Dose-dependent requirement of patched homologue 1 in mouse pancreatic beta cell mass

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Abstract

Aims/hypothesis Ectopic activation of hedgehog (HH) signalling in pancreas induces various abnormal morphogenetic events in the pancreas. This study analysed the dose-dependent requirement of patched homologue 1 (PTCH1), a negative regulator of HH signalling on pancreatic development.

Methods We used a recessive spontaneous mutant mouse denoted as *mes* which carries a mutated *Ptch1* resulting in deletion of the most carboxy-terminal cytoplasmic domain of the PTCH1 protein. In this study, we analysed pancreatic morphology in *Ptch1*^{+/+}, *Ptch1*^{+/mes}, *Ptch1*^{+/-}, *Ptch1*^{mes/mes} and *Ptch1*^{-mes} mouse embryos, as well as the islet mass in adult *Ptch1*^{+/+}, *Ptch1*^{+/mes} and *Ptch1*^{+/-} mice.

Results Until embryonic day (E) 12.5, no obvious abnormality of pancreas was observed in any of the *Ptch1* mutants. The levels of PDX1 and glucagon were also not evidently different among the mice genotypes studied. Thereafter, morphological abnormalities appeared in the *Ptch1* mutant mice. The beta, alpha and exocrine cell masses decreased at E18.5 in parallel with increased HH signalling, with beta cell

mass showing the highest sensitivity to HH signalling with a significant decrease even in *Ptch1*^{+/mes} mice. Adult *Ptch1*^{+/+} mice also showed a significant decrease in beta cell mass compared with wild-type mice.

Conclusions/interpretation Our findings indicate that the carboxy-terminal domain of *Ptch1* is essential for pancreatic development. In addition, the loss of *Ptch1* function decreases both the endocrine and exocrine cell mass in a dose-dependent manner, with beta cells particularly sensitive to changes in HH signalling.

Keywords Hedgehog · Insulin · Islets · Patched 1 · Ptc1 · Ptch1 · Type 2 diabetes

Abbreviations

alpha-SMA	alpha-smooth muscle actin
E	embryonic day
EMT	epithelial to mesenchymal transition
HH	hedgehog
IPGTT	intraperitoneal glucose tolerance test
PTCH1	patched homologue 1
SHH	sonic hedgehog

Introduction

Hedgehog (HH) signalling regulates morphogenesis and cell differentiation in various tissues [1]. There are at least three secreted mammalian HH ligands, including sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH) [1]. While their binding affinities differ slightly, all HH proteins bind to transmembrane receptors, patched homologue 1 (PTCH1) and 2 (PTCH2) in target cells. The expression of PTCH1 is more prominent and its function has been analysed in detail [2]. In the absence of

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ligand, PTCH1 represses the activity of *Smo*, a G-protein coupled-like receptor. Upon ligand binding, repression of *Smo* activation is alleviated, allowing nuclear translocation of *Gli* transcription factors and subsequent expression of HH target genes including *Gli* itself [3].

During early endoderm differentiation, both *Shh* and *Ihh* are expressed strongly throughout the endodermal epithelium, with the exception of pancreatic primordia [4–6]. Similarly, *Ptch1* is expressed in the mesenchyme adjacent to the pancreas, but expression has not been confirmed in the pancreas area of mouse embryos before embryonic day (E) 12.5 [4]. Thereafter, the developing tissue starts to express several HH components [7, 8]. The expression of *Ptch1* has been confirmed by RT-PCR assay after E13.5, and mature pancreas expresses *Ihh*, *Dhh*, *Hhip*, *Ptch1* and *Smo* in islet and ductal cells [8–11]. Several groups have investigated the effect of increased HH signalling on pancreatic morphogenesis. Ectopic expression of SHH under the control of the *Pdx1* promoter results in almost complete organ ablation [4, 12]. In addition, ectopic expression of both SHH and IHH under the control of the *Pax4* promoter induces a decreased pancreatic mass [12]. These findings suggest that the limited HH signalling in the pancreas plays a key role in pancreatic organogenesis.

A previous study suggested that HH signalling affects pancreatic development dose-dependently [10]. Loss of the HH pathway inhibitor, *Hhip*, increased the formation of pancreatic mesenchyme and decreased the size of the pancreatic epithelium. The additional loss of one *Ptch1* allele in *Hhip* mutant mice caused a more profound loss of epithelial cells, indicating that pancreatic cells respond to different levels of HH activity [10]. However, the dose-dependency of HH activity in later-stage pancreatic development could not be fully analysed using these models because loss of one *Ptch1* allele in the *Hhip* mutant was lethal before E13.

