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Research Article

Origin of pancreatic endocrine cells from biliary duct epithelium

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Abstract. We describe an explant culture system to study the formation of pancreatic-type endocrine cells by the biliary tract. In this model, β-cells and other endocrine cells appear in the biliary duct epithelium and their number increases. Evidence for an origin from the duct epithelium is threefold. Firstly, differentiating cells transiently co-express insulin and bind *Dolichos* lectin. Secondly, β-cells in cultures isolated from *Alb-Cre-R26R-LacZ* mice are β-galactosidase positive. Thirdly, co-culture of biliary epithelium and ROSA26 pancreatic buds shows that endocrine cells do not migrate from the pancreas. The expression of the pancreatic transcription factors Pdx1, HNF6 and Sox9 is widespread, as is Hes1, which represses endocrine development, while that of Ngn3, which is a proendocrine transcription factor, is transient, consistent with an early stage of endocrine cell differentiation. Nicotinamide will increase the number of β-cells formed, while EGF+LIF completely inhibits their formation.

Keywords. Pancreas development, liver development, β-cells, Pdx1, Hes1, Ngn3, extrahepatic biliary system, gall bladder development.

Introduction

The pancreas of higher vertebrates develops from a ventral and dorsal bud of the foregut endoderm [1–4]. These buds subsequently fuse and give rise to all pancreatic cell types including exocrine acini and ducts and endocrine islets of Langerhans containing five cell types secreting insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), pancreatic polypeptide (PP-cells) or ghrelin (ε-cells) [5, 6]. Whereas cells secreting the last four hormones can also be found in the gut, and ectopic expression of insulin has been described in some pathological conditions [7], the development of β-cells was thought to be restricted to the pancreas alone. However, we have recently described another population of endocrine cells closely resembling or identical to those the pancreas, which is located in the extrahepatic biliary system of the mouse and arising in late embryonic development [8].

The extrahepatic biliary system consists of a set of extrahepatic bile ducts coming out of the liver, together with the gall bladder, the cystic duct connecting the gall bladder to the main duct system, and the common bile duct which joins the liver and pancreas to the small intestine. For clarity, we refer to this whole group of structures as the "extrahepatic biliary system", while we reserve the term "extrahepatic bile ducts" just for those ducts that lie between

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the cystic duct and the liver (Fig. 1). Together with the liver and the ventral pancreas, the extrahepatic biliary system arises from a contiguous region of the ventral endoderm [9–11]. The insulin expressing cells were found as individuals or clusters in and along the biliary epithelium, but not in the gall bladder epithelium. They are likely to be genuine β-cells, as they process insulin (C-peptide positive), contain typical electrodense insulin granules visible by electron microscopy, and are glucose responsive [8]. These findings suggest that the embryonic extrahepatic biliary system has the potential to produce β-cells. Indeed, the transcription factors Pdx1 (pancreatic and duodenal transcription factor 1, [12], HNF6 [13] and Hes1 (hairy enhancer of split) [14] which are expressed during pancreatic development are also expressed in the extrahepatic biliary system. Most strikingly, in mice lacking *Hes1*, the extrahepatic biliary epithelium converts to pancreatic tissue [14, 15], indicating that Hes1 represses pancreatic development in this region.

In the present study we examine the mechanism of production of endocrine cells by the extrahepatic biliary tract. For this purpose we have devised a new explant culture system of the liver hilar region, which is the region where major blood vessels enter and the bile ducts exit the liver. The cultures form a small amount of liver parenchyma, the main extrahepatic bile ducts, the gall bladder, the cystic duct and part of the common bile duct. We have identified individual cells in the duct epithelium which express pro-pancreatic transcription factors and are presumably the precursors for the endocrine cells. These cells are not found in the gall bladder. Two different types of cell labelling experiment show that the endocrine cells arise from the ducts *in situ* and do not migrate from the ventral pancreas. Additionally, we have studied the effect of exogenous factors and identified some that increase and some that decrease the number of β-cells arising from the ducts.

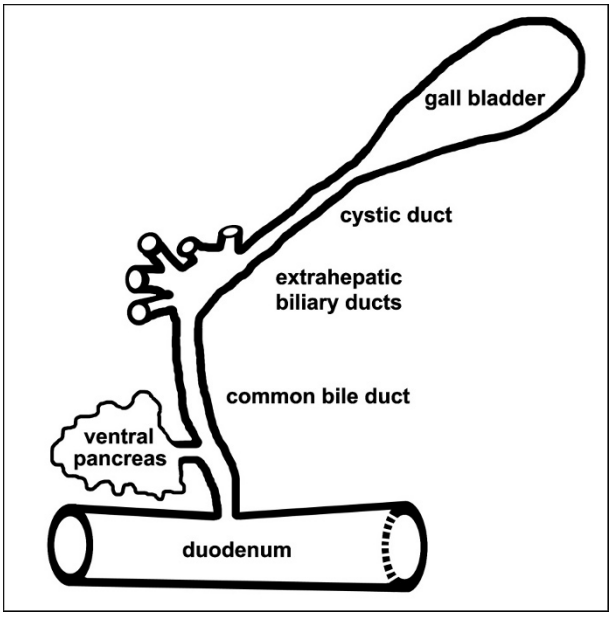

Figure 1. Anatomy of the extrahepatic biliary duct system.

Materials and Methods

Isolation of embryonic extrahepatic biliary system. Animal husbandry and embryo isolation were carried out in accordance with UK Home Office regulations. E15.5 stage mouse embryos were isolated from CD1 females which were sacrificed by cervical dislocation. Embryos were removed from the uterus and transferred to cold Minimum Essential Medium (MEM) with Hanks' salts (Sigma), 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma, P4333) and 20 μg/ml gentamicin (Invitrogen) and the liver with a part of the duodenum attached to the extrahepatic biliary system was dissected free. The components of the biliary system were then separated from liver tissue and adjacent tissues.

Biliary ducts were cultured on coverslips subbed with 3-aminopropyltriethoxysilane (APTES, Sigma) then coated with bovine plasma fibronectin (50 μg/ml, Invitrogen). The medium was DMEM (Gibco, 41966, 4.5 g/l glucose), 20% FBS, 1% penicillin/streptomycin (Sigma, P4333) and 20 μg/ml gentamicin. A cloning ring was placed over the fibronectin-coated area during the first two days to support attachment of the explant on the substrate. The medium was changed every two days. The cultures were grown at 37°C, 95% air/5% CO_2 in a humidified incubator for up to eight days.

Soluble factors/growth factors. To test the potential of various exogenous factors on the development of insulin-positive cells, per experiment, 6–8 ducts were randomly chosen on the day of isolation and cultured in high glucose DMEM medium containing a soluble factor (for concentrations see list below). Simultaneously, an equal number of ducts were grown in medium without factors as a control group. Inhibitors were added the following day, as we found they inhibit attachment of the ducts to the fibronectin matrix. The media with the factors was changed every day. After six days in culture, the average number of insulin-positive cells in treated and untreated ducts was determined. Each experiment was repeated three to five times and the average increase and S. E. M. was calculated and plotted.

The following concentrations were used: 10 ng/ml betacellulin (Sigma Aldrich), 50 ng/ml EGF (R&D Systems), 100 ng/ml FGF10 (R&D Systems), 100 ng/ml activin A (R&D Systems), 1 nM exendin-4 (Sigma Aldrich), 20 μM γ-secretase inhibitor (Sigma Aldrich, S2188), 20 mM nicotinamide (Sigma Aldrich), 40 ng/ml leukaemia inhibitory factor (LIF, Sigma Aldrich), 10 μM AG1478 (EGRF inhibitor, Calbiochem), 10 μM LY294002 (PI3K inhibitor, Calbiochem), 100 μM AG490 (JAK2 inhibitor, Calbiochem, cat.658401), 5 μM MEK I/II inhibitor (Calbiochem), 20 μM JNK II inhibitor (Calbiochem) and 10nM VEGF (R&D Systems).

