# Title
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## Author(s)
Hosokawa, Shinichi

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Impact of Sox9 Dosage and Hes1-mediated Notch Signaling in Controlling the Plasticity of Adult Pancreatic Duct Cells in Mice

Shinichi Hosokawa1,3, Kenichiro Furuyama1,3, Masashi Horiguchi1,3, Yoshiki Aoyama1,3, Kunihiko Tsuboi1,3, Morito Sakikubo1,3, Toshihiro Goto1,3, Koji Hirata1,3, Wataru Tanabe3,5, Yasuhiro Nakano3, Haruhiko Akiyama2, Ryoichiro Kageyama4, Shinji Uemoto1 & Yoshiya Kawaguchi3

1Department of Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan, 2Department of Orthopaedics, Gifu University, Gifu, Japan, 3Department of Clinical Application, Center for iPS cell Research and Application, Kyoto University, Kyoto, Japan, 4Institute for Virus Research, Kyoto University Graduate School of Medicine, Kyoto, Japan, 5Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan.

In the adult pancreas, there has been a long-standing dispute as to whether stem/precursor populations that retain plasticity to differentiate into endocrine or acinar cell types exist in ducts. We previously reported that adult Sox9-expressing duct cells are sufficiently plastic to supply new acinar cells in Sox9-IRES-CreERT2 knock-in mice. In the present study, using Sox9-IRES-CreERT2 knock-in mice as a model, we aimed to analyze how plasticity is controlled in adult ducts. Adult duct cells in these mice express less Sox9 than do wild-type mice but Hes1 equally. Acinar cell differentiation was accelerated by Hes1 inactivation, but suppressed by NICD induction in adult Sox9-expressing cells. Quantitative analyses showed that Sox9 expression increased with the induction of NICD but did not change with Hes1 inactivation, suggesting that Notch regulates Hes1 and Sox9 in parallel. Taken together, these findings suggest that Hes1-mediated Notch activity determines the plasticity of adult pancreatic duct cells and that there may exist a dosage requirement of Sox9 for keeping the duct cell identity in the adult pancreas. In contrast to the extended capability of acinar cell differentiation by Hes1 inactivation, we obtained no evidence of islet neogenesis from Hes1-depleted duct cells in physiological or PDL-induced injured conditions.

During organogenesis, the plasticity of embryonic cells gradually decreases as lineage separation proceeds and cells differentiate into mature cell types. However, the generation of iP cells and the direct reprogramming of some cell types into others clearly show the astonishing plasticity that is retained in adult cells1,2. The reprogramming can be created by artificially introducing a few transcription factors, and the plasticity of adult cells is shown in several physiological and pathological conditions, including organ maintenance, tissue regeneration and carcinogenesis. Indeed, organ-specific stem/progenitor cells have been identified in adult organs that continuously supply new cells, such as the skin and gut, where they maintain physiological organ homeostasis3,4. Other reports have shown the dedifferentiation of mature cells into an immature status during the regeneration process after injury5,6,7. In addition, pathological metaplasia of mature cell types sometimes causes malignant transformation8,9,10. However, in contrast with our understanding of the cell differentiation machinery during embryonic stages, details of the mechanism that controls adult cell plasticity in vivo largely remain to be elucidated.

There has been long-standing debate as to whether physiologically functioning stem/progenitor cell populations exist in the adult ductal compartment of the pancreas11. Several lineage-tracing experiments have been conducted to follow the fate of adult pancreatic duct cells in vivo, but conflicting results have left this question unanswered. Solar et al. demonstrated that adult Hnf1β+ cells do not differentiate into acinar or endocrine cells12. In addition, a cell-tracking experiment of adult Hes1+ cells found no evidence of acinar or endocrine differentiation13. However, these reports do not completely refute the existence of stem/progenitor cells in the adult ductal structure, because the expression of neither Hnf1β nor Hes1 represents the entire adult ductal epithelium. We have previously reported that Sox9 is expressed throughout the adult ductal tree and used in lineage-tracing...
experiments to demonstrate the continuous supply of new acinar cells from the adult Sox9-expressing ductal component in Sox9-CreER knock-in (Sox9CreERT2) mice. Considering the pancreatic exocrine structure, centroacinar cells that localize at the tip of the Sox9-expressing ductal structure are thought to be the best candidate for acinar progenitors that function in keeping physiological homeostasis in Sox9CreERT2 mice. However, another lineage-tracing experiment using BAC Sox9-CreER transgenic mice provided no evidence of acinar cell differentiation from adult Sox9+ cells. Therefore, exploration of the mechanism by which new acinar cells are supplied from the Sox9-expressing cells in Sox9CreERT2 mice should provide insights into the plasticity of adult pancreatic duct/centroacinar cells.

