Research Article

β cells occur naturally in extrahepatic bile ducts of mice

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Summary

Insulin-secreting β cells were thought to reside only in the pancreas. Here, we show that β cells are also present in the extra-hepatic bile ducts of mice. They are characterised by insulin and C-peptide content, the presence of secretory granules that are immunoreactive for insulin, and the ducts exhibit glucose-stimulated insulin secretion. Genetic lineage labelling shows that these β cells arise from the liver domain rather than the pancreas and, by histological study, they appear to be formed directly from the bile duct epithelium in late embryogenesis. Other endocrine cell types (producing somatostatin and pancreatic polypeptide) are also found in close association with the bile-duct-derived β cells, but exocrine pancreatic tissue is not present. This discovery of β cells outside the mammalian pancreas has implications for regenerative medicine, indicating that biliary epithelium might offer a new source of β cells for the treatment of diabetes. The finding also has evolutionary significance, because it is known that certain basal vertebrates usually form all of their β cells from the bile ducts. The mammalian bile-duct-derived β cells might therefore represent an extant trace of the evolutionary origin of the vertebrate β cell.

Key words: β cell, Insulin, Bile duct, Pancreas, Liver, Diabetes, Cre-lox

Introduction

In recent years the development of the pancreas has received much attention, largely in response to the challenge of diabetes mellitus as a major problem of human health (Edlund, 1999; Murtaugh and Melton, 2003; Slack, 1995; St-Onge et al., 1999). The pancreas contains five types of endocrine cell, mostly assembled into the islets of Langerhans, of which the insulin-producing β cells are the most numerous. Some of the other endocrine cell types, producing glucagon, somatostatin, pancreatic polypeptide or ghrelin, may be found elsewhere in the body, but until now the β cells were thought to be confined to the pancreas.

Insulin therapy for diabetes has been highly successful and has become very sophisticated, but the disease still produces a considerable burden of distressing complications and represents a substantial cost to health budgets around the world (Zimmet et al., 2001). One possible route forward, especially for type 1 diabetes, is through an induced augmentation of β-cell mass. Some success has been achieved in recent years using islet grafts into the liver (Kendall et al., 2001; Shapiro et al., 2002), but at present the main limitation to the wider application of this technique is the limited supply of islets from human organ donors. This has encouraged clinicians and developmental biologists to think of ways in which the mass of β cells might be augmented either through expansion in vitro, or differentiation from embryonic stem cells, or differentiation from other tissue types (Roche et al., 2003).

One potential source of new β cells is the liver. The pancreas and liver arise from adjacent areas in the anterior endoderm of the developing embryo (Wells and Melton, 1999). This suggests that the developmental commitment of the liver and pancreas differs by the expression of only a few genes, and so by manipulating the expression of those genes it might also be possible to convert liver into pancreas. A number of labs have shown that it is possible to induce expression of a variety of endocrine and exocrine pancreatic genes in the liver by overexpression of transcription factors such as pancreatic and duodenal homeobox gene 1 (Pdx1, also known as insulin promoter factor 1, IPF1) and neurogenin 3 (Ber et al., 2003; Horb et al., 2003; Sapir et al., 2005; Zalzman et al., 2003; Zalzman et al., 2005). In the course of some experiments attempting to introduce various genes into the liver, we discovered that our control animals already had some β cells in their livers: more specifically they were associated with the large bile ducts found in the hilar region of the liver, which is the region near the gall bladder where the liver lobes come together around the bile ducts and major blood vessels.

The biliary system in mouse and in humans originates in the liver parenchyma, where bile is secreted by hepatocytes into a system of intrahepatic ducts. These drain into larger extrahepatic ducts, which join with the cystic duct from the gall bladder to form the common bile duct. This is joined by the main pancreatic duct shortly before entry into the duodenum. The epithelium of the intrahepatic bile ducts is derived from bipotential precursor cells within the hepatic parenchyma (Lemaigre and Zaret, 2004; Zhao and Duncan, 2005). The extrahepatic biliary tract develops separately from the intrahepatic ducts and becomes anastomosed to them by an unknown mechanism (Lemaigre, 2003; Shiojiri, 1997). A recent study by Sumazaki and colleagues demonstrated the
formation of foci of ectopic pancreatic tissue in the extrapancreatic bile ducts of mice homozygous for a null allele of \(Hes1\) (hairy and enhancer of split), a mediator of Notch signalling (Sumazaki et al., 2004). This suggests that within the liver-domain-derived tissues, the extrapancreatic bile ducts may be particularly closely related to pancreas in terms of the combination of transcription factors whose expression controls differentiation.