Previous studies have revealed that the carboxy-terminal cytoplasmic domain of Patched plays an essential role in blocking HH signalling in wing disc [13], as well as regulating Patched degradation and trafficking [14] in *Drosophila*. *mes* encodes a mutant PTCH1 protein lacking most of the carboxy-terminal 220 amino acids [13] and is considered a hypomorphic allele. Organ development phenotypes in compound heterozygotes of *mes* and *Ptch1* null alleles (*Ptch1^{mes/-}*) are generally more severe than those for *mes* homozygotes (*Ptch1^{mes/mes}*), but milder than those for homozygotes of *Ptch1* null alleles (*Ptch1^{-/-}*) [13]. The *Ptch1^{mes/-}* mice survive longer than *Ptch1* null homozygotes, making them a useful tool to study the biological roles of PTCH1 in later development. Interestingly, although *mes* is considered to be a hypomorphic allele, *Ptch1^{mes/-}* mice show no patterning defect of the developing spinal cord where titration of HH activation plays an essential

role [13, 15, 16]. Thus, the carboxy-terminal domain of PTCH1 has a qualitatively distinct function depending on tissue or developmental stage [15, 17].

Here, we used *mes* mice to investigate the role of the PTCH1 carboxy-terminal domain in pancreatic development and the dose-dependent effects of increased HH signalling. We generated *Ptch1^{+/+}*, *Ptch1^{+/mes}*, *Ptch1^{+/-}*, *Ptch1^{mes/mes}* and *Ptch1^{-/-mes}* mice to analyse pancreatic morphology during development in each mouse. Our data implicated the carboxy-terminal domain of PTCH1 in pancreatic development.

Methods

Animals The Animal Care and Use Committee of Juntendo University, School of Medicine reviewed and approved the study protocol. All mice were housed in specific pathogen-free barrier facilities, maintained in a 12 h light–dark cycle and had free access to standard rodent food (Oriental Yeast, Osaka, Japan) and water. *Ptch1* knockout mice, ROSA26-lacZ mice and Ptf1a-Cre mice were generated as described previously [18–20]. *Ptch1^{+/mes}* mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). *Ptch1* mutant mice used to investigate embryonic development were maintained in a CD1 background. To analyse the adult phenotype, the *Ptch1* mutant mice were backcrossed with C57BL/6J mice for a minimum of ten generations. Mutant mice and embryos were genotyped as described previously [15, 18–20].

RNA preparation and RT-PCR analysis Dissected E14.5 pancreas was lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was synthesised using 1 µg of total RNA with oligo dT primers and superscript reverse transcriptase (Invitrogen). For quantitative Quanti-ProbePCR (Qiagen, Hilden, Germany), cDNA was analysed using an ABI 7700 PCR machine (Applied Biosystems Japan, Tokyo, Japan) with 2× QuantiTect Probe PCR Master Mix (Qiagen) and specific probes. The thermal cycle for amplification was as follows: 2 min at 50°C, 15 min at 95°C and 40 cycles of 94°C for 15 s, 56°C for 30 s and 76°C for 30 s. The relative mRNA expression levels were calculated by the cycle threshold method with standard cDNA. The threshold is manually adjusted to a value above the background and significantly below the plateau of an amplification plot. The cycle at which amplification plot crosses the threshold is determined as threshold cycle. Quantification of target amounts was calculated by comparative cycle threshold method [21]. β-Actin mRNA served as an internal invariant standard. The primers and probes used are shown in Electronic supplementary material (ESM) Table 1.

Table 1 Relative *Gli1* mRNA expression and body weight in each *Ptch1* mutant

	<i>Ptch1</i> ^{+/+}	<i>Ptch1</i> ^{+/mes}	<i>Ptch1</i> ^{+/-}	<i>Ptch1</i> ^{mes/mes}	<i>Ptch1</i> ^{-/-}
<i>Gli1</i>	1.0±0.2	2.8±0.4	4.7±1.6	77.8±26.5	294±168
Body weight (g)	1.51±0.04	1.49±0.03	1.56±0.07	1.68±0.06	1.95±0.04

Data are mean±SEM. *Gli1* mRNA expression levels were measured by real-time RT-PCR analysis using E14.5 pancreas of each mice (*n*=6 each). Values are shown relative to the level of β -actin mRNA. To facilitate comparison, the expression in wild-type pancreas was adjusted to 1. Body weight was measured at E18.5 (*n*=5 each)