Immunohistochemistry. Depending on the antibody to be used, duct cultures were either fixed in 4% paraformaldehyde at room temperature for 30 min, or for 5 min in acetone/methanol (1:1 ratio) at -20°C. Subsequently, cultures were washed in PBS and stored in PBS at 4°C. For immunostaining, ducts were permeabilised with 1% Triton X-100 (Sigma) in PBS for 30 min and non-specific binding sites were blocked for at least half an hour in 2% Blocking Reagent (Roche). Staining with primary antibodies was performed overnight at 4°C. After three 15 min washes in PBS, the fluorescent secondary antibody was applied for three hours at room temperature and the cultures counterstained with DAPI (Sigma). Samples were finally mounted in Gel/Mount (Biomeda corp). The antibodies and lectins were obtained and diluted as follows: Mouse monoclonal anti-cytokeratin 7 (Abcam, 1/100); DBA (*Dolichos biflorus* agglutinin FITC conjugated, Vector, 1/200); DBA (*Dolichos biflorus* agglutinin TRIC conjugated, Vector 1/200); mouse monoclonal anti-E-cadherin (BD Biosciences, 1/200); Guinea pig polyclonal anti-insulin (DAKO, 1/200); Mouse monoclonal anti-glucagon (Sigma, 1/100); Goat polyclonal anti C-peptide (Linco Research Inc., 1/100); rabbit polyclonal anti-somatostatin (Dako, 1/100); rabbit polyclonal anti-pancreatic polypeptide (Zymed, 1/50); Rabbit polyclonal anti-grehlin (Phoenix, 1/100); rabbit polyclonal anti-amylase (Sigma, 1/100); mouse monoclonal anti-PECAM (CD31, Pharmingen, 1/100), mouse anti β-galactosidase (Promega, 1/200); rabbit alpha fetoprotein (Dako, 1/100); rabbit polyclonal anti-Pdx1 (J. Slack, 1/100); rabbit polyclonal anti-Ngn3 (M. German, 1/100); rabbit polyclonal anti-Hes1 (Santa Cruz, 1/50); rabbit polyclonal anti-p48 (Abcam, 1/100); rabbit polyclonal HNF6 (Santa Cruz, 1/50); rabbit Anti-Sox9 (Chemicon, 1/100); Anti- guinea TRITC conjugated IgG (Sigma, 1/200); Rabbit anti-goat FITC conjugated IgG (Vector, 1/200); Goat anti-rabbit biotin IgG (Vector, 1/200); horse anti-mouse biotin IgG (Vector, 1/100); Avidin DCS FITC (Vector, 1/100). Specimens stained for cytokeratin 7 and E-cadherin were fixed in acetone/methanol. All other specimens were fixed in 4% paraformaldehyde.

Specimens were analysed using a Leica DMRB microscope and images were taken using a colour SPOT RT camera (Diagnostic Instruments) operated with Advanced Spot RT 3.0 software. A Zeiss LSM510 confocal microscope was used to take high resolution images of optical sections. The images were finally arranged using Adobe Photoshop 7.0. 3D reconstructions were calculated from z-scans using algorithms from the Zeiss LSM Image browser.

Lineage tracing. Cultures from 15.5 dpc embryos of *C57BL/6 Alb-Cre x R26R LacZ* mice (heterozygous for both transgenes) were used to trace the origin of the insulin-positive cells [16, 17]. For the X-gal staining, the cultured extrahepatic biliary duct explant systems were fixed in 2% formaldehyde in PBS for 30 min at 4°C. Subsequently, the tissue was rinsed in PBS and stained at 37°C in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCI_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS (pH 7.3). Cultured pancreas of *Alb-Cre x R26R LacZ* reporter mice were used as controls to exclude unspecific labelling. For lineage tracing, ducts were stained with both β-galactosidase and insulin antibodies. The galactosidase staining was enhanced using a biotinylated secondary and an avidin FITC DCS antibody.

To analyse the possible migration of pancreatic cells into the bile duct explants, E15.5 CD1 biliary ducts were co-cultured with E15.5 β-galactosidase expressing Rosa26 pancreas [18]. The dorsal pancreas of a Rosa26 embryo was isolated and cut into 5–6 small pieces. One piece of pancreas (including mesenchyme) was placed in contact with the blunt cut ends of the extra hepatic biliary ducts in a cloning ring using a Tungsten needle. After six days in culture, they were fixed and stained in X-gal for 2–3 h at 37°C. Subsequently, a combined insulin and DBA stain was performed and the distribution of blue β-galactosidase positive and fluorescent β-cells was analysed. Under the conditions used, the FITC signal was not obscured by the X-gal stain.

Results

Extrahepatic biliary duct culture. To reveal the anatomy of the hilar region of E15.5 mouse embryos, the upper abdomen including a segment of duodenum, the ventral pancreas and the extrahepatic biliary tract were stained with the duct marker *Dolichos*