Here, we show that there is a small population of extrapancreatic endocrine cells within or adjacent to bile ducts in the hilar region of the adult liver. At least four of the normal pancreatic endocrine types are present, and the insulin-positive cells appear to be genuine \(\beta\) cells rather than some other cell population that is making insulin. We have used albumin-Cre mice to label the liver domain during development and show that the ectopic \(\beta\) cells originate from this region rather than migrating from the pancreas. On the basis of the morphological analysis the cells appear to be formed directly from the bile duct epithelium in late embryogenesis and to multiply slowly thereafter to form small clusters in the duct mesenchyme. Other endocrine cell types are also found in close association with the bile-duct-derived \(\beta\) cells but exocrine pancreatic tissue is not present.

Although the extrapancreatic endocrine cells are not numerous, their existence is highly significant for two distinct reasons. First, the fact that they arise spontaneously suggests that the extrapancreatic bile ducts must have a similar transcription factor code to the pancreatic endocrine cells, and this means that the biliary epithelium may offer a new source from which to make \(\beta\) cells for the treatment of diabetes. Second, they provide a clue concerning the evolutionary origin of the vertebrate \(\beta\) cell. The agnatha, which are the most basal living vertebrates, have no exocrine pancreas and appear to normally form all their \(\beta\) cells from the bile ducts (Youson, 2000). The bile-duct-derived \(\beta\) cells of mice may therefore represent an extant trace of this primordial method of \(\beta\)-cell development in modern mammals.

**Results**

Endocrine cells are found in bile ducts of the hilar region

The hilar region is the location in the body where the liver lobes meet with the principal blood vessels, bile ducts and the gall bladder (Fig. 1A). During an analysis of the livers of some experimental mice we found insulin-positive cells in this region and it soon became clear that these cells existed also in normal control mice. They are found where the extra-hepatic bile ducts branch to individual liver lobes (Fig. 1A,B). Insulin-positive cells or cell clusters were found in bile ducts of most mice with those examined ranging in age from embryonic day 18.5 (E18.5) up to 6 months.

Sections showing insulin-positive cells associated with large bile ducts are shown in Fig. 1C-F, and the locations are further documented in an analysis of serial transverse sections from 30 mice shown in Fig. 1H. The cells are not seen in mid-gestation embryos but first become visible in the late embryo, from E17.5. At E17.5 most of the cells are isolated individuals and about 50% lie within the ductal epithelium or touch it (Fig. 1C). At postnatal stages they are mostly found in the duct connective tissue that surrounds the epithelium and very few are in the epithelium itself, consistent with the view that, once formed, they migrate from the epithelium (Fig. 1D-F). A few cells are actually found within the parenchyma of the liver (Fig. 1G) so they can presumably migrate this far from the duct. With advancing age, the total number of foci remains constant and the proportion of isolated cells falls while the proportion of small clusters increases (Fig. 2A-C). This morphological study suggests that the endocrine cells arise from the duct epithelium in late embryonic life, migrate into the neighbouring connective tissue and then divide slowly to yield small cell clusters.

Hilar region insulin-positive bile duct cells are not a peculiarity of one individual mouse strain because they were found in both CD1 and C57BL/6 mice. The cells were found in both sexes and in most but not every individual mouse (31 of 35 strain CD1s and 5 of 7 strain C57BL/6s), but because it is not practical to examine every single section of the extended regions of the duct it is quite possible that all mice contain them.