Immunohistochemical analysis Isolated pancreas or pancreas-containing tissues were fixed for 4 h at 4°C in 4% paraformaldehyde in phosphate-buffered saline (wt/wt). The fixed tissue was embedded in paraffin and then cut into 5 μ m thick sections and mounted on microscope slides. Immunohistochemical analysis was carried out as described previously [22] using the following primary antibodies at the indicated dilutions: mouse anti-vimentin antibody (1:200; Sigma-Aldrich Japan, Tokyo, Japan); rabbit anti-PDX1 antibody [23], 1:5,000; guinea pig anti-insulin antibody (1:1,000; Linco Research, St Charles, MO, USA); rabbit anti-glucagon antibody (1:1,000; Dako, Carpinteria, CA, USA); rabbit anti-amylase antibody (1:2,500; Sigma-Aldrich); mouse anti-somatostatin antibody (1:1,000; Biogenesis, Sandown, NH, USA); rat anti-pancreatic polypeptide antibody (1:500; Linco Research); rabbit anti-musculoaponeurotic fibrosarcoma oncogene family, protein A (MAFA) antibody (1:2,500; Bethyl Laboratories, Montgomery, TX, USA); mouse anti-E-cadherin antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and rat anti-bromodeoxyuridine antibody (1:50; OBT, Oxford, UK). Secondary antibodies were as follows: biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), biotinylated goat anti-rat IgG (Cedarlane, Hornby, ON, Canada), biotinylated goat anti guinea pig IgG (Chemicon International, Temecula, CA, USA), goat anti-mouse IgG conjugated with Alexa 488 (Molecular Probes, Eugene, OR, USA) and goat anti-rabbit IgG conjugated with Alexa 568 (Molecular Probes). To identify proliferating cells in the E14.5 pancreas, BrdU was injected and viewed as described previously [24]. With regard to all antibodies, we performed immunostaining without first or secondary antibodies and confirmed that the immunostaining shown in this study was not artefactual.

The positively-stained cell area was determined as a percentage on insulin-, glucagon- and amylase-stained sections viewed on a bright-field microscope (E800; Nikon, Tokyo, Japan) connected to an XYZ controller and digital camera (Sony, Tokyo, Japan). Each area was determined on four to six immunostained sections of three to seven mice each, with each section separated by at least 100 μ m. The cell areas were analysed using image analysis software

(Image Pro4.5J; Planetron, Tokyo, Japan). Non-specific staining was excluded from the quantification. The percentage of the cell area was calculated by the following formula: cell area (%) = the area stained by antibody/the total pancreatic area. Glucagon-positive area/exocrine area and insulin-positive area/exocrine area were calculated by the area stained by glucagon or insulin divided by exocrine area judged by haematoxylin staining.

X-gal staining X-gal staining was carried out as described previously [25].

Glucose and insulin tolerance tests Intraperitoneal glucose tolerance test (IPGTT) was carried out on 12-week-old male *Ptch1*^{+/+}, *Ptch1*^{+/mes} and *Ptch1*^{+/-} mice with 2.0 g/kg glucose. The insulin tolerance test was carried out with 0.75 units/kg insulin as described previously [26].

Data analysis Results are presented as mean±SEM. Differences between two groups were examined for statistical significance using the unpaired Student's *t* test. Statistical differences among three or more groups were examined by one-way ANOVA followed by Scheffé's post hoc test. A *p* value <0.05 was considered significant.

Results

Role of carboxy-terminal domain of PTCH1 in pancreatic development To investigate the involvement of carboxy-terminal domain of PTCH1 in the development of pancreas, we generated *Ptch1*^{+/+}, *Ptch1*^{+/mes}, *Ptch1*^{+/-}, *Ptch1*^{mes/mes} and *Ptch1*^{-/-} mice. We quantified HH signalling in the developing pancreas from the expression of HH target gene *Gli1* mRNA, using total RNA isolated from E14.5 pancreas. The presence of *mes* alleles, together with loss of wild-type *Ptch1* alleles, led to a stepwise increase in HH signalling in the pancreas (Table 1). These results clearly demonstrate that deletion of the carboxy-terminal domain of PTCH1 increases HH signalling during pancreatic development. In parallel with the increased HH signalling, we noted a dose-dependent increase in body weight at E18.5 (Table 1), consistent with previous findings [15].