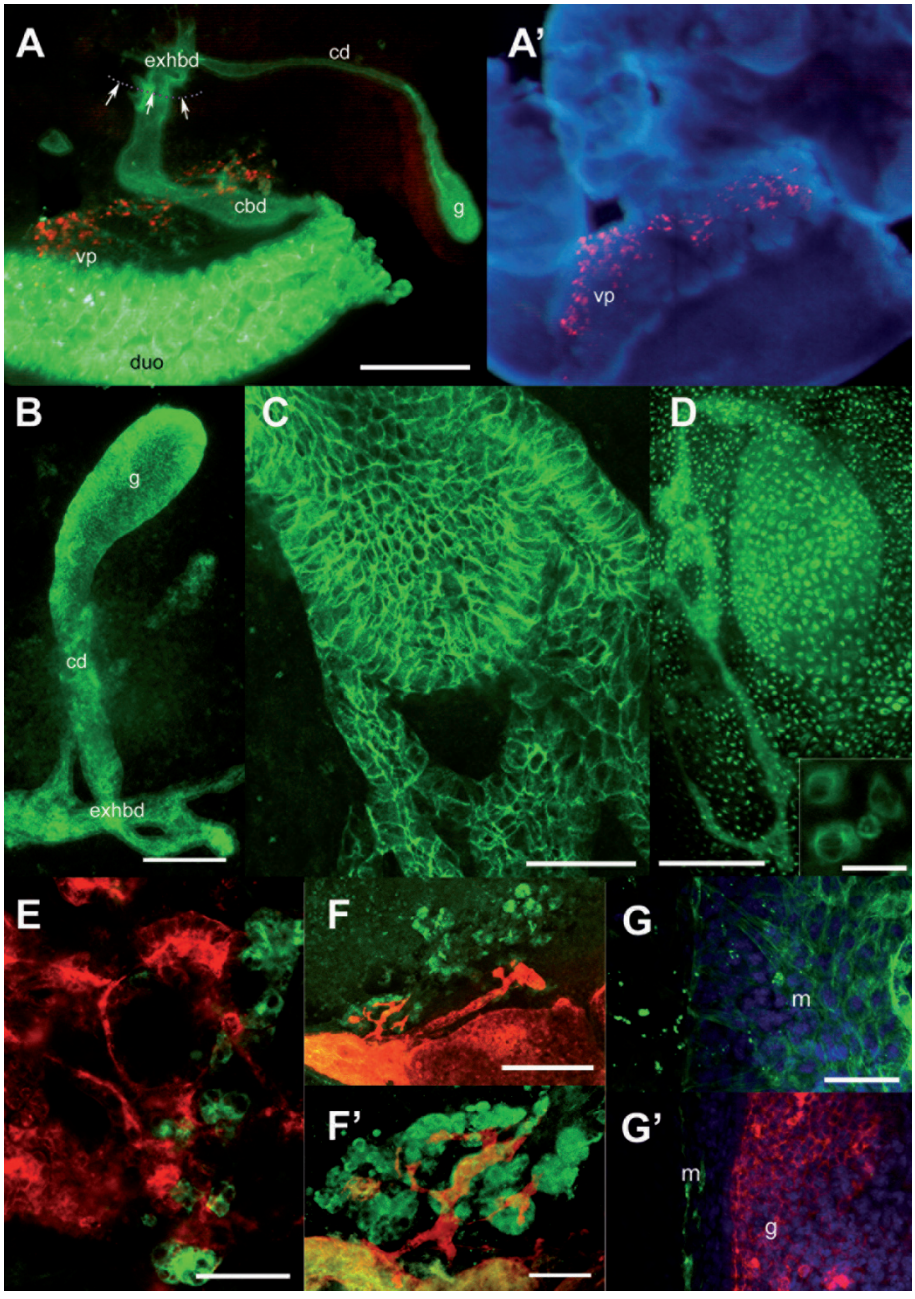

Figure 2. Extrahepatic biliary cultures.
A, *A'*; Extrahepatic biliary system of an E15.5 mouse embryo with liver removed, stained with DBA (green) to show the anatomy of the extrahepatic biliary system and connections to the small intestine. For the hilar cultures the region comprising extrahepatic bile ducts (exhbd), gall bladder (g) and cystic duct (cd) was retained. The common bile duct (cbd) and neighbouring ventral pancreas (vp: insulin, red) was removed, as indicated by the dotted line.
B; Extrahepatic biliary explant cultured for six days and stained for DBA (green).
C; Extrahepatic biliary tract cultured for six days and stained for E-cadherin (green).
D; Extrahepatic biliary system cultured for six days and stained for CK7 (green). Inset: Magnification of CK7 positive cells in the gall bladder epithelium.
E, Staining for AFP (green) reveals hepatoblasts in the hilar region (DBA, red) in d6 cultures. There was no co-localization of AFP and DBA.
F, In a few cases amylase positive cells (green) were visible near the extrahepatic bile ducts (DBA, red), and in one specimen the region contained an outgrowth comprising ductal (DBA red) and acinar cells (amylase, green) (*F'*, Magnification of *F*; Other acinar cells belong to the same pancreatic outgrowth, but the connecting ducts are not in the plane of focus.
G, G'; Smooth muscle actin (SMA) positive mesenchymal cells surrounding epithelial explant core. *G*, Layer of mesenchymal (m) smooth muscle actin positive cells (green) growing on top of the gall bladder (g) epithelium (DBA, red) seen in *G'* (blue, nuclear stain). *G, G'* are images from two different planes using confocal microscopy.
cd, cystic duct; cbd, common bile duct; duo, duodenum; g, gall bladder; exhbd, extrahepatic bile ducts; vp, ventral pancreas.
Scale bars, *A, A'* 500 μm; *D*, 250 μm; *B, F,* 200 μm; *E, F', C, G, G',* 50 μm.

biflorus Agglutinin (DBA lectin, [11]) (Fig. 2A, A'). The extrahepatic biliary system is visible as a branching structure connected to the cystic duct (leading to the gall bladder) and the common bile duct (leading to the gut). The ventral pancreas invests the distal part of the common bile duct. It is marked by a band of insulin-positive cells and is clearly separated from the hilar region (Fig. 2A, A').

To study the bile duct β-cell development *in vitro*, the extrahepatic ducts were cleared of surrounding liver tissue and cultured. To exclude contamination with pancreatic endocrine cells, the common bile duct and the ventral pancreas were removed (separation border indicated by dotted line in Figure 2A, A'). Small adhering masses of hepatic tissue were left attached to protect the duct system and to orient the specimen. Explants were placed into cloning rings to facilitate attachment to the substrate and cultured for up to eight days.

The explants adhered well to fibronectin, collagen I, IV or Matrigel substrates within 24 h and survived well in several media including DMEM, BME, CMRL or RPMI. After 48 h a monolayer of mesenchymal cells and cuboidal shaped cells surrounded the core biliary explants. The cut epithelial ends of the ducts anastomosed with each other or expanded in length while there was no obvious expansion of the gall bladder and the cystic duct, (Figs. 2, 3). Cell division was examined by staining for phospho-histone 3. This showed that the labelling index is higher for duct outgrowths (20.5 %) than for large ducts (3.7 %), or for the gall bladder (1.2 %). Insulin-positive cells appeared after a few days in culture (see below).

Immunostaining was performed to monitor the differentiation of the duct epithelium in culture and to identify different cell types in the cultures (Fig. 2B-G). Biliary epithelial differentiation was maintained for at least six days as shown by staining for the duct marker DBA receptor (Fig. 2B), and the general epithelial markers E-cadherin (Fig. 2C) and cytokeratin 7 (CK7, Fig. 2D). The culture core was surrounded and covered by a layer of smooth muscle actin (SMA) positive mesenchymal cells (Fig. 2G, G'). Some cells surrounding the ducts were also positive for CK7 (Fig. 2D), but not for DBA or E-Cadherin.

Although most of the hepatic tissue present in the cultures detached during the first days in culture, some α-fetoprotein (AFP) positive hepatoblasts remained attached around the biliary epithelium of the hilar region (Fig. 2E). These AFP positive cells did not stain with DBA.

Occasionally, small amylase-positive cell patches were observed attached to the basal side of the bile duct epithelium. In one specimen, an outgrowth with amylase positive acinar cells was observed, resembling a tiny ectopic pancreas (Fig. 2F). Amylase positive cells were not found in the gall bladder, and the extrahepatic biliary epithelium itself was amylase negative.

Development of bile duct β-cells. To study the development of insulin-positive cells in the extrahepatic biliary region we cultured hilar region explants for up to eight days and co-stained with DBA and antibody to insulin (Fig. 3A-D). Insulin-positive cells were found in the extrahepatic bile ducts from E15.5 onwards and their number increased considerably during the culture period (Fig. 4A). The insulin-positive cells were confined to the extrahepatic bile ducts and, in cultures where the common bile duct was not removed, also along the common bile duct (Fig. 3B). They were not found in the gall bladder, and only two insulin-positive cells were found in the cystic duct out of more than one hundred cultures analysed (Fig. 3B). Some insulin-positive cells were located within the plane of the biliary epithelium as observed by co-staining for DBA and insulin (Fig. 3E-E'', reconstruction, supplemental movie 1). But most of them were attached to the basal side of the biliary epithelium and did not stain with DBA. The duct system, including the insulin positive cells is located between upper and lower layers of mesenchymal cells staining positive for smooth muscle actin (supplemental movie 2).