**Expression of other pancreatic hormones**

We wanted to know whether bile ducts also contain the other pancreatic endocrine cell types, i.e. \(\alpha\) cells (producing glucagon), \(\delta\) cells (producing somatostatin, SS), pancreatic-polypeptide-producing (PP) cells and \(\epsilon\) cells (producing ghrelin), hereafter referred to as glucagon-, SS-, PP- and ghrelin-cells. SS- and PP-cells were often found, glucagon cells rarely and ghrelin cells were not found at all. None of the other types were as numerous as the insulin-positive cells. Fig. 3A shows a single SS cell in the bile duct epithelium, suggesting that they too arise directly from the extrapancreatic biliary epithelium. A typical cluster is shown in Fig. 3B, containing insulin and SS cells. Large clusters are very rare (examples are shown in Fig. 3C,D), resemble pancreatic islets and contain many SS and PP cells. This high content of PP cells is reminiscent of the islets formed from the ventral pancreatic bud, which is developmentally and anatomically much closer to the biliary system than is the dorsal pancreatic bud. Fig. 3E describes a model for the formation of the endocrine cells that is consistent with the data obtained.

**Characterisation of bile duct derived \(\beta\) cells**

Next, we wished to know whether a cell that is immunopositive for insulin is really a \(\beta\) cell or simply a cell that has accumulated some insulin protein. A set of criteria has been devised for examining this question, comprising evidence for synthesis and secretion of insulin as well as its presence in cells (Rajagopal et al., 2003). Measurement of insulin mRNA from isolated hilar region bile ducts by quantitative reverse transcriptase (RT)-PCR (Table 1) indicates about seven times more insulin mRNA in the hilar ducts than in liver parenchyma. This is consistent with insulin mRNA synthesis by insulin-positive cells. To detect proteolytic processing of the insulin precursor molecule we immunostained for the presence of C-peptide, which was present in – and only in – the insulin-positive bile-duct-derived cells (Fig. 4A,A'). \(\beta\) cells possess characteristic electron-dense secretory granules that are immuno-positive for insulin. To examine the secretory apparatus we processed hilar region ducts for electron microscopy. Although the endocrine cells are not numerous they can be located by staining semi-thin (1 \(\mu\)m) hilar-region-duct sections with basic fuchsin, followed by examination of the adjacent thin sections. We found that some, but not all, of the bile duct endocrine cells have dark-centered
secretory granules characteristic of β cells (Fig. 4B). When stained for insulin by immunogold labelling, these cells show the presence of insulin within the granules (Fig. 4C). There are also cells with secretory granules that are not immunoreactive for insulin, but these granules are not the typical dark-centered secretory granules characteristic of β cells and therefore presumably represent one of the other endocrine cell types. At present there is no possibility of isolating this small bile duct endocrine cell population for physiological studies, but we were able to examine secretion of insulin provoked by glucose stimulation of isolated ducts, with explants of normal gall bladder and liver as controls. This showed an approximately sevenfold increase in the release of insulin provoked by exposure to 25 mM glucose (Table 2). From this set of studies we conclude that the insulin-positive cells in the bile ducts really are β cells rather than some other cell type expressing the insulin gene.

Fig. 1. Insulin-positive cells in the hilar region. (A) Bile duct system of an adult mouse made visible by injection of the gall bladder with 0.5% Trypan Blue. The boxed area is the hilar region where the ectopic cells are located. gb, gall bladder; panc, pancreas; duo, duodenum. (B) Whole-mount stain of the ducts in the hilar region of an adult mouse using Dolichos biflorus lectin (red), and antibody against insulin (green). The insulin-positive cells are in clusters in the connective tissue surrounding the ducts. Bar, 500 μm. (C-F) Insulin-positive cells (green), associated with bile ducts: (C) 18.5-day embryo, (D) 1 day postnatal, (E) 7 days postnatal, (F) 6 months. (G) Two insulin-positive cells just within the liver parenchyma of a 3-week-old mouse. Bars, 20 μm. (H) Analysis of the hilar duct system in 30 mice of different ages (E18.5 to age 6 months). Each vertical line represents a series of serial sections from one mouse of which every fifth slide (five sections) was examined. The horizontal bars indicate those sections containing one or more insulin-positive cells.

