by slow speed centrifugation at 50 x g. The remaining cells were then pelleted and red cells lysed using Red Cell Lysis buffer (Gibco). Following lysis, cells were blocked in PBS containing BSA and sodium azide for 30 min on ice. Following blocking, cells were incubated in blocking buffer with CD11b-PE and CD45-PE-Cy7 (Biolegend) antibodies for 1 h at room temperature and were then sorted using a FACS Aria II. Dead cells were excluded from sorting using DAPI.

Table 1 qPCR primers used in this study.

| Provider | Catalogue number |
|----------|------------------|
| Mouse    |                  |
| Axin2    | QIAGEN            |
| Ccd1l1   | QIAGEN            |
| Ctgf     | QIAGEN            |
| Lef1     | QIAGEN            |
| Mmp7     | QIAGEN            |
| Myc      | QIAGEN            |
| Ppia     | QIAGEN            |
| Ptk7     | QIAGEN            |
| Vang1    | QIAGEN            |
| Vang2    | QIAGEN            |
| Wnt5a    | QIAGEN            |
| PPA      | QIAGEN            |
| VANG1L   | SA Biosciences    |
| VANG1L2  | SA Biosciences    |
| WNT5A    | SA Biosciences    |
Western blotting: Isolated bile ducts or organsoids were lysed in RIPA buffer containing phosphatase (Thermo Fisher) and protease (MiniComplete, Roche) inhibitors. Protein quantification was determined using Pierce BCA reagent (Pierce) and quantified using a nanodrop. The standard curve for protein quantification was derived from the BCA reagent handbook using Albumin standards provided. In all, 20 μg of total protein was loaded onto a 4–12% NuPage Bis-Tris gel (Thermo Fisher). Prior to running, proteins were reduced with NuPage LDS sample buffer (4×) and NuPage Sample Reducing Agent (10×). All gels were run gel (Thermo Fisher). Prior to running, proteins were reduced with NuPage LDS sample buffer (4×) and NuPage Sample Reducing Agent (10×). All gels were run

| Primary antibodies | Provider | Catalogue number | Conditions |
|--------------------|----------|------------------|------------|
| Alpha smooth muscle actin (1A4) | Sigma Aldrich | A2547 | 15 m Citrate. 1/2000 dilution O/N |
| β-actin | Cell Signalling Technologies | 8457P | 1/5000 O/N (western blot) |
| β-catenin | BD Transduction Laboratories | 610154 | 1h Citrate 1/50 O/N |
| Calnexin | Enzo | ADI-SPA-860-D | 1/2000 O/N |
| CD45-PE | BioLegend | 103105 | 1 μl per 1 x 10^6 cells, 30 m RT |
| CD11b-PECy7 | BioLegend | 101215 | 1 μl per 1 x 10^6 cells, 30 m RT |
| CD68 (KP1) | Dako | M081401-2 | 30 m Citrate. 1/100 dilution O/N 1/1000 O/N (western blot) |
| c-JUN (total) (60A8) | Cell Signalling Technologies | 9165 | 30 m Citrate. 1/100 dilution O/N 1/1000 O/N (western blot) |
| Collagen-1 | Southern Biotech | 1310-01 | 5 m Citrate. 2/2000 dilution 72 h |
| Ctgf | Abcam | ab6992 | No antigen retrieval 1/100 O/N |
| Desmin (Y66) | Abcam | Ab32362 | 15 m Citrate. 1/100 dilution O/N |
| E-cadherin DECMA-1 | Genetex | GTX11512 | 1/100 O/N |
| GPP | Abcam | ab13970 | 5 m Tris-EDETA. 1/500 O/N |
| Glutamine Synthetase | Abcam | ab64613 | 10 m Citrate 1/100 |
| Keratin-19 (TROMA-III) | Developmental Studies | Troma-III | 10 m Citrate. 1/200 dilution O/N |
| p-cJUN (ser73) (D479G) | Cell Signalling Technologies | 3270 | 30 m Citrate. 1/100 dilution O/N |
| p-cJUN (ser63) (54B3) (western blot) | Cell Signalling Technologies | 2361 | 1/1000 O/N |
| JNK (total) | Cell Signalling Technologies | 9252 | 30 m Citrate. 1/100 dilution O/N 1/1000 O/N (western blot) |
| βJNK (Thr183/Tyr185) (81E11) | Cell Signalling Technologies | 4668 | 30 m Citrate. 1/100 dilution O/N |
| PTK7 | Source Bioscience | LS-B10725 | 10 m Tris-EDETA 1/100 O/N |
| RFP | Abcam | ab62341 | 10 m Citrate. 1/100 dilution O/N |
| Vangl1 | Sigma Aldrich | HPA025235 | 10 m Tris-EDETA 1/100 O/N |
| Vangl2 | Sigma Aldrich | HPA027043 | 10 m Tris-EDETA 1/100 O/N |
| Vangl2 (western blot) | R&D Systems | AF-4815 | 1/500 O/N |
| Wnt5a | LS Bio | LS-B4565 | 10 m Tris-EDETA 1/100 O/N |