During embryonic stages, several transcription factors and signals control cell differentiation machineries in pancreas organogenesis. For example, the amounts of expressed Sox9 and Ptf1a have been shown to influence the differentiation of endocrine and exocrine lineages, respectively. In addition, many reports have revealed the pivotal role of Notch signaling in pancreas formation: overexpression of the Notch intracellular domain (NICD) suppresses endocrine and exocrine differentiation, while inactivation of Hes1, the main effector of Notch signaling, causes inadequate expansion of pancreatic progenitors and early premature differentiation resulting in hypoplastic pancreas formation. While the effect of the dosage of transcription factors such as Sox9 and Ptf1a has not been fully investigated in the adult organ, that pancreatic regeneration after cerulein-induced pancreatitis requires the reactivation of Notch signaling in mice. Hes1-depletion accelerates acinar cell differentiation from Sox9-expressing duct cells in Sox9CreERT2 mice, whereas NICD induction suppresses it. In addition, we show that Notch signaling positively regulates Sox9 and Hes1 in parallel. Based on these findings, we propose that the strength of Hes1-mediated Notch signaling and the dosage of Sox9 expression function cooperatively to control the plasticity of adult pancreatic duct cells.

Results
Pancreatic Sox9 expression is not altered in neonates but is reduced in adult Sox9-IRES-CreER knock-in mice. In Sox9CreERT2 mice, the IRES-CreER cassette is inserted in the 3’UTR of the Sox9 locus, thus the altered structure of the Sox9 locus potentially disrupts the control machinery of Sox9 expression in Sox9CreERT2 mice. At postnatal day 1 (P1), Western blotting and quantitative PCR analyses showed no difference in the expression of Sox9 between wild-type and Sox9CreERT2 heterozygous mice (Fig. 1A). However, at the adult stage, Sox9 expression in Sox9CreERT2 heterozygous mice was reduced to 80 ± 4.3% and 59 ± 12% of that in age-matched wild-type mice at the protein and mRNA level, respectively (Fig. 1B). The expression of Sox9 was restricted to CK19-expressing duct/centroacinar cells and the ratio of Sox9-expressing cells among total pancreatic epithelial cells was the same in wild-type and Sox9CreERT2 mice both at the neonatal stage and adulthood (Fig. 1A, B bottom right panels and Supplementary Table S1), indicating that pancreatic duct/centroacinar cells in Sox9CreERT2 mice express an equal amount of Sox9.

In the present study, we aimed to analyze how the differentiation ability of Sox9+ cells into acinar cells is controlled in Sox9CreERT2 mice. We revealed that Sox9 expression is decreased but that Hes1-mediated Notch signaling is normally conserved in the pancreas of adult Sox9CreERT2 mice. Hes1-depletion accelerates acinar cell differentiation from Sox9-expressing duct cells in Sox9CreERT2 mice, whereas NICD induction suppresses it. In addition, we show that Notch signaling positively regulates Sox9 and Hes1 in parallel. Based on these findings, we propose that the strength of Hes1-mediated Notch signaling and the dosage of Sox9 expression function cooperatively to control the plasticity of adult pancreatic duct cells.