The effect of dysfunctional *PTCH1* on early stage of pancreatic differentiation Macroscopic analysis of E10.5 and E12.5 pancreas from each *Ptch1* mutant revealed no obvious reduction in pancreas organ size (data not shown). In the early stage of pancreatic differentiation, all pancreatic epithelial cells express PDX1 [27], which is thus considered to be a good marker of pancreatic epithelial cells. We compared the expression of PDX1 in E10.5 and E12.5 pancreas from each *Ptch1* mutant. However, no obvious differences were observed (Fig. 1a). Next, we investigated the differentiation of endocrine cells in E10.5 and E12.5 pancreas. In E10.5 pancreas, we easily detected glucagon-positive cells in each *Ptch1* mutant and did not find any obvious differences in the number of glucagon-positive cells in E10.5 and E12.5 pancreas (Fig. 1b). On the other hand, in E10.5 pancreas, we hardly detected any insulin-positive cells in any of the *Ptch1* mutants (data not shown). In E12.5 pancreas, we were able to detect insulin-positive cells to a similar degree to that seen in each *Ptch1* mutant (data not shown). These results suggest that the dysfunction

of *Ptch1* does not result in obvious changes in cell number of epithelial cells and in the differentiation into endocrine cells in pancreas until E12.5.

The effect of dysfunctional *PTCH1* on exocrine pancreas at late stage of pancreatic differentiation Macroscopic analysis of E15.5 and E18.5 pancreas from each *Ptch1* mutant revealed an obvious reduction in pancreas organ size in *Ptch1*^{mes/mes} mice, while the size of the surrounding duodenum was comparable among the *Ptch1* mutants. The effect was more remarkable in *Ptch1*^{−/mes} mice (Fig. 2a). Vimentin and amylase double-staining of E18.5 pancreas revealed an increase in the mesenchyme and a concurrent decrease in acinar cell area in *Ptch1*^{mes/mes} mice and *Ptch1*^{−/mes} mice, the result being more striking in the latter (Fig. 2b).

To quantitatively assess the effect of dysfunctional *Ptch1* on the number of acinar cells in the pancreas, we investigated the levels of amylase in E15.5 and E18.5 pancreas (ESM Fig. 1). As early as E15.5, a decreased number of pancreatic acinar cells in *Ptch1* mutant was

Fig. 1 The effect of dysfunctional *PTCH1* on early stages of pancreatic differentiation. **a** PDX1 immunostaining of pancreas at E10.5 and E12.5 in mice as indicated. Scale bar, 50 μ m. **b** Glucagon staining of pancreas at E10.5 and E12.5. Scale bar, 50 μ m

Fig. 2 Exocrine cell mass in *Ptch1* mutants. **a** Macroscopic analysis of E15.5 and E18.5 pancreas from *Ptch1*^{+/+}, *Ptch1*^{+/-}, *Ptch1*^{+mes}, *Ptch1*^{mes/mes} and *Ptch1*^{-mes} mice. The black dotted line marks the pancreas. dp, dorsal pancreas; sp, spleen; vp, ventral pancreas. **b** Vimentin (green) and amylase (red) staining of E18.5 pancreas from each *Ptch1* mutant as indicated. **c** Percentages of amylase-immunopositive areas in E18.5 pancreas from each *Ptch1* mutant. To facilitate comparison, amylase-immunopositive areas in wild-type pancreas quantifications were adjusted to 1. Data are mean±SEM. *Ptch1*^{+/+}, *n*=8; *Ptch1*^{+/-}, *n*=8; *Ptch1*^{+mes}, *n*=7; *Ptch1*^{mes/mes}, *n*=5; *Ptch1*^{-mes}, *n*=6; **p*<0.05 vs *Ptch1*^{+/+}; †*p*<0.05 vs *Ptch1*^{+/-}; ‡*p*<0.05 vs *Ptch1*^{+mes}

observed. Quantitative analysis in E18.5 pancreas confirmed a marked decrease in amylase-positive cell area in *Ptch1*^{mes/mes} and *Ptch1*^{-mes} mice, as well as in *Ptch1*^{+/-} mice (Fig. 2c). These results indicate that the exocrine cell mass decreases in parallel with an increase in HH signalling.