There was some variation in the number of the insulin-positive cells between individual cultures, each of which was established form one embryo. In explants cultured for four days, ~81 % of the cultures contained some insulin-positive cells with the number varying from 1 to 17 cells per explant. The mean number of insulin-positive cells per culture approximately quadruples from two days, corresponding to E17.5, to six days of culture, corresponding to P2 of mouse development. We have considered whether this increase indicates ongoing neogenesis from the ducts, or whether it is due to division of already formed insulin-positive cells. Although the majority of the cells are single, there are two reasons for thinking that most of the neogenesis takes place in the late embryo. Firstly, with each day of culture the proportion of the insulin-positive cells which are located within the duct epithelium decreases, and the proportion found in the adjacent mesenchymal tissue increases, consistent with the idea that the cells migrate out of the epithelium once they have formed (Fig. 4B). Secondly, there is an overall shift towards higher clump sizes with time. This is indicated by Figure 4C, which shows the proportion of insulin-positive cells appearing as single cells or in clusters of different sizes at 2 d and 6 d of culture. A χ^2 test on this data (comparing the

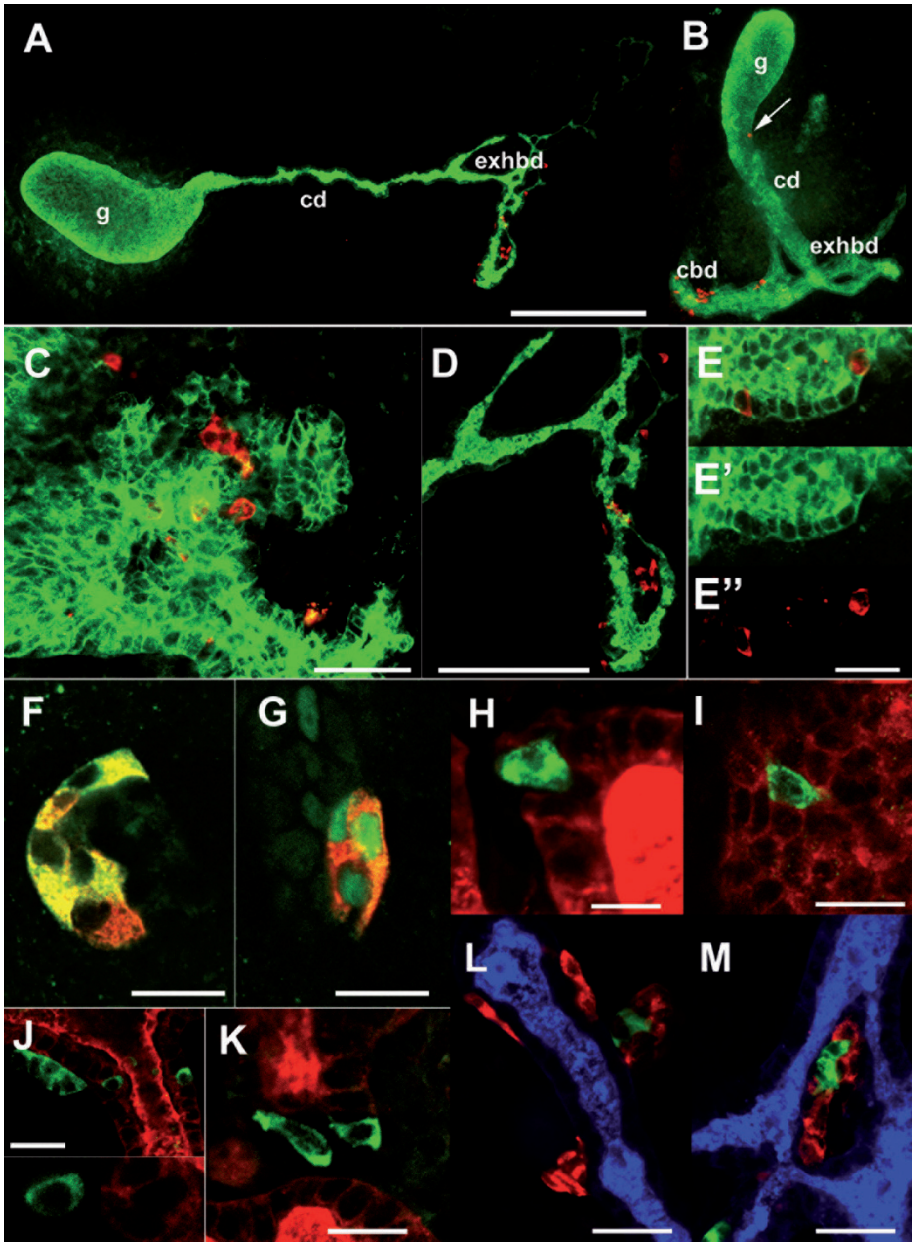

Figure 3. Pancreatic endocrine cells in the extrahepatic biliary tract.
A; Extrahepatic biliary tree explant cultured for eight days. The bile ducts (green, DBA positive) show a high degree of branching. Insulin-positive cells (red) are attached to the biliary epithelium (see E for magnification)
B; Extrahepatic biliary explant with a small part of the common bile duct cultured for six days. The arrow indicated a rare insulin-positive cell (red) in the cystic duct (cd).
C; Insulin-positive cells in bile ducts of a day six culture (green, DBA, red insulin).
D, Anastomosing bile ducts of a day eight culture harbouring insulin-positive cell clusters (red), Magnification of *A*.
E-E'', Examples of biliary epithelial cells (DBA stained, green) co-staining for insulin (red).
F; Insulin-positive cells (red) also expressing C-peptide (green).
G, Cells co-staining for insulin (red) and Pdx1 (green).
H-K. Presence of other types of endocrine cell. All stained DBA red, hormone green.
H, Glucagon-positive cell.
I, Somatostatin and DBA positive cell in the lining of the duct epithelium.
J, Pancreatic polypeptide positive cells seen as DBA negative clusters attached to the duct and within the lining of the biliary cells (DBA positive): Inset: enlargement of pancreatic polypeptide and DBA positive cell.
K. Ghrelin positive cells
L, Insulin (green), somatostatin (red) and DBA (blue) positive endocrine cell clusters
near extrahepatic bile ducts.
M; Insulin (green), pancreatic polypeptide (red) and DBA (blue) positive endocrine cell clusters near extrahepatic bile ducts.
g, gall bladder; cd, cystic duct; cbd, common bile duct; exhbd extrahepatic bile ducts.
Scale bars, *A*, 500 μm; *D*, 200 μm; *C* 50 μm; *E, J,* 25 μm; *F, G, I, K, L, M* 20 μm; *H*, 10 μm.

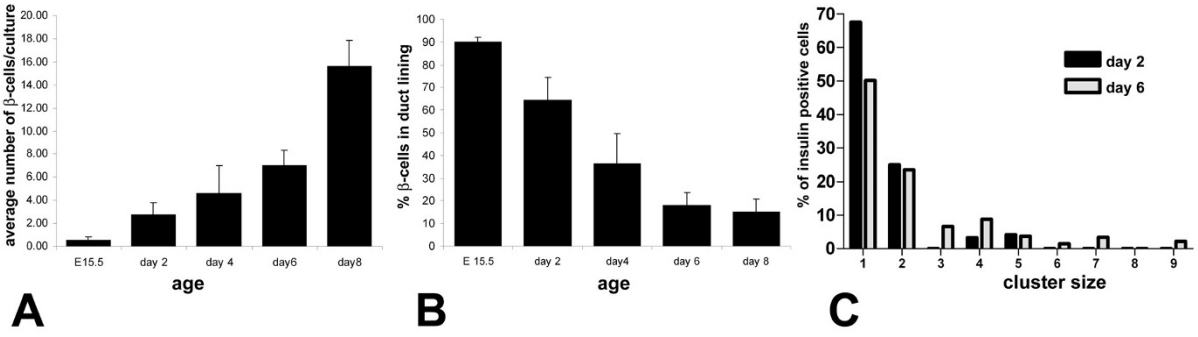

Figure 4. Quantitative data.
A, Average number of insulin-positive cells in the hilar region of uncultured biliary tree explants at E15.5 and from hilar regions cultured for 2, 4, 6 and 8 days (n = 20, 16, 15, 22, 13)
B, Percentage of insulin-positive cells in the biliary epithelium lining. Error bars represent standard errors.
C, Percentage of insulin-positive cells at two and six days of culture found as individuals (first bar) or in clusters of different cell number. For day 2 cultures, 25 ducts from six independent experiments and containing 120 insulin-positive cells were analysed. For day 6 cultures, 46 cultures from three independent experiments and containing 408 cells were analysed. Most insulin-positive cells were found as individuals, i.e. 67.5 % (81 of 120 cells) in day 2, and 50.2 % (205 of 460 cells) in day 6 cultures, respectively.

frequency of single cells and clusters of 2, 3–5, and 6–9 cells between the two culture periods) gives a value of 22.2, which is highly significant ($p_{0.05} = 7.8$ for $f = 3$). Clumps are only found in the mesenchyme and so must arise from division or aggregation of already formed cells.