Figure 1 | Reduced Sox9 expression in the adult pancreas of Sox9CreERT2 mice. At the neonatal stage (A), the relative expression level of Sox9 in the pancreata of Sox9CreERT2 mice is the same as that of wild-type mice as shown by Western blot (upper panels, n = 3 each) and qPCR (bottom left panel, n = 3 each). In the adult pancreas (B), Sox9 expression is less in Sox9CreERT2 mice than in wild-type mice (Western blot shown in upper panels, n = 3 each and qPCR in bottom left panel, n = 3, 4). The ratio of Sox9-expressing cells among total DAPI+ epithelial cells is not significantly different between wild-type and Sox9CreERT2 mice at P1 or the adult stage (bottom right panels in A and B, n = 3 each, refer to Supplementary Table S1). *P < 0.05. Data are expressed as means ± s.e.m.
Sox9 in the newborn stage, but less in adulthood than do wild-type mice.

Expression of Hes1 is the same in adult wild-type and Sox9-IRESCreER knock-in mice. Considering a previous report that showed the inactivation of Rbpj, a main effector of Notch signaling, causes rapid differentiation of adult Hes1-expressing duct/centroacinar cells into the acinar cell type26, we questioned if Notch activity is reduced in the adult pancreas of Sox9creERT2 mice and evaluated the activity by Hes1 expression, another main effector of Notch signaling. Consistent with previous findings on wild-type mice19,20, our immunohistochemical analyses revealed that Hes1 is periodically expressed in the Sox9+ ductal compartment, including the centroacinar cells in the adult pancreas of Sox9creERT2 mice (Fig. 2A). Quantitatively, up to 63.6 ± 1.57% of CK19+ duct/ centroacinar cells were positive for Hes1 in Sox9creERT2 mice, which was not significantly different from wild-type mice (Fig. 2B, C and Supplementary Table S2). Hes1 expression was also observed in a subset of Pecam+ endothelial cells (Fig. 2B) and islets (data not shown), but we detected no Hes1 expression in amylase+ acinar cells (Fig. 2B). Quantitative PCR analyses showed no difference in Hes1 mRNA amounts between adult wild-type and adult Sox9creERT2 mice (Fig. 2C). Taken together, we concluded that Hes1 expression is reduced no more in the pancreas of adult Sox9creERT2 mice than it is in the pancreas of wild-type mice.

Notch signaling controls the differentiation ability of Sox9+ duct/centroacinar cells into the acinar cell type in adult Sox9-IRESCreER knock-in mice. We next questioned whether the modulation of Notch activity influences the differentiation ability of adult Sox9+ duct/centroacinar cells in Sox9creERT2 mice. For this purpose, we designed two sets of tamoxifen-inducible Cre-mediated experiments and evaluated the number of lineage-labeled acinar cells (Fig. 3A). First, we bred Sox9creERT2; Hes1loxp/loxp; Rosa26R mice, in which Sox9+ cells have Hes1 null status due to tamoxifen-induced recombination. The second experiment inducibly forced the expression of NICD, which constitutively activates Notch signaling in Sox9+ cells from Sox9creERT2; RosaNICD; Rosa26R; Rosa26R mice. In both experiments, tamoxifen treatment caused nuclear localization of the CreERT2 protein, and CreERT2-mediated recombination excised out the STOP cassette of the Rosa26R allele and activated lacZ expression to enable the detection of the recombined cells and their progeny as X-gal-positive cells (Fig. 3A). Without tamoxifen administration, no X-gal positive cells were detected in all experimental settings (Supplementary Fig. S1), thus the difference in the number of X-gal positive acinar cells is expected to represent the difference in the differentiation ability of lineage-labeled duct/ centroacinar cells provided that both the Rosa26R allele and Hes1loxp or NICD allele are recombined (see below).