Fig. 2. Quantitative data. (A) Average number of foci per animal. (B) Average number of cells per focus. (C) Percentage of insulin-positive cells on the sections found within 10 μm of the ductal epithelium. n=5 mice per group, except data for 2-month-old mice, where n=10. For the postnatal mice insulin-positive cells were counted on every fifth slide (where one slide contains 4×4 μm or 5×4 μm sections) of serial sections through the hilar region. For embryos, every other slide was examined (approximately 8-μm-intervals). The number of animals with insulin-positive cells was five of five for 1-day- and 6-month-old animals, four of five for 7-day- and 3-week-old animals and for embryos at E18.5, and nine of ten for 2-month-old animals. Error bars represent standard errors.
Lineage tracing of β cells of the hilar region bile duct

The morphological study suggests that the β cells arise from the hilar region bile duct epithelium (indicated in Fig. 3E). However, because some cells may migrate long distances during development it is not possible to prove cell lineage from morphological data alone. We have therefore used a Cre-lox system to label the origin of the cells. It is known that the albumin promoter is active in the liver rudiment from an early stage of development (Yakar et al., 1999). Matings were made between mice in which Cre recombinase is driven by the albumin promoter (Alb-Cre) (Postic and Magnuson, 2000), and reporter mice in which β-galactosidase is expressed in cells that experience Cre-mediated recombination (R26R lacZ) (Soriano, 1999). Fig. 5A shows a view of the gall bladder and duct system of a newborn mouse, which are positive for β-galactosidase, and the pancreas, which is negative. Fig. 5B shows a section of hilar region bile duct stained for β-galactosidase and insulin; insulin-positive cells are also β-galactosidase positive. Of insulin-positive cells found in five newborn mice, 60% stained positive for β-galactosidase compared with 64% of duct cells and 0.7% of pancreatic β cells (Table 3). These results match those expected for cells derived from albumin-positive regions rather than from pancreas and clearly show that these cells are derived from the bile duct epithelium and have not migrated from the developing pancreas.

Discussion

This study is the first report of mammalian β cells that exist and are formed outside of the pancreas. The cell population we describe does not migrate from the pancreas although a few cells do probably migrate postnatally into the adipose tissue associated with the distal part of the common bile duct. From our combination of morphological study and lineage tracing we believe that the β cells described here arise from the bile duct epithelium in late embryonic life. Neogenesis of β cells from pancreatic ducts has been observed in many situations (Bonner-Weir, 2000; Gu et al., 1994), but this is the first observation of their formation from bile ducts (excepting cases of ectopic pancreas, see below). Since the number of foci seems to be constant with age we believe that the cells do not arise postnatally as metaplasias, but develop during embryonic life and then persist with slow multiplication, thereby increasing the cell number per focus. They are common among individuals, arising in most or perhaps all mice, but they are rare at the cellular level, accounting for a tiny proportion of all the epithelial cells in the ducts.

Possible mechanisms

The region in which the endocrine cells arise lies near the junction between the intra- and extra-hepatic bile ducts. Epithelial junctions are often regions of developmental instability, as witnessed by the frequent occurrence of metaplasia and cancer at the oesophageo-gastric or cervico-vaginal junctions of humans (Slack, 1986). So the endocrine cells probably represent rare spontaneous instances of altered transcription factor expression, such that occasional duct cells

Table 1. RT-PCR of mRNA in hilar and lobe regions

| mRNA      | Hilar region* | Liver lobe* | Mean difference | s.d. | t value | Significance |
|-----------|---------------|-------------|----------------|------|---------|--------------|
| Insulin 1 | 32.32         | 35.40       | 3.08           | 1.85 | −3.72   | P<0.02       |
| Insulin 2 | 31.28         | 33.97       | 2.69           | 1.06 | −5.67   | P<0.005      |
| β actin   | 19.26         | 18.63       | −0.62          | 1.42 | 0.98    | 0.382, NS    |

Five separate measurements, t calculated by paired test with 4 d.f. *, average number of cycles; s.d., standard deviation.

Fig. 3. Formation and migration of endocrine cells. (A) Single somatostatin-positive cell in the duct epithelium. (B) Typical cluster showing SS- and insulin-positive cells. (C,D) Triple-stained large endocrine cell clusters: (C) insulin (green), SS (red), glucagon (blue); (D) insulin (green), PP (red), glucagon (blue). Bars, 20 μm. (E) Hypothetical mechanism for endocrine cell formation from duct epithelium.