We stained the cultures for C-peptide and Pdx1 to verify that the insulin-positive cells are in fact β-cells rather than some other cell type expressing the *insulin* genes. Indeed, overlapping staining for insulin and C-peptide indicated ongoing insulin processing (Fig. 3F) and all the insulin-expressing cells were positive for Pdx1, also a characteristic of genuine pancreatic β-cells (Fig. 3G). The intensity of Pdx1 staining was similar to that in insulin-positive cells of pancreatic buds cultured in parallel. Staining with the endothelial marker PECAM revealed that 89 % of the β-cells were connected to blood vessels (3d reconstruction supplemental movie 3). Thus it is likely that the β-cells have a connection to the circulation *in vivo*.

The duct system also produced cells expressing the other hormones: glucagon, somatostatin, pancreatic polypeptide and ghrelin, all characteristic of endocrine cells in the Islets of Langerhans of the pancreas (Fig. 3H-K). Like β-cells, these cells were only present in the extrahepatic bile duct region and were either part of the biliary epithelial lining (DBA stained) or attached to the epithelium (DBA unstained). Co-staining of SS or PP cells with insulin showed that these cells could be present in the same clusters. Although the clusters are very small compared to typical pancreatic islets, there is a tendency for the β cells to be internal, as is the pattern in rodent islets (Fig. 3L, M).

β-cells derive from the bile duct epithelium. Two types of lineage tracing experiment were performed to find if the observed β-cells originate from the biliary epithelium or from the pancreas or both. The *albumin* promoter is expressed in the whole early liver domain, including the region destined to form the extrahepatic bile ducts [11]. We generated offspring from the cross *Albumin-Cre* x *stopfloxed R26 LacZ reporter* (*Alb-Cre* x *R26R LacZ*) in which the progeny of *albumin* positive cells irreversibly express β-galactosidase following the Cre-recombinase mediated excision of a transcription stop sequence. The *Alb-Cre* x *R26R LacZ* were bred on a C57Bl/6 background, which has a lower average number of β-cells in the biliary system than the CD1 s used for the data in Figure 4 above (at six days they have a mean of about 1 cell per culture rather than 7, although some cultures have several cells). Figures 5A, B demonstrates the presence of labelled biliary epithelial cells expressing β-galactosidase. The labelling is not 100 % due to incomplete Cre-mediated recombination. For example there were some unlabelled cells in the gall bladder epithelium (Fig. 5A). Mosaic labelling has also been reported in other transgenic mice expressing Cre recombinase under the control of albumin regulatory elements and α-fetoprotein enhancers [19]. The labelling of bile duct cells was 65.9 % (517 cell counted from five mice). A very similar proportion (56 %; 26 of 46 cells counted) of the β-cells present in the bile duct cultures were β-galactosidase positive. By contrast, no β-galactosidase positive cells were observed in pancreatic bud cultures from the same embryos following X-gal staining. These data show that the β-cells are derived from the liver (*albumin*) domain.

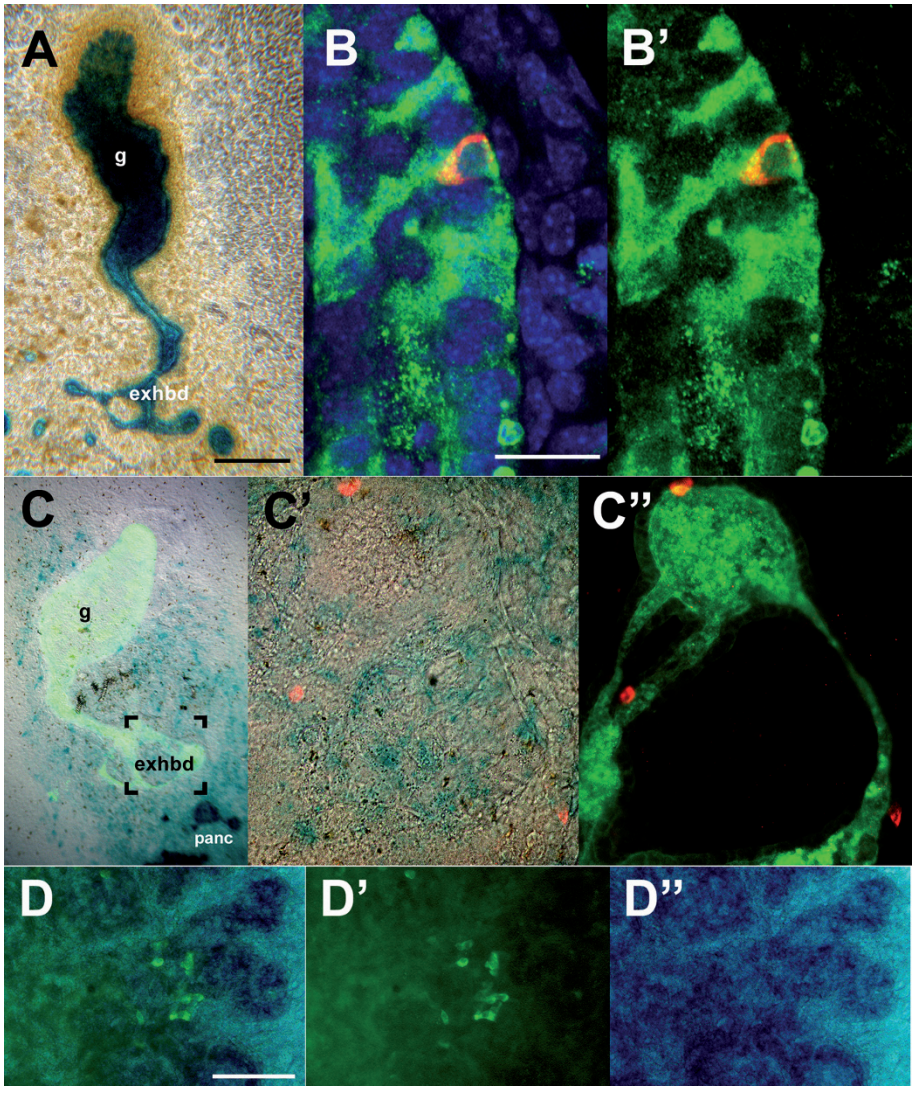

Figure 5. Lineage tracing.
A; *Alb-Cre x R26R LacZ* biliary explant culture stained with X-gal. Note the unlabelled cells at the tip of the gall bladder.
B, B'; Example of insulin positive (red) and β-galactosidase (anti-β-galactosidase, green) stained cell in a 15.5 dpc duct (*Alb-Cre x R26R LacZ*) cultured for 6d. *B*, merge of insulin, β-galactosidase and DAPI stain; *B'*, β-galactosidase and insulin stain only.
C; Merge of DBA (green, staining the biliary epithelium) and X-gal co-stain of an unlabelled biliary explant co-cultured with a Rosa26 (lacZ labelled) pancreas ("panc").
C'-C''; Pancreatic mesenchyme migrates into the bile duct region, but no dark blue epithelial cells migrate and β cells were always unlabelled (*C'*, X-gal, *C''*, DBA (green) plus insulin (red), *C'* and *C''* are the same field).
D-D''; 15.5 dpc pancreatic bud of Rosa26 mouse cultured for six days showing β-galactosidase labelling of β-cells. *D*, X-gal stain and insulin immunostaining, *D'*, insulin stain; *D''*, X-gal stain only. g, gall bladder. exhbd, extrahepatic biliary duct. Scale bars: *A*, 200 μm; *E*, 100 μm *B*, 50 μm; *C*, 25 μm