As we have previously demonstrated14, the number of X-gal-positive acinar cells gradually increases after a single injection of tamoxifen (0.2 mg/kg body weight) in Sox9creERT2; Hes11/1+; Rosa26R mice, suggesting a continuous acinar cell supply from the Sox9-expressing duct/centroacinar cell compartment (Fig. 3B, C, E). While a 50% reduction of Hes1 dosage caused no apparent change in the number of labeled acinar cells in Sox9creERT2; Hes11/2+; Rosa26R mice, conditional Hes1 depletion in Sox9creERT2; Hes11/2+/−; Rosa26R mice resulted in accelerated acinar cell labeling (Fig. 3B, C). Quantitatively, the average number of X-gal-positive acinar cells was 1140 and 2859 at days 3 and 10 after tamoxifen injection, respectively, in Sox9creERT2; Hes11/2+/−; Rosa26R mice, which was larger than the 594 cells and 1741 cells in control Sox9creERT2; Hes11/1−/−; Rosa26R mice (Fig. 3C and Supplementary Table S3). On the contrary, forced expression of NICD caused a decrease in the number of lineage-labeled acinar cells (Fig. 3D, E), as the average number of X-gal-positive acinar cells in Sox9creERT2; RosaNICD; Rosa26R mice was 308 and 857 at days 3 and 10 (Fig. 3E and Supplementary Table S3).

Results of the lineage tracing experiments above indicate that Notch signaling negatively regulates the differentiation ability of Sox9+expressing progenitors into the acinar cell type in adult Sox9creERT2 mice. However, this conclusion requires further examination of whether lineage-labeled cells eventually depleted Hes1 and induced NICD expression in Sox9creERT2; Hes11/1+/loxp; Rosa26R mice and Sox9creERT2; RosaNICD; Rosa26R mice, respectively. Since Hes1 is not expressed in acinar cells at steady state (Fig. 2B), it is impossible to dismiss the existence of lineage-labeled acinar cells that escaped the recombination of the Hes1loxp allele in Sox9creERT2; Hes11/1+/loxp; Rosa26R mice by immunostaining of Hes1. However, even if the existence of these escaper cells is taken into consideration, activated differentiation into the acinar cell type is supported by the following observation: Hes1-X-gal double positive cells were not detected in the duct/centroacinar compartment, whereas Hes1 expression was preserved in X-gal negative ducts at the same frequency as that in wild type or Sox9creERT2 mice without tamoxifen treatment (Fig. 2 and Supplementary Fig. S2A). In addition, the ratio of cell proliferation to cell death remained the same in Sox9creERT2; Hes1loxp/loxp; Rosa26R mice (data not shown). In Sox9creERT2; RosaNICD; Rosa26R mice, we tested EGFP expression, which was co-induced with NICD by Cre-mediated activation of the NICD-IRESCreER cassette (Fig. 3A), and found that lineage-labeled acinar cells contained EGFP-negative cells (Supplementary Fig. S2B). Therefore, the results in Fig. 3E underestimate the impact of suppressed acinar differentiation by NICD induction. On the other hand, the existence of EGFP/X-gal double positive acinar cells indicates that NICD induction does not completely block the differentiation of duct/centroacinar cells into the acinar cell type in Sox9creERT2; Rosa26R mice (Supplementary Fig. S2B). This notion was confirmed by the existence of Hes1-X-gal double positive acinar cells (Supplementary Fig. S2B). These findings indicate that activated Notch activity suppresses but does not completely block the acinar cell supply from the duct/centroacinar compartment in adult Sox9creERT2 mice, a property that contrasts the complete acinar cell agenesis by NICD induction during embryonic stages19,20.

It was reported that rapid differentiation into acinar cell types caused by Rbpj depletion in Hes1-expressing cells is accompanied by the loss of a centroacinar cell population26. However, Hes1 seems to be dispensable for the long-term maintenance of organ homeostasis in Sox9creERT2 mice, as centroacinar cells were not lost and the normal acinar structure was maintained in Sox9creERT2; Hes11/1+/loxp; Rosa26R mice, suggesting the existence of a compensatory machinery. In fact, our lineage-tracing experiments using Sox9creERT2; Hes11/1+/loxp; Rosa26R mice showed that both the duct and acinar cells retained their labeling status up to one year after tamoxifen injection (Supplementary Fig. S3A), indicating that Sox9-expressing duct/centroacinar cells could self-duplicate and continuously supply new acinar cells after Hes1 depletion. In addition, X-gal-labeled endocrine cells were not observed, indicating that Hes1 inactivation did not induce transdifferentiation of duct cells into endocrine cells in the adult pancreas (Supplementary Fig. S3B).