Secondary antibodies

- Anti-goat Alexa 594 | Thermo Fisher | A-27016 | 1/500 1 h RT |
- Anti-goat Biotinylated | Vector Laboratories | BA-5000 | 1/500 1 h RT |
- Anti-mouse Alexa 488 | Thermo Fisher | A32723 | 1/500 1 h RT |
- Anti-rabbit Alexa 594 | Thermo Fisher | A-21207 | 1/500 1 h RT |
- Anti-Rabbit Biotinylated | Vector Laboratories | BA-1000 | 1/500 1 h RT |
- Anti-rabbit HRP | Vector Laboratories | PI-1000 | 1/500 1 h RT |
- Anti-rat Alexa 488 | Thermo Fisher | A-21208 | 1/500 1 h RT |
- Anti-rat Alexa 594 | Thermo Fisher | A-21209 | 1/500 1 h RT |
- Tyramide Alexa 594 | Thermo Fisher | B40957 | 1/50 10 m RT |
- Tyramide Alexa 488 | Thermo Fisher | B40953 | 1/50 10 m RT |

Plasmid preparation. Both the pT3-EF1αH N90-beta-catenin and PT2/C-Luc/PGK-SB13 plasmids were grown in LB media, containing 100 μg/ml ampicillin overnight. Cells were pelleted and plasmids were prepared with a Qiagen endotoxin-free maxiprep kit, as per the manufacturer's instructions. Plasmid concentration was determined using a Nanodrop.

Image analysis. Image analysis was conducted using ImageJ and macros written by Dr Tim J Kendall. Macros are available on request.

Statistics. All experimental groups were analysed for normality using a D’Agostino–Pearson omnibus test. Groups that were normally distributed were compared with either a two-tailed Student’s t test (for analysis of two groups) or using one-way ANOVA to compare multiple groups, with a post hoc correction for multiple testing. Non-parametric data were analysed using a Wilcoxon–Mann–Whitney U test when comparing two groups or a Kruskall–Wallis test when comparing multiple non-parametric data. Throughout p < 0.05 was considered significant. Data are represented as mean ± S.E.M. for parametric data or median with S.D. for non-parametric data.

Study approval. All animal experiments were approved by the University of Edinburgh local ethics committee and were licensed by the UK Home Office. All patient material contained in this paper was approved by the NHS Lothian Bio resource ethics committee. No prospective tissue was collected in this study.

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

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. The source data underlying Figs. 1b, d, e, 2b–d, 3a, c–f, 4a, c, d, 5b–g and 6c, e, and supplementary Figs. 1a, e–g, 2a, 3a, f, 4a, d, e, b, 5a and 6b, c are provided as a Source Data file.

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Author contributions

D.H.W., E.I.J., R.P.M., M.L.W., S.H.W., P.T., A.T.R.N., N.T.Y. and A.R. performed experiments and analysed data. S.R.B. and D.O.R. bred, maintained and provided transgenic lines used in this study. S.W.O.D. and F.G.S. provided tissue and expertise in bile duct biology, they also provided sponsorship and financial support for A.T.R.N. P.C. provided advice and the Vangl2GFP knock-in mouse line through collaboration. C.H.D. collaborated on provision of the Vangl2S464N mouse line and Vangl2S464N tissues. D.I.H. generated and provided the Vangl2Glow/RFP mouse line. J.M.B. generated and provided the normal human cholangiocyte (NHC-3) primary cultures. O.J.S. provided RNAscope data and funding support for A.R. T.J.K. provided pathological support, wrote the macros for image analysis, contributed to the direction of the project and edited the paper. L.B. conceived and provided funding for the project, conducted experiments, analysed data, compiled, wrote the paper and led the project.

Competing interests

The authors declare no competing interests.

Additional information

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