In the second experiment we examined the possibility of migration of cells from the ventral pancreas into the duct system of the hilar region, E15.5 pancreata of Rosa26 mice which ubiquitously express β-galactosidase under the *rosa26* promoter [18] were attached to extrahepatic bile duct region explants from unlabelled CD1 mouse embryos of the same stage. X-gal staining of 6 day combination cultures revealed extensive migration of mesenchymal cells from the pancreas around the biliary explant (Fig. 5C-C'), but β-galactosidase positive cells were not found within the duct epithelium. None of the insulin positive cells, whether within the duct epithelium or adjacent to it, were labelled with β-galactosidase (Fig. 5C'-C''). On the other hand, all insulin positive cells in the Rosa 26 pancreas were labelled with β-galactosidase (Fig. 5D-D'').
Both of these labelling experiments support the conclusion that the endocrine cells arise from the biliary duct epithelium and do not migrate from the pancreas.

Transcription factor domains. We were interested to know if the endocrine cells formed by the extrahepatic bile ducts arise by the same mechanism as their counterparts in the pancreas. For an initial indication of the developmental mechanism we examined the expression of various transcription factors known to be required for the formation of pancreatic endocrine cells.
Pdx1 is expressed in the entire endodermal region forming the pancreatic buds and is required for their development [12, 20, 21]. A scattered population of extrahepatic bile duct cells was Pdx1 positive (Fig. 6A'-B') the number falling somewhat with stage. They are seen only very rarely in the gall bladder (3 Pdx1 positive cells in just one sample,

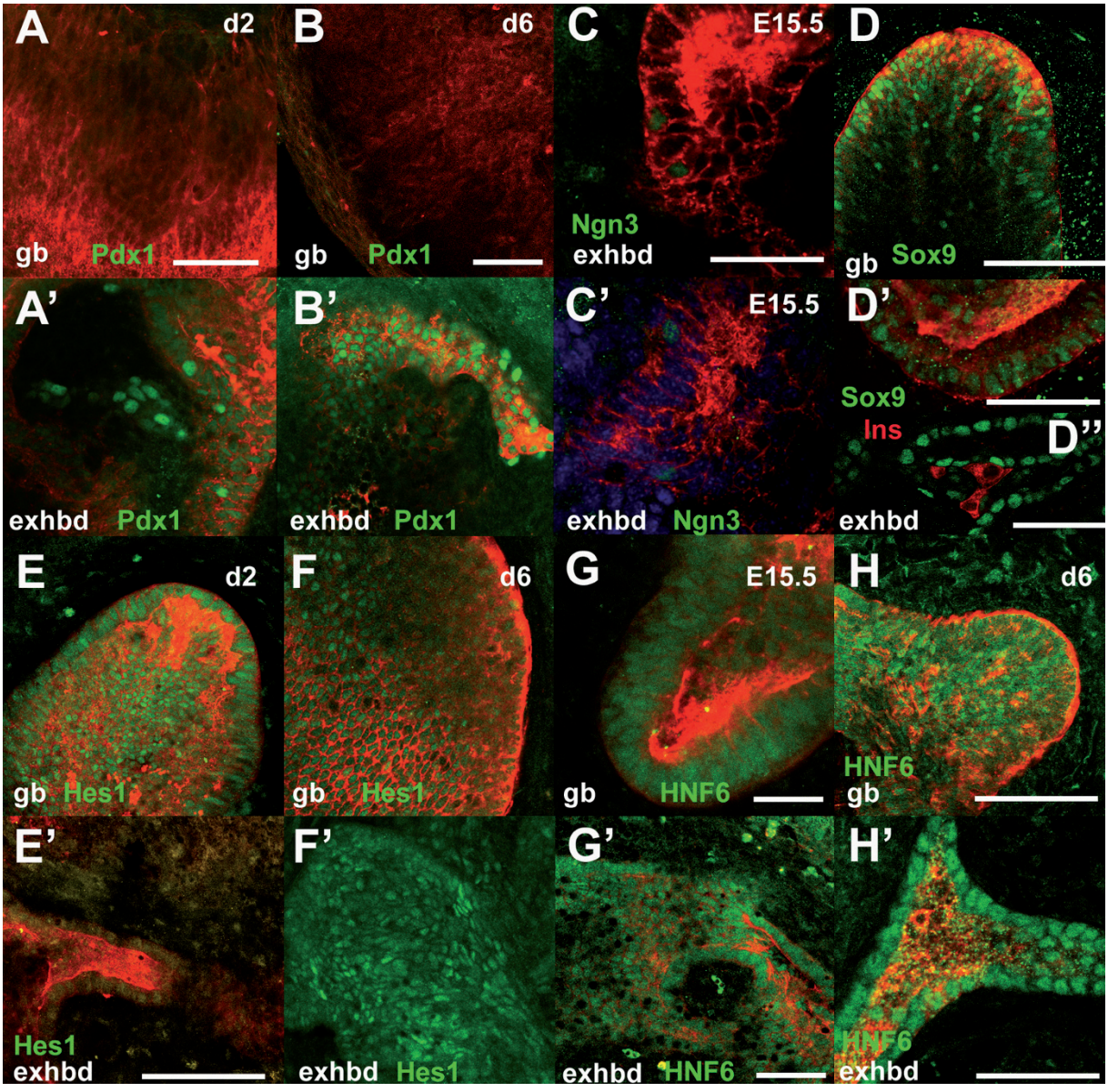

Figure 6. Transcription factor domains in the embryonic extrahepatic biliary system.
A-B'; Pdx1 positive nuclei (green) in DBA positive (red) extrahepatic bile ducts of day 2 (*A'*) and day 6 (*B'*) cultures. The Pdx1 positive, DBA negative cells in A' represent an endocrine cluster. Pdx1 is absent in the gall bladder epithelium at any stage analysed, e.g. day 2 (*A*) and 6 (*B*).
C, Ngn3 positive cells (green) within the biliary epithelium (DBA, red) of E15.5 extrahepatic bile ducts. *C'*, Ngn3 (green), DBA (red), DAPI co-staining.
D, D'', Sox9 positive cells (green) in the gallbladder (*D*; DBA, red) and extrahepatic bile ducts (*D'*) of a day 6 culture. *D''*, Insulin positive cells (red) do not express Sox9 (green).
E-F'; Hes1 (green) positive nuclei in day 2 and day 6 biliary epithelium cultures (DBA, red), *E*, d2 extrahepatic bile ducts; *E'* d2 gall bladder, *F*, d6 gall bladder; *F'*. d6 extrahepatic bile ducts.
G-H', HNF6 (green) in E15.5 (*G, G'*) and d6 staining of biliary epithelium (DBA, red) cultures (*H, H'*).
gb, gallbladder; exhbd, extrahepatic biliary duct. Scale bars, *G*, 20 μm; *A=A', B=B', C, D', D'', G', H'* 50 μm; *D, E=E'=F=F', H* 100 μm.

Figure 6A, B). A recent report indicates that Pdx1 expression may persist into adulthood [22].