Ptf1a expression is not influenced by the modulation of notch signaling in the adult pancreas. While its role in the adult pancreas is still unknown, Ptf1a is indispensable for acinar cell differentiation27,28 and Notch signaling negatively regulates Ptf1a expression28,29 during pancreaticogenesis. Considering the continuous acinar cell supply from the Sox9+ component in adult Sox9creERT2 mice, we questioned whether Ptf1a is ectopically expressed in Sox9+ cells, especially in centroacinar cells in adult Sox9creERT2 mice and whether modulation of Notch activity affects Ptf1a expression. However, as shown in Fig. 4A, Ptf1a expression is confined to acinar cells and not detected in the CK19-positive ductal structure, which includes centroacinar cells at steady state (Sox9creERT2; Rosa26R mice).
Figure 2 | Unaltered Notch signal in adult Sox9CreERT2 mice at steady state. (A) In the adult pancreas of Sox9CreERT2 mice, Hes1 is expressed in a subpopulation of Sox9+ duct/centroacinar cells. White and yellow arrowheads indicate Sox9 single positive and Sox9/Hes1 double positive duct/centroacinar cells, respectively. The same section was used for both images. Scale bars = 25 μm. (B) Periodical expression of Hes1 in the ductal tree is confirmed by co-immunostaining of Hes1 and CK19 (upper panels). Note that Hes1 is expressed in a subset of Pecam-expressing endothelial cells (white arrowheads in the middle panels) but not in amylase+ acinar cells (bottom panels) in the adult pancreas of Sox9CreERT2 mice. Scale bars = 25 μm. (C) In adult Sox9CreERT2 mice, the ratio of Hes1+ cells to total CK19+ duct/centroacinar cells is unaltered compared with that of wild-type mice (left, n = 3 each, refer to Supplementary Table S2). qRT-PCR revealed that the Hes1 mRNA amount is the same in adult Sox9CreERT2 and wild-type mice (right, n = 3 each). Data are expressed as means ± s.e.m.
Hes1 activity was affected by neither Hes1 depletion nor NICD induction, indicating that Notch-mediated Ptf1a suppression is less robust at adult stages than at embryonic stages. Indeed, Ptf1a expression was not detected in X-gal-positive duct cells of Sox9CreERT2; Hes1loxp/loxp; Rosa26R mice, confirming Hes1 depletion (Supplementary Fig. S2A). Furthermore, we treated mice with a higher dosage of tamoxifen (four injections of 0.2 mg/g body weight), which achieved more efficient labeling (approximately 60%, data not shown) of Sox9+ duct cells (Supplementary Fig. S4A). Efficient recombination was confirmed by observing a decrease and
increase in the number of Hes1-expressing cells in Sox9CreERT2; Hes1loxp/loxp; Rosa26R mice and Sox9CreERT2; RosaNICD; Rosa26R mice, respectively (Supplementary Fig. S4B). Again, we detected no ectopic Ptf1a expression, at least by immunostaining, in the Sox9 ductal component of Sox9CreERT2; Hes1loxp/loxp; Rosa26R mice (Fig. 4B). In Sox9CreERT2; RosaNICD; Rosa26R mice, Ptf1a expression was confined to the Sox9-negative acinar cells (Fig. 4B), including the Hes1-expressing acinar cells that were formed by NICD induction (Supplementary Fig. S5). Thus, the expression pattern of Ptf1a was unperturbed by modulating Notch activity in Sox9CreERT2 mice, as neither Sox9/Ptf1a double positive cells nor structural alterations were observed.