The effect of dysfunctional PTCH1 on endocrine pancreas at late stage of pancreatic differentiation Next, we investigated the endocrine pancreatic cell area at E15.5 and E18.5. At E15.5, the glucagon-positive cell area was decreased in pancreas of *Ptch1* mutant mice (Fig. 3a). Quantitative analysis of E18.5 pancreas revealed a correlation between the decrease in glucagon-positive cell area and the increase in HH signalling (Fig. 3b). To investigate whether dysfunctional PTCH1 specifically altered glucagon-positive area, we calculated glucagon-positive area: exocrine area ratio in pancreas of wild-type, *Ptch1*^{+/-} and *Ptch1*^{+mes} mice. As shown in Fig. 3c, there were no differences in the glucagon-positive area:exocrine cell area ratio among any of the mice. These results suggest that dysfunctional PTCH1 results in a proportional decrease of glucagon-positive area with a decrease of exocrine cell area.

At E15.5, the insulin-positive cell area was also decreased in pancreas of *Ptch1* mutant mice (Fig. 4a). Quantitative analysis of E18.5 pancreas revealed a correlation between the decrease in insulin-positive cell area and the increase in HH signalling. The reduction of insulin-positive cells even in *Ptch1*^{+mes} mice suggested that the beta cell mass was particularly sensitive to changes in HH signalling (Fig. 4b). To investigate whether dysfunctional *Ptch1* specifically altered the insulin-positive area, we calculated the insulin-positive area:exocrine cell area ratio in pancreas of wild-type, *Ptch1*^{+/-} and *Ptch1*^{+mes} mice. As shown in Fig. 4c, the insulin-positive area:exocrine cell area ratio in pancreas was associated with increased HH signalling. These results suggest that the subtle dysfunction of PTCH1 results in a specific decrease of insulin-positive area in pancreas.

Immunostaining of somatostatin and pancreatic polypeptide in the pancreas of each *Ptch1* mutant at E18.5 revealed that delta and pancreatic polypeptide cells also tended to decrease according to the increase in the HH signal, although quantitative analysis was not performed (data not shown). Thus, increased HH signalling induces a decrease

Fig. 3 Alpha cell mass in each *Ptch1* mutant. **a** Glucagon staining of pancreas at E15.5 and E18.5 in mutants as indicated. Scale bar, 50 μ m. **b** Percentages of glucagon-immunopositive areas in pancreas of each *Ptch1* mutant at E18.5. To facilitate comparison, glucagon-immunopositive area in wild-type pancreas was adjusted to 1. Data are mean $\pm$ SEM. *Ptch1*^{+/+}, *n*=7; *Ptch1*^{+/*mes*}, *n*=6; *Ptch1*^{+/-}, *n*=7; *Ptch1*^{*mes/mes*}, *n*=5; *Ptch1*^{-/*mes*}, *n*=3; **p*<0.05 vs *Ptch1*^{+/+}; †*p*<0.05 vs *Ptch1*^{+/*mes*}; ‡*p*<0.05 vs *Ptch1*^{+/-}; §*p*<0.05 vs *Ptch1*^{*mes/mes*}. **c** Glucagon-positive area:exocrine cell area ratio in pancreas of each *Ptch1* mutant at E18.5. Data are mean $\pm$ SEM, *n*=7

in the mass of all four endocrine pancreas cell types in a dose-dependent manner. Among them, the number of insulin-positive cells seemed to be specifically decreased in the *Ptch1* mutants.

Effect of dysfunctional PTCH1 on transcription factors implicated in beta cell differentiation *Ngn3* (also known as *Neurog3*) is a presumed marker of endocrine precursor cells [28, 29]. We next investigated whether the differentiation

Fig. 4 Beta cell mass in each *Ptch1* mutant. **a** Insulin staining of pancreas at E15.5 and E18.5 in mutant mice as indicated. Arrowheads indicate insulin-positive cells. Scale bar, 50 μ m. **b** Percentages of insulin-immunopositive areas in pancreas of each *Ptch1* mutant at E18.5. To facilitate comparison, insulin-immunopositive area in wild-type pancreas has been adjusted to 1. Data are mean $\pm$ SEM. *Ptch1*^{+/+}, *n*=7; *Ptch1*^{+/*mes*}, *n*=6; *Ptch1*^{+/-}, *n*=7; *Ptch1*^{*mes/mes*}, *n*=5; *Ptch1*^{-/*mes*}, *n*=3; **p*<0.05 vs *Ptch1*^{+/+}; †*p*<0.05 vs *Ptch1*^{+/*mes*}; ‡*p*<0.05 vs *Ptch1*^{+/-}; §*p*<0.05 vs *Ptch1*^{*mes/mes*}. **c** Insulin-positive area:exocrine cell area ratio in pancreas of each *Ptch1* mutant at E18.5. Data are mean $\pm$ SEM (*n*=7 each); **p*<0.05 vs *Ptch1*^{+/+}