Fig. 4. Characterisation of β cells. (A, A') C-peptide (red) and insulin (green). Bar, 20 μm. (B) TEM showing cells with β-cell-like secretory granules (arrowheads). Bar, 2 μm. (C) Immuno-EM showing a positive signal for insulin in the lower right cell. The upper right cell has secretory granules that are not insulin-positive. Bar, 0.5 μm.
The transcription factor combination that encodes pancreatic endocrine cell rather than the one that encodes biliary epithelial cell. The chances of such an event arising are obviously greater if the two tissues have similar transcription factor codes. This is probable because the distinction in development between the hepatic rudiment, forming the liver and biliary system, and the ventral pancreatic bud, forming the proximal pancreas, is controlled by a single inductive event involving fibroblast growth factors and bone morphogenetic proteins from the adjacent mesoderm (Zhao and Duncan, 2005). Although one signalling process may activate or repress several transcription factors, this suggests that the transcription factor codes for the two tissue types are probably rather close. Furthermore, we already know of one gene product whose expression seems to distinguish between pancreatic and biliary tissue. The bHLH transcription factor Hes1 is activated by Notch signalling and normally functions as a repressor of genes controlling endocrine cell differentiation. In the Hes1-knockout mouse it has been shown that foci of pancreatic tissue replace the bile ducts (Sumazaki et al., 2004). It is therefore plausible that, in normal development, the distinction between biliary and pancreatic epithelium is actually controlled by the expression of Hes1 (Burke et al., 2004) and this possibility is under current investigation in our lab. The reason that the ectopic endocrine cells are found in the extrahepatic but not the intrahepatic bile ducts may be related to the embryonic activity of transcription factor Pdx1, whose gene is expressed in the developing extrahepatic but not intrahepatic ducts (Offield et al., 1996; Sumazaki et al., 2004). However, the fact that we see small groups of endocrine cells without exocrine pancreas is probably because the PTF1α transcription factor, needed for exocrine development, is not normally expressed in the bile ducts (Fukuda et al., 2006).

We currently do not know whether bile-duct-derived β cells are also present in humans. Small but macroscopic foci of heterotopic pancreatic tissue have occasionally been described in literature of human pathology as an occurrence in gall bladder or bile ducts (Branch and Gross, 1935; Jarvi and Meurman, 1964). However, apart from being extremely rare, these cells also differ from the cells we describe in that they, like the Hes1 knockout mouse, contain ductal and acinar structures. It will be of interest to look for the presence of β cells in the normal human hilar region.

**Evolution**

The finding that β cells develop naturally in mouse bile ducts has an evolutionary significance because it is thought that primitive jawless vertebrates usually form all their β cells from the bile ducts. In the hagfish, there is no exocrine pancreas but β-like cells cluster at the base of the bile duct, with some cells located within the biliary epithelium (Youson, 2000). In lampreys the entire biliary system becomes resorbed during metamorphosis, in the course of which much of the ductal epithelium appears to become transformed into β-like cells. In jawed fish and all higher vertebrates the situation is similar to mammals, with the pancreas developing from two buds that produce exocrine and endocrine cells (Ober et al., 2003).

### Table 2. Glucose-stimulated insulin release

| Sample          | Glucose (mM) | Insulin release (ng/mg total protein) | Fold increase of secretion |
|-----------------|--------------|--------------------------------------|---------------------------|
| Bile duct       | 0.0          | 1.7                                  |                           |
|                 | 25.0         | 11.8                                 | 6.9                       |
| Bile duct       | 5.5          | 4.0                                  | 12.7                      |
|                 | 25.0         | 50.8                                 |                           |
| Bile duct       | 5.5          | 1.7                                  | 2.5                       |
|                 | 25.0         | 4.3                                  |                           |
| Liver           | 5.5          | 0.49                                 |                           |
| Gall bladder    | 25.0         | 0.0                                  |                           |

Three separate experiments are shown for bile duct. Liver and gall bladder showed no glucose-stimulated secretion and representative results are shown.