**Notch signaling regulates Sox9 expression in adult pancreatic duct cells.** We found that the Sox9/CK19-expressing ductal

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**Figure 4 | Ptf1a expression is not altered by Notch modulation in adult Sox9CreERT2 mice.** Expression of Ptf1a is confined to acinar cells and not affected by the modulation of Notch activity. (A) Ptf1a is expressed in lineage-labeled acinar cells (white arrows) and surrounding non-labeled acinar cells but not in CK19-expressing duct/centroacinar cells (dotted line and yellow arrowheads) in Sox9CreERT2; Hes1loxp/loxp; Rosa26R mice (left panels), Sox9CreERT2; Hes1loxp/loxp; Rosa26R mice (middle panels) and Sox9CreERT2; RosaNICD; Rosa26R mice (right panels). The same section was used for X-gal staining and immunostaining in each column. Scale bars = 25 μm. (B) Ptf1a expression is confirmed by the co-staining of Ptf1a and Sox9 using sections obtained after high-dose tamoxifen treatment (refer to Supplementary Fig. S4). Scale bars = 25 μm.
component was structurally impaired by neither Hes1 depletion nor forced expression of NICD (Fig. 5A), even at the higher dosage of tamoxifen treatment (four injections of 0.2 mg/g body weight). Further, we did not detect any ectopic Sox9 expression in acinar cells, and the ratio of Sox9+ cells among total DAPI+ epithelial cells remained constant with modulating Notch activity (Fig. 5B and Supplementary Table S4). Notably, our Western blotting and quantitative PCR analyses revealed that the expression of Sox9 was elevated by the forced expression of NICD (Fig. 5B). On the other hand, Hes1 depletion did not influence the amount of Sox9 or the number of Sox9-expressing cells, reflecting the asymmetric division of Sox9+ cells into acinar and Sox9+ cells in Sox9CreERT2 mice. Thus, considering the increased number of Hes1-expressing cells by NICD induction (Supplementary Fig. S4 and S5), our findings show that Notch positively regulates Hes1 and Sox9 in parallel in adult pancreatic duct cells of Sox9CreERT2 mice and that the regulation of Sox9 expression by Notch is mediated in a Hes1-independent manner in the adult pancreas.

Hes1-depleted duct cells do not show endocrine neogenesis after pancreatic duct ligation. Another long-standing debate is whether adult pancreatic duct cells possess plasticity to differentiate into the endocrine lineage. In particular, possible islet neogenesis after pancreatic duct ligation (PDL) has attracted the interest of researchers.4,5 We previously reported that beta cell neogenesis from Sox9-expressing duct cells was observed in neither the physiological condition nor the regeneration process after injury, including partial pancreatectomy, cerulein-induced acute pancreatitis, streptozotocin diabetes or PDL, in Sox9CreERT2 mice.4,5 Because Hes1 inactivation in the embryonic pancreas accelerates endocrine differentiation,23 we tested whether Hes1 depletion combined with PDL prompts adult Sox9-expressing cells to differentiate into endocrine lineages in Sox9CreERT2 mice. However, lineage tracing analyses detected no X-gal-positive cells in the islets at days 7 or 60 after PDL in Sox9CreERT2; Hes1loxp/loxp; Rosa26R mice (Fig. 6), indicating that adult Sox9-expressing duct/centroacinar cells are not capable of differentiating into endocrine lineages even by the combination of Hes1-inactivation and PDL.

Discussion
In this study, we demonstrated that the plasticity of adult pancreatic duct cells is controlled by the strength of Notch signaling. The differentiation ability of Sox9-expressing duct/centroacinar cells into acinar cell types was accelerated by Hes1 inactivation and suppressed by NICD induction in Sox9CreERT2 mice. This effect is consistent with two reports by Kopinke et al. that show Hes1-expressing centroacinar/terminal duct cells do not normally differentiate into acinar cells but will do so rapidly upon the inactivation of Rbpj in Hes1-CreER knock-in mice, which suggests that Notch signaling functions to maintain the duct cell identity in the adult pancreas.12,23 Adding to that our previous report that showed Sox9-expressing duct/centroacinar cells continuously supply new acinar cells in adult Sox9CreERT2 mice,4,5 we hypothesized Hes1-negative/Sox9-positive centroacinar cells are best candidates for the origin of continuous acinar cell supply in Sox9CreERT2 mice. However, Notch signaling alone cannot explain why Sox9-expressing cells were reported not to differentiate into acinar cells in adult BAC Sox9-CreER transgenic mice.4 We therefore predicted the existence of one or more other mechanisms that control adult duct cell plasticity.