from immature pancreatic epithelial cells to *Ngn3*-expressing endocrine precursor cells is affected by increased HH signalling. Quantification of *Ngn3* expression using RNA isolated from E14.5 pancreas revealed a decrease in parallel with the increased HH signalling (Fig. 5a). However, the reduction in *Ngn3* expression was relatively modest compared with the reduction in alpha and beta cell mass. Thus, these results suggest that increased HH signalling could induce both a decreased fate commitment into endocrine cells and a reduced expansion of endocrine cells. In the late stage of pancreas differentiation, the expression of PDX1 is gradually restricted to pancreatic beta and delta cells. Reflecting the decreased insulin-positive cell number, PDX1-positive cells decreased in *Ptch1^{mes/mes}* and *Ptch1^{-mes}* mice at E15.5 and E18.5 (ESM Fig. 2). In addition, the expression of *MAFA* at E18.5, a marker of fully mature beta cells [30], also decreased in parallel with the increased HH signalling (Fig. 5b).

Increased mesenchyme induced by dysfunctional *PTCH1* is not derived from pancreatic epithelial cells The observed increase in mesenchymal cells and concurrent decrease in epithelial cells in the pancreas is a well-known phenotype of mice with increased HH signalling in pancreas [4, 10, 11]. Previous studies have suggested that HH signalling attenuates fibroblast growth factor 10 signalling, which regulates the proliferation of pancreatic epithelial cells [11, 31]. Thus, increased HH signalling results in a reduction of the pancreatic epithelial cells. On the other hand, no studies have investigated the possibility that the increased mesenchyme is formed by an epithelial to mesenchymal transition (EMT) in pancreas of *Ptch1* mutants [32–34]. To investigate whether the increased mesenchymal cells are indeed the result of an EMT, we performed Cre-lox recombination studies based on in vivo genetic lineage tracing. Pancreas specific transcription factor, 1a (PTF1A) is expressed on all epithelial cells. In the pancreas, it localises in pancreatic buds, but not in neighbouring epithelial or mesenchymal cells. We crossed *Ptf1a*-Cre

mice with *ROSA26-lacZ* mice, which carry a modified *lacZ* gene driven by the cell type-independent *ROSA26* promoter. In the progeny from this cross, *Ptf1a*-driven expression of CRE excises a stop cassette upstream of *lacZ*, thereby activating the expression of beta-galactosidase. Therefore, the expression of beta-galactosidase marks PTF1A-expressing cells and their progeny [20]. This strategy showed that PTF1A-lineage cells differentiate into duct, acinar and endocrine cells, i.e. all pancreatic epithelial cell types, but not pancreatic mesenchymal cells. We then crossed the *Ptf1a*-Cre:*ROSA26-lacZ* mice with *Ptch1^{-mes}* mice to examine whether the increased mesenchymal cell mass expressed beta-galactosidase. Figure 6 shows that the beta-galactosidase-expressing cells in E15.5 pancreas of *Ptch1^{-mes}* mice comprised only epithelial cells, as in wild-type mice. Accordingly, lineage-tracing analysis revealed that the extra mesenchymal cells in *Ptch1* mutants were not formed via EMT; rather, these cells represented increased mesenchymal cell proliferation. An increased number of BrdU-positive mesenchymal cells in the *Ptch1^{-mes}* mice provided further evidence of such proliferative activity (data not shown). Previously, forced expression of *Shh* under the control of the *Pdx1* promoter induced transdifferentiation of pancreatic mesenchyme into duodenum mesoderm, which expresses alpha-smooth muscle actin (alpha-SMA) [4]. Immunostaining for alpha-SMA in *Ptch1^{-mes}* mice pancreas in this study showed staining only in the cells surrounding blood vessels (data not shown). Thus, disruption of *Ptch1* in this study did not induce transformation of pancreatic mesenchyme into duodenum mesoderm.

Interestingly, the lineage-tracing analysis of *Ptch1^{-mes}* mice revealed beta-galactosidase-expressing epithelial cells in the stomach. This unexpected finding suggests that deficiency in *Ptc1* function causes a cell fate switch from pancreatic progenitors to stomach epithelial cells.