### Table 3. Alb-Cre labelling

| % labelled    | n    |
|---------------|------|
| Duct β cells  | 60   | 63   |
| Duct cells    | 64   | 1359 |
| Pancreatic cells | 0.7 | 838  |

Five newborn mice were counted.

Fig. 5. Alb-Cre lineage labelling. (A) Newborn Alb-Cre/R26R lacZ mouse stained with X-gal. The gall bladder (g) and bile duct system are labelled, as is a piece of liver left attached in the hilar region (h). The pancreas (p) is virtually unlabelled, the visible blue being surface background stain and a small part of the pancreatic duct; d, duodenum. (B, B', B'') Section showing an insulin-positive cell labelled with X-gal in the duct epithelium. Bar, 20 μm.
Youson and Al-Mahrouki, 1999). These observations suggest that the evolutionary origin of the vertebrate β cell actually lies within the biliary system, as seen in the present day hagfish and lamprey. Our discovery of β cells in the murine biliary system indicates that the primordial system still persists on a small scale in modern mammals, representing a residue of the original method of endocrine cell formation in the vertebrate ancestor.

Significance for regenerative medicine

Diabetes has become a vast global problem. Its complications cause enormous suffering and consume 10-15% of national health care budgets (Zimmet et al., 2001). Consequently, cell therapy to bring about β-cell augmentation or replacement is a key goal of regenerative medicine. Although islet transplantation protocols have had growing success (Kendall et al., 2001; Shapiro et al., 2002) they will always be limited by the supply of donor organs. There is currently a worldwide effort to find ways of making β cells from embryonic stem cells (Roche et al., 2003) from adult stem cells and other tissue types, such as pancreatic exocrine tissue or liver (Ber et al., 2003; Sapir et al., 2005; Zalzman et al., 2005; Zalzman et al., 2003a, 2003). So far, the cells that have been produced have been insulin-producing cells of mixed phenotype rather than true β cells. We believe that the existence of β cells derived from biliary epithelium means that this tissue too should be considered a candidate for reprogramming to β cells. Given the existence of the natural bile-duct-derived β-cell population the chances for making genuine β cells from isolated bile duct epithelium should be high. Furthermore, the gall bladder is lined with very similar epithelium to the bile ducts, and because it is a relatively dispensable organ this may serve as a suitable source of tissue for reprogramming either in vivo or in vitro.

Methods and Materials

Immunohistochemistry

CD1 or C57BL/6 mice were killed by cervical dislocation. Hilar regions were dissected in PBS then fixed in MEMFA buffer (0.15 M MOPS pH 7.5; 2 mM EGTA; 1 mM MgSO4; 3.7% formaldehyde) at 4°C overnight under constant agitation. Tissue was then processed and embedded in paraffin. 4-μm serial sections were made through the hilar region. Sections were dewaxed in histoclear and dehydrated in an ethanol dilution series. 4-μm sections were permeabilised in 1% Triton-X-100 and blocked in 2% Roche blocking buffer (both for 1 hour) at room temperature. Samples were incubated in guinea-pig anti-insulin antibody (DAKO) 1:500 (1:200 for embryos) overnight at 4°C; subsequent steps were carried out at room temperature. Sections were washed 3×15 minutes in PBS, incubated in secondary antibody, anti-guinea-pig FITC antibody (Vector) 1:100 for 2 hours, washed 2×15 minutes in PBS, incubated in DAPI (1:10000) for 15 minutes, washed for 2×5 minutes in PBS and then mounted with GELMOUNTTM (Biemeda Corp.). Other primary antibodies were: rabbit anti-human somatostatin (DAKO), rabbit anti-pancreatic polypeptide (Zymed Laboratories Inc.), monoclonal anti-glucagon (SIGMA), goat anti-rat C-peptide serum (Linco Research Inc.) All were used at 1:100.