We propose that the amount of Sox9 expression is pivotal in controlling adult duct cell plasticity (Fig. 7). The dosage requirement of Sox9 in the formation of the embryonic endocrine pancreas has been previously suggested by autopsy samples from three human cases of campomelic dysplasia, an autosomal dominant disorder caused by SOX9 haploinsufficiency that has been described as ‘‘less densely packed epithelial cells within the mesenchymal stroma and less clearly formed islets.’’ The dosage requirement has also been experimentally supported previously; a Pdx1-Cre-mediated 50% reduction of Sox9 expression caused a decrease in the number of neurogenin 3 (Ngn3)-expressing endocrine progenitors and less endocrine formation in Pdx1-Cre; Sox9loxp/loxp mice.17 However, in the human campomelic dysplasia cases, little attention was given to the exocrine tissue, and exocrine pancreatic formation was not impaired in Pdx1-Cre; Sox9loxp/loxp mice, suggesting that Sox9 dosage has less effect on exocrine pancreas formation during the embryonic stages. In contrast, our results suggest the involvement of Sox9 dosage in controlling the plasticity of adult pancreatic duct cells. In the present study, we demonstrated that pancreatic Sox9 expression in neonates was the same in wild-type and Sox9CreERT2 mice, whereas adult pancreatic duct cells express less Sox9 in Sox9CreERT2 mice than in adult wild-type mice. It should be noted that two previously reported lineage-tracing experiments using Sox9CreERT2 and BAC Sox9-CreER transgenic mice are consistent in that embryonic Sox9+ duct cells were sufficiently plastic to differentiate into acinar and endocrine lineages, an ability that persisted until the perinatal stages up to one week after birth.4,17 Provided that the amount of Sox9 expression in BAC Sox9-CreER transgenic mice was kept at the same level as that of wild-type mice at the newborn stage and adulthood, reduced Sox9 expression may allow duct cells to differentiate into acinar cells in adult Sox9CreERT2 mice, whereas higher Sox9 expression in BAC Sox9-CreER transgenic mice possibly functions to maintain the duct cell identity, thereby preventing continuous differentiation into acinar cell types.

We also show that the induction of NICD caused elevated Sox9 expression, indicating that Sox9 expression is regulated by Notch signaling in adult Sox9CreERT2 pancreata. This result is consistent with Shih et al., who reported that Notch signaling regulates Sox9 expression in the embryonic pancreas.2 An in vitro tissue culture experiment using wild-type pancreas at e12.5 further showed that Sox9 expression was reduced by the addition of a low-dose γ-secretase inhibitor, while Sox9 expression was decreased at a higher dosage, indicating that Notch regulates Hes1 and Sox9 in parallel in embryonic pancreata. Nevertheless, we hypothesized that the reduced Sox9 expression in the adult pancreas of Sox9CreERT2 mice at steady state is unconnected with Notch-mediated regulation. Notch activity seemed normal based on the ratio of Hes1-expressing cell numbers to CK-positive ductal epithelia and the amount of Hes1 mRNA was the same between wild-type and Sox9CreERT2 mice.4,5,18 In Sox9CreERT2 mice, the IRES-CreERT2 cassette is inserted in the 3’UTR of the Sox9 locus.2 We therefore speculate that the altered structure of the Sox9 locus is related to the reduction of Sox9 expression in the adult pancreas, though the precise machinery has yet to be revealed. Recent reports showed a conserved sequence among species very near the site of the IRES-CreERT2 cassette insertion, suggesting the existence of a regulatory element in this region.4,18,29 Because the Sox9 expression was normal at the perinatal stage in Sox9CreERT2 mice, Notch-independent Sox9 regulation using this element, if it exists, would be silent until adulthood, when it maintains the Sox9 expression and thereby maintains the identity of the duct cell type. Future studies to dissect the complete regulatory machinery of Sox9 are required.