Decreased beta cell mass in *Ptch1^{-/+}* mice Finally, we investigated whether subtle reductions in *Ptc1* function

Fig. 5 Expression of key transcription factors for differentiation into pancreatic beta cells in each *Ptch1* mutant mice. **a** *Ngn3* mRNA expression levels are shown relative to β -actin mRNA. To facilitate comparison, expression in wild-type pancreas has been adjusted to 1. Data are mean $\pm$ SEM. *Ptch1^{+/+}*, $n=6$; *Ptch1^{+mes}*, $n=8$; *Ptch1^{+/-}*, $n=9$; *Ptch1^{mes/mes}*, $n=6$; *Ptch1^{-mes}*, $n=8$; * $p<0.05$ vs *Ptch1^{+/+}*. **b** MAFA immunostaining of pancreas at E18.5. Scale bar, 50 μ m

Fig. 6 The origin of increased mesenchyme in *Ptc1*^{-/-} mice. **a** Macroscopic phenotypes of E15.5 pancreas from *Ptf1a*-Cre; *ROSA26*-lacZ mice (*n*=7) and the trigenic *Ptf1a*-Cre; *Ptc1*^{-/-}; *ROSA26*-lacZ mice (*n*=5) stained with X-gal. The black dotted line represents the pancreas. d, duodenum; dp, dorsal pancreas; S, stomach; vp, ventral pancreas. **b** X-gal staining of pancreatic sections from E15.5 *Ptf1a*-Cre; *ROSA26*-lacZ mice and the trigenic *Ptf1a*-Cre; *Ptc1*^{-/-}; *ROSA26*-lacZ mice. Blue staining for LacZ production marks cells that have undergone CRE-mediated recombination. Scale as indicated

could alter the beta cell mass in adult pancreas, thereby affecting glucose tolerance. For rigorous comparison of glucose tolerance, we used wild-type, *Ptc1*^{+/-} and *Ptc1*^{-/-} mice with a C57BL/6 background (backcrossed more than ten generations). At 12 weeks of age, the *Ptc1*^{+/-} and *Ptc1*^{-/-} mice showed glucose and insulin levels during IPGTT that were comparable to those of wild-type mice (Fig. 7). Insulin sensitivity of these mice was also similar to wild-types, as assessed by the insulin-tolerance test. However, the area of pancreatic beta cells in *Ptc1*^{+/-} mice was lower than in wild-type mice. These results confirm that reduced expression of *Ptc1* can cause decreased beta cell mass in the pancreas.

Fig. 7 Glucose tolerance and beta cell mass in *Ptc1* mutants. **a** Blood glucose concentrations after intraperitoneal glucose injection (2 g/kg glucose) in 12-week-old, male *Ptc1*^{+/-} (squares, *n*=6), *Ptc1*^{+/-} (triangles, *n*=4) and wild-type littermates (circles, *n*=5). **b** Serum insulin levels during IPGTT in each group. **c** Reduction in blood glucose levels after intraperitoneal injection of insulin (0.75 U/kg) in 15-week-old male *Ptc1*^{+/-} (squares, *n*=13), *Ptc1*^{+/-} (triangles, *n*=12) and wild-type littermates (circles, *n*=13). Data are mean±SEM. **d** Beta cell areas in 15-week-old *Ptc1*^{+/-} mice (*n*=6), *Ptc1*^{+/-} (*n*=6) and wild-type littermates (*n*=6), shown as a percentage relative to the whole pancreatic area. Data are mean±SEM; **p*<0.05 vs *Ptc1*^{+/+}

Discussion

In this study, we clearly found that *mes* functions as a hypomorphic allele of *Ptch1* in the developing pancreas and that these *Ptch1* mutant mice are useful in ascertaining the dose-dependent effect of increased HH signalling on pancreatic development.

A previous study suggested that increased HH signalling inhibits early pancreatic development [4]. It has also been reported that the levels of PDX1 in pancreas at E9.5 in *Ptch1*^{−/−} mice was decreased, although *Ptch1* knockout mice die early during development, making it impossible to perform a detailed analysis of *Ptch1* function in them [8]. In this study, the levels of PDX1 and glucagon were not decreased at E10.5 and E12.5 in *Ptch1* mutants. Thus, we confirmed that early pancreatic development is disturbed only by total loss of PTCH1 function.