Neurogenin 3 is a proendocrine transcription factor required for endocrine development and its expression is thought to be regulated by Notch signalling through a lateral inhibition process [23]. We stained E15.5 and cultured explants for the presence of Ngn3 as positive cells might represent the endocrine progenitor cells of the biliary epithelium. Indeed, a small population (<10) of Ngn3 positive cells was observed in the DBA stained duct epithelium of freshly isolated E15.5 biliary explants (Fig. 6C, C'). Ngn3 positive cells

were no longer found in the duct epithelium following culture, indicating a transient expression in the duct system, as is also observed in the developing pancreas [24]. Again, the gall bladder epithelium was negative for Ngn3 (not shown).

In the pancreas, Hes1 expression is activated by Notch signalling and is thought to repress expression of Ngn3 [25]. Hes1 was expressed throughout the bile duct and gallbladder epithelium at all culture stages, e.g., in d 2 to d 6 (Fig. 6E-F'). In the less mature cultures, staining for Hes1 staining was only obtained by amplifying the staining signal using Avidin-Biotin, suggesting that the level of expression builds up over this period.

The onecut transcription factor HNF6, and the HMG box factor Sox9, are both thought to be required for continued growth of pancreatic precursor cells, and are downmodulated during endocrine cell differentiation [26, 27]. Both of these factors are present in biliary epithelial cells at all stages (Fig. 6D-D', G-H'), although in the gall bladder, Sox9 expression was restricted to a subpopulation of epithelial cells. Insulin positive cells were Sox9 negative (Fig. 6D'') as previously reported for pancreatic β-cells [27]. Consistent with previous linage tracing experiments suggesting that biliary cells do not express the pancreatic transcription factor Ptf1a [15], we could not find any Ptf1a expressing cells at any stage in the duct cultures (not shown).

Effects of soluble factors on β-cell number. Because only very few biliary duct cells become endocrine cells and the numbers are somewhat variable between individual cultures, it is likely that the cells that do so are at the extreme end of a random distribution, for example a small minority of cells with high Pdx1 and low Hes1. We investigated the potential of exogenous factors to alter the number of endocrine progenitors, using a variety of substances that have been considered to affect β-cell differentiation in other contexts. Extrahepatic biliary duct explants were treated for six days in culture and compared to untreated duct controls. Only a few factors showed an effect on the number of β-cells in the duct region, and as usual the gall bladder did not contain any β-cells (Fig. 7). Interestingly, treatment with 20 mM nicotinamide reproducibly increased insulin-positive numbers about 4-fold compared to untreated ducts (Fig. 7, supplemental Fig.1). At least some of this effect must be due to neogenesis rather than extra division, as there is a twofold increase of β cells by day two of culture in the nicotinamide-treated cases. These also show no increase of cluster size relative to 2d controls, which would be expected if the primary effect were on division. We also studied EGF and the combination EGF+ LIF [28] on duct β-cells. Surprisingly their

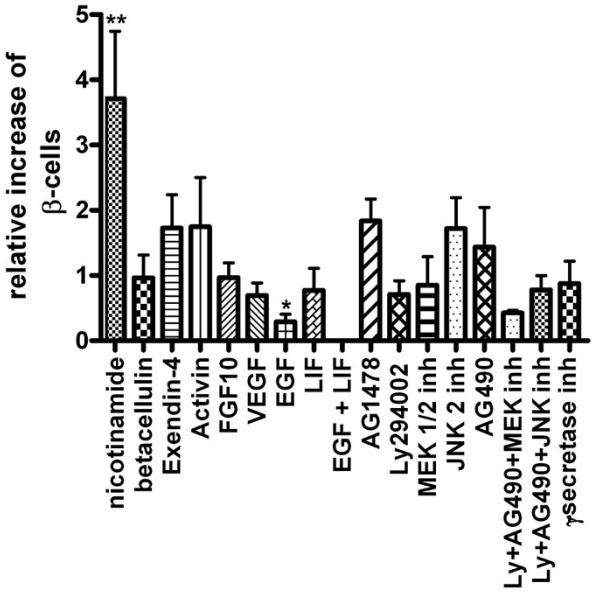

Figure 7. Effect of exogenous factors on the formation of insulin positive cells by embryonic biliary ducts.
Extrahepatic duct explant cultures of E15.5 embryos were treated with various factors for six days. For each experiment 6–8 treated ducts were compared to an equal number of untreated ducts cultured simultaneously. The histogram shows the ratio of the number of β-cells developing in treated vs. untreated cultures. Each experiment was performed between three and five times. For example for nicotinamide, a total of 33 explants was cultured in five independent experiments. For the others the following total number of explants was cultured, with the number of experiments given in brackets: betacellulin, 27 (3), exendin-4, 22 (3),: Activin A, 21 (3); FGF10, 21 (3) ; EGF, 29 (4); LIF, 20 (3); EGF+ LIF, 26 (4); AG1478, 21 (3); LY294002, 20 (3); MEK I/II inhibitor, 21 (3); JNK II inhibitor, 18 (3), AG490, 33 (4); LY+AG490+MEK inh, 21 (3); LY+AG490+JNK inh, 21 (3), VEGF, 21 (3); γ-secretase inhibitor, 16 (3). Error bars represent SEM. Significance levels were calculated using an unpaired t-test. $p = 0.006$ (nicotinamide), 0.043 (EGF). Because of the 0 value, no p can be calculated for EGF+LIF, but this is evidently more significant than the EGF alone result.

effects were negative. EGF-treated ducts only contained 20% of the usual number of β-cells. This remained true even when the cultures were kept a further six days after withdrawal of EGF to allow differentiation. No β-cells at all were found in EGF + LIF treated ducts, although ducts treated with LIF alone showed the normal number of β-cells. Since these substances were inhibitory, we considered it possible that a tonic stimulation of the EGF pathway and/or LIF pathway might inhibit β-cell growth, and this negative effect might be relieved by treatment with inhibitors known to inhibit the EGF and LIF pathway. However, there was no significant change in number of β-cells when the cultures were treated with AG490 (JAK2 inhibitor), AG1478 (EGFR inhibitor) or combinations of these with other inhibitors (Fig. 7). As it was considered that a decrease in Hes1 levels may activate the proendocrine Ngn3 expression, the

ducts were also treated with two γ-secretase inhibitors (MG132 and Sigma S2188) at various concentrations. These interfere with Notch signalling and may thereby reduce Hes1 expression. In fact, there was no obvious effect on Hes1 expression (not shown) so it may be that this is not controlled by the Notch pathway in this system. These inhibitors produced no change in β-cells numbers and high concentrations were toxic.

Discussion

We have devised a culture model to study the development of naturally occurring endocrine cells in the embryonic extrahepatic biliary epithelium. This system enabled us to analyse endocrine cell appearance, movement and growth, observe transcription factor domains, study the effects of exogenous factors and perform linage tracing. We believe that the insulin-expressing cells in the biliary system are genuine β-cells since they are characterised by insulin and C-peptide content, contain secretory granules with a characteristic morphology that are immunoreactive for insulin, and exhibit glucose-stimulated insulin secretion [8].

There are three lines of evidence that these cells originate from the biliary ducts. At early stages most of the cells lie within the biliary epithelium as shown in Figures 3C,E and 4B. At this stage the cells co-stain for insulin and for the duct marker DBA. Subsequently the cells are found in the adjacent connective tissue, but they do not move far and remain associated with the bile ducts. Our genetic lineage tracing experiments using *Alb-Cre x R26R LacZ* mice give an equal proportion of labelling of the duct cells and the insulin positive cells, while giving no labelling of pancreatic endocrine cells from the same embryos. This shows that the duct associated β-cells must derive from the *albumin* domain of the endoderm, i.e. the liver bud, which forms both hepatocytes and biliary cells [10, 11, 29]. Because pancreatic endocrine cells have some migratory capability we felt it important to do an independent experiment to exclude an origin from the nearby ventral pancreas. In combinations of hilar explants with ROSA26 pancreatic explants there was abundant migration of X-Gal positive mesenchymal cells to surround the biliary duct explant, but endocrine cells did not migrate very far and were never found in the biliary ducts.