Electron microscopy

Bile ducts from 3-month-old CD1 mice were fixed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.3) containing 0.5% glutaraldehyde, 4% paraformaldehyde and 2.5 mM CaCl2. On the following day, the tissue was rinsed in PBS and then incubated in 0.5% BSA in PBS overnight at 4°C. The grids were washed 3×10 minutes in PBS, then incubated with secondary antibody (10-20 nm gold-labelled goat anti-guinea pig IgG) 1:50 in 1% normal goat serum and 0.5% BSA in PBS for 2 hours at room temperature. To remove excess secondary antibody, the grids were then washed in 0.1 M PIPES buffer (pH 7.3) 3×10 minutes. Sections were postfixed in 1% glutaraldehyde in 0.1 M PIPES buffer pH 7.3, rinsed in distilled water, and then finally stained in 2% uranyl acetate and lead citrate. Grids were viewed under a Jeol 1200EX TEM.

For immuno-transmission electron microscopy (TEM), aldehyde sites were blocked by incubating in 0.05 M glycine in PBS for 15 minutes. The grids were rinsed in PBS, blocked with 10% normal goat serum and 0.5% bovine serum albumin (BSA) in PBS. The grids were again briefly rinsed in PBS and then incubated in guinea pig anti-insulin antibody (1:500) in 1% normal goat serum and 0.5% BSA in PBS overnight at 4°C. The grids were washed 3×10 minutes in PBS, then incubated with secondary antibody (10-20 nm gold-labelled goat anti-guinea pig IgG) 1:50 in 1% normal goat serum and 0.5% BSA in PBS for 2 hours at room temperature. To remove excess secondary antibody, the grids were then washed in 0.1 M PIPES buffer (pH 7.3) 3×10 minutes. Sections were postfixed in 1% glutaraldehyde in 0.1 M PIPES buffer pH 7.3, rinsed in distilled water, and then finally stained in 2% uranyl acetate and lead citrate. Grids were viewed under a Jeol 1200EX TEM.

Quantitative RT-PCR analysis

Tissues from 2-month-old mice were frozen on dry ice. RNA was prepared using Trizol (Invitrogen Life Technologies, Paisley, UK). 2 μg of RNA was DNase treated and checked for DNA removal by GAPDH PCR. Samples were reverse transcribed using Oligo dT primer and SSII reverse transcriptase RT (Invitrogen).

Real-time PCR was performed on a lightcycler (Roche) using Quagen’s Quantitect Custom Assay system on samples of 2 μl denatured cDNA. Cycling conditions were: denaturation at 95°C for 15 minutes, annealing at 56°C for 30 seconds, extension at 76°C for 30 seconds, for 45 cycles.

Glucose-stimulated insulin release

Bile ducts from the hilar region, gall bladders or liver explants were dissected in glucose-free PBS. Both male and female CD1 mice were used and the ducts were inspected to ensure no pancreatic tissue was contaminating the samples. The tissue explants were incubated in 125 μl PBS supplemented with high or low concentration of glucose at room temperature for 2 hours. In the first experiment the low glucose was zero, in subsequent ones 5.5 mM. The high glucose was 25 mM. Aliquots of medium were then assayed for insulin by ELISA (Mercodia ultra-sensitive anti-rat insulin plate).

Lineage tracing

Tissue of neonatal C57BL/6 Alb-Cre × R26R LacZ mice (heterozygous for both transgenes) was used to trace the origin of the ectopic insulin-positive cells. The liver hilar region and the common bile duct attached to a small piece of intestine was dissected and fixed in 2% formaldehyde in PBS overnight at 4°C. Subsequently, the tissue was rinsed in PBS and stained overnight at 28°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 MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS (pH 7.3). Tissue of R26R LacZ reporter mice were used as controls to monitor endogenous β-galactosidase activity. The stained tissue was finally refluxed in 4% PFA overnight at 4°C, dehydrated and embedded in paraffin. Sections (5 μm) were stained for insulin as described above, to localise ectopic insulin-positive cells. Under the conditions used, the FITC signal was not obscured by the X-gal stain. Newborn Alb-Cre × R26R LacZ mice showed virtually no labelling of pancreatic β cells, although labelling appeared in older mice, presumably because of limited albumin promoter activity. The studies with live mice were carried out with approval from the ethics committee of the University of Bath under a UK Home Office project licence.

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