In this study, *Ptch1* mutants showed a decreased exocrine cell mass in parallel with increased HH signalling in the pancreas. Similar changes have been reported in PDX1-*Shh* mice and PAX4-*Hh* mice [4, 10]. PDX1-*Shh* mice had only small pancreatic remnants in the duodenal tissue and the phenotype was evidently more severe than in the *Ptch1* mutants analysed in this study. PDX1-*Shh* mice have SHH ectopically expressed very early during pancreatic development. In addition, while PTCH1 was only weakly expressed in pancreatic epithelial cells, the promoter activity of *Pdx1* in pancreas was relatively strong. Thus, HH signalling in the pancreas of these mice might have exceeded that in our *Ptch1* mutant mice. The PAX4-*Hh* mice in the other study [10] showed a decreased pancreatic cell mass, but the pancreatic tissue was macroscopically visible. The PAX4-*Hh* phenotype was therefore less severe than in the PDX1-*Shh* mice and was relatively similar to our *Ptch1*^{−/mes} mice. In addition, HH activity was only upregulated later in development of the PAX4-*Hh* mice, when expression should be restricted within limited cell lineages such as endocrine cells. While it is possible that increased HH signalling during early pancreatic development might affect the differentiation of exocrine cells more adversely, the exocrine cell mass in *Ptch1* mutant mice decreased in parallel with increasing HH activation in this study, implying that the phenotype severity might depend on HH signalling intensity.

All pancreatic endocrine cell types decreased in accordance with the increased HH signalling in our mutant mice. However, beta cells seemed to be most sensitively affected by the increased HH activity among the pancreatic epithelial-derived cells. This finding suggests that subtle increases in HH signalling might induce a decrease in beta cell mass. A decrease in beta cell mass is a typical feature of the onset of type 2 diabetes [35, 36]. Thus, we investigated the glucose tolerance and beta cell mass in

our *Ptch1*^{+/mes} and *Ptch1*^{+/−} mice after adjusting for genetic background. No differences in glucose tolerance were apparent among wild-type, *Ptch1*^{+/mes} and *Ptch1*^{+/−} mice. However, a previous study showed that *Ptch1*^{+/−} mice are glucose-intolerant [8]. This discrepancy could be attributed to the different background of mice used. On the other hand, beta cell mass diminished in a stepwise fashion in parallel with the increased HH signalling, although the difference between wild-type and *Ptch1*^{+/mes} mice was not statistically significant. A recent genome-wide association study identified *EXT2* as a candidate gene causing type 2 diabetes [37]. The fact that extoses genes modulate HH signalling [38] implicates these proteins in the development of type 2 diabetes via a decrease in beta cell mass induced by increased HH signalling. Further studies are needed to investigate the relationship of extoses genes and pancreatic beta cell mass.

The *Ptch1*^{mes/mes} and *Ptch1*^{−/mes} mice in this study showed increased mesenchyme at least in the pancreas, as observed with PAX4-*Hh* mice [12]. The major phenotypic difference between the mesenchyme of PAX4-*Hh* mice and *Ptch1*^{−/mes} mice is the presence of alpha-SMA, a marker for duodenal mesenchyme in pancreas. While alpha-SMA was produced in ordinary mesenchymal cells of PAX4-*Hh* mice, we saw immunostained cells only in the cells surrounding blood vessels within the pancreas [12]. In this regard, the mesenchyme phenotype of *Ptch1*^{−/mes} mice might be more similar to the previously described *Hhip*^{−/−}; *Ptch1*^{+/−} mice, which showed a subtle increase in mesenchymal cells and no presence of alpha-SMA in mesenchyme [10].

EMT is a process by which epithelial cells lose their mature characteristics and acquire properties associated with mesenchymal cells. While two studies have suggested that under certain conditions pancreatic beta cells can undergo EMT into fibroblast-like cells under culture [32, 33], a recent study used lineage tracing to demonstrate that islet-derived fibroblast-like cells were not EMT-derived [34]. The increased pancreatic mesenchyme observed in our *Ptch1* mutants resembled an EMT event. However, lineage-tracing analysis also revealed here that the increased mesenchyme in the *Ptch1*^{−/mes} mice was not derived via EMT. BrdU staining confirmed that the increased mesenchymal mass reflected proliferation of pancreatic mesenchymal cells. Interestingly, the lineage tracing also revealed a cell fate switch from pancreatic epithelial cell progenitors to stomach epithelial cells in the *Ptch1*^{−/mes} mice. This cell fate change warrants further detailed characterisation.

In conclusion, our study demonstrated that dysfunctional PTCH1 decreases pancreatic endocrine and exocrine cell mass in a dose-dependent manner, with the beta cell mass particularly responsive to subtle reductions in PTCH1 function and expression. Decreased beta cell mass is a common pathophysiological feature of type 2 diabetes.

Together with data on *EXT2* as a candidate gene causing type 2 diabetes, our data functionally link HH signalling with the development of the disease.

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