The bile ducts are morphologically and biochemically similar to the pancreatic ducts, and an origin of β-cells from pancreatic ducts has been a controversial topic for some time. A recent study of *insulin-Cre* labelled mice has indicated that in normal growth most β-cells arise from pre-existing β-cells [30]. However, there is also considerable morphological evidence of an origin of β-cells from ducts, especially in pathological situations of inflammation or regeneration [31–33]. A recent study has shown, by perdurance of the product of a *neurogenin 3* reporter, that β-cells can arise from pancreatic ducts in a situation of duct ligation [34]. The situation we are examining here is not the same as the pancreas but does nonetheless shows that small numbers of β-cells can arise naturally from ducts. Our morphological studies indicate that this only occurs in the last quarter of embryonic life. Postnatally the cells are mostly located outside the epithelium and the increase in cell number which occurs at this stage is presumably by self-replication, as in the pancreas. The fact that the endocrine cells are mostly adjacent to blood vessels may suggest that signals from blood vessels are important in their formation, as is believed to be the case in the pancreas [35]. However the blood vessel network is so extensive that it would contact many cells just by random distribution, and we found that treatment of the cultures with VEGF did not increase the number of β-cells (Fig. 7).

Transcription factor domains. The number of endocrine cells in the extrahepatic biliary system is too few to have a significant physiological role; furthermore the numbers vary between mouse strains and between individual mice. But their formation is of great interest from the point of view of developmental biology. The fact that a few endocrine cells arise at random among a large number of duct cells suggests that they result from slight random perturbations of the transcription factor code of the duct cells. This could only occur if the transcription factor codes of the duct cells and pancreatic endocrine cells were fairly similar. We do not know which transcription factors are perturbed but can make some guesses based on the considerable knowledge that has recently been acquired of the molecular genetics of pancreatic endocrine cell development [1, 3, 36, 37]. One likely possibility is the bHLH transcription factor Hes1, which is activated by Notch signalling and usually functions as a repressor of genes like *ngn3* controlling endocrine cell differentiation [5, 23, 24]. In *Hes1* knock-out mice de-inhibition of *ngn3* leads to the conversion of much of the extrahepatic biliary primordium to ectopic pancreas [14,15]. It should be emphasized that we very rarely see actual ectopic pancreas, containing exocrine and endocrine tissue with a duct supply (only 1 case out of 8 examined by amylase staining). In the present work we have shown that Hes1 is expressed throughout the extrahepatic biliary epithelium with the level building up over the six-day culture period. It is possible that occasional random reduction of Hes1

expression in an individual cell de-represses *ngn3* and consequently activates the endocrine program. Since the level of Hes1 is normally increasing this would be more likely to happen early on, consistent with the observed time of appearance of the cells. However, we did not find it possible to lower Hes1 level by treating the ducts with a γ-secretase inhibitor, which should prevent cleavage of Notch protein and block activity of the Notch pathway.

Sox9 is another transcription factor important in pancreatic development. In the developing pancreas, its expression is restricted to mitotically active, Pdx1 positive progenitors and it is one of the factors regulating Hes1 [27]. Thus, fluctuations of Sox9 may also cause de-stability and activation of the pro-endocrine Ngn3 in a subset of cells, and would not depend on Notch signalling.

Additionally we observed some significant differences between the extrahepatic bile ducts and the gall bladder. In particular the pancreatic key master regulator Pdx1 is highly expressed in the hilar region ducts, but is not detectable by immunostaining in the gallbladder (this study and [12]). This might explain why endocrine cells do not develop in the gall bladder.

Growth factors. Several factors have been successfully been used to enhance β-cell maturation and neogenesis [38], and we considered that exposure of the developing ducts to these factors might affect the number of β-cells formed. In fact none of the factors tested showed a significant effect, with two exceptions. One is nicotinamide, which showed a reproducible 3–4–fold increase. Previous studies have shown that nicotinamide is a potent inducer of endocrine development in the human fetal pancreas [39] and promotes, in conjunction with KGF, endocrine differentiation from expanded human pancreatic duct cultures [40, 41]. It was also shown to promote the transdifferentiation of adult rat hepatic oval "stem" cells to pancreatic endocrine hormone-producing cells [42]. The mechanism is not known but may involve the inhibition of polyADP ribose polymerase, and activation of expression of the MafA transcription factor [43].

The other significant effect was a negative one. Embryonic ducts cultured with EGF exhibited a decreased number of β-cells, an effect that was enhanced by adding LIF. In fact the cultures treated with LIF and EGF produced no β-cells at all. This was a surprise, since EGF and LIF are known to enhance β-cells production from rat pancreatic exocrine tissue [44]. In general the evidence relating to a role for EGF in normal development suggests that it should increase β-cell formation. In mice lacking the EGF receptor branching morphogenesis and islet cell differentiation were impaired and the proportion of insulin-positive cells was significantly lowered [45]. Transgenic mice overexpressing EGF under the *insulin* promoter showed an increased islet size and frequency of insulin positive cells in murine pancreatic ducts [46]. However, similar experiments on EGF treated pancreatic buds suggested that EGF might stimulate epithelial proliferation and thereby inhibit differentiation of pancreatic precursors to β-cells [47, 48]. LIF is known to be expressed in pancreatic ducts [28], but evidence of its endogenous functions in pancreatic development are lacking.

Implications for a cell based diabetes therapy

Islet transplantation therapies for type 1 diabetes have been explored for many years and have become quite successful, but transplant numbers are limited due to donor supply [49,50]. The way forward is to produce β-cells for transplantation *in vitro*. This approach may eventually become possible with one or more of several approaches: islet progenitor cells, adult stem cells, embryonic stem cells or expansion of existing β-cells [51, 52]. An alternative possibility is to generate insulin producing β-cells from other available cells of the body, especially from "sister" tissues of the pancreas arising from the foregut endoderm. Hepatocytes [53–56] and pancreatic acinar cells [44] have been converted to β-like cells by adding soluble factors and/or overexpressing pancreatic transcription factors *in vivo* or *vitro* [52]. Also, non-pancreatic stem cells, like oval cells which reside in the liver, have been reprogrammed to insulin producing cells and were able to reverse hyperglycemia in diabetic mice [42]. The presence of β-cells in the extrahepatic ductal epithelium suggests it may also be possible to reprogram biliary epithelium to β-cells for the treatment of diabetes, although to do so successfully may require more knowledge of the underlying mechanism than is available at present.

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Supplementary Material

Supplementary Movie 1. Three dimensional model of insulin expressing cells (red) in bile ducts (DBA, green). Only one insulin positive cell is DBA negative and attached to the duct (top right).

Supplementary Movie 2. Three dimensional model of β-cells (red) located between two smooth muscle actin positive mesenchymal cell layers (green) in the extrahepatic biliary system.

Supplementary Movie 3. Three dimensional model of β-cells (red) attached to PECAM positive cells (green) in the extrahepatic biliary system.

Supplementary Figure 1. Example of insulin positive cells in the extrahepatic biliary ducts (DBA, green) of untreated (*A*) and 20 mM nicotinamide treated ducts (*B*) cultured for six days. In this example, the untreated duct contains six and the nicotinamide treated duct 21 cells (see arrows). Scale bars, 100 μm.