Islet neogenesis has long been believed to occur in the ductal compartment during the tissue restoration process, but we did not observe evidence of islet regeneration from Sox9-expressing cells in a PDL model. Xu et al. showed that the number of Ngn3-positive endocrine precursor cells increases after PDL.4 Furthermore, in BAC Sox9-CreER transgenic mice, it was reported that de novo Ngn3-expressing cells are of duct cell origin, but that they do not express other pivotal transcription factors, such as Nkx6.1 and Pax6, and thus are unable to drive the entire differentiation program into endocrine lineage.4,5 Consistent with this observation, Xiao et al. showed that Ngn3-expressing cells originating from the ducts after PDL do not differentiate into mature endocrine cells.4
Figure 5 | Notch signaling regulates Sox9 expression in adult Sox9<sup>CreERT2</sup> mice. Sox9 immunostaining, Western blotting, qPCR and cell counting were carried out after high-dose tamoxifen treatment (see Supplementary Fig. S4). (A) Neither Hes1 inactivation nor NICD induction causes ectopic Sox9 expression. Sox9 expression is confined to CK19<sup>+</sup> duct/centroacinar cells. Scale bars = 25 μm. (B) Western blotting (upper panels) and the real-time qRT-PCR analyses (bottom, left panel) show that the expression level of Sox9 is increased by NICD induction (n = 3, P = 0.0027 and 0.0003, respectively) but not influenced by Hes1 inactivation (n = 3, P = 0.38 and 0.49, respectively). The ratio of Sox9<sup>+</sup> cells to total DAPI<sup>+</sup> epithelial cells is not altered by the modulation of Notch activity (bottom right panel, refer to Supplementary Table S4). *P < 0.05, **P < 0.01. Data are expressed as means ± s.e.m.
Recent reports have focused more on the plasticity of adult acinar cells. Inducible Cre-mediated cell tracking using Ptf1a-CreERTM knock-in mice showed that adult acinar cells differentiate into endocrine cells and migrated into the islet structure after PDL35. Moreover, the plasticity of adult acinar cells has been experimentally demonstrated in the process of malignant transformation. The induction of oncogenic KrasG12D in the adult murine acinar cell type caused PanIN formation via acinar-to-ductal metaplasia (ADM) and

Figure 6 | Hes1-deleted pancreatic duct cells do not contribute to islet neogenesis after PDL. (A) Schematic presentation of the experimental design. Two days before pancreatic duct ligation (PDL), a single injection of tamoxifen was given. Mice were analyzed 7 days and 60 days after PDL. (B) X-gal-stained pancreas section at 7 days after PDL. PDL induced acinar cell devastation and a duct-like structure in the ligated portion but not in the unligated portion. Scale bars = 50 μm. (C) Immunostaining revealed that neither insulin+ nor glucagon+ cells are labeled at 7 days or 60 days after PDL (n = 4 for day 7, n = 3 for day 60). Islets are encircled with dotted lines. Scale bars = 50 μm.
developed into pancreatic ductal adenocarcinoma, whereas KrasG12D activation in the duct cell type did not. Interestingly, Sox9/CPA1 developed into pancreatic ductal adenocarcinoma, whereas KrasG12D

Figure 7 | Proposed machinery for the regulation of duct-to-acinar differentiation in adult Sox9<sup>CreERT2</sup> mice. Notch upregulates Hes1 and Sox9 in parallel. Hes1 and Sox9 seem to function cooperatively to maintain the identity of the duct/centroacinar cell type. Continuous acinar differentiation from the duct/centroacinar cells in adult Sox9<sup>CreERT2</sup> mice correlates with reduced Sox9 expression. The existence of one or more unknown factors that regulate Sox9 expression in a Notch-independent manner is predicted.

**Methods**

**Mice.** To obtain Hes1-conditional knock-out mice, Hes1<sup>Cre<sub>T2</sub></sup>- mice<sup>11</sup>, Sox9<sup>Cre</sup>-<sup>ERT2</sup> mice<sup>15</sup> and Rosa26<sup>YFP</sup> mice<sup>45</sup> were interbred. Rosa26<sup>LacZ</sup> (Rosa26<sup>R</sup>) mice were crossed to produce 10<sup>10</sup>. Morris, J. P., Cano, D. A., Sekine, S., Wang, S. C. & Hebrok, M. Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions. Proc Natl Acad Sci U S A 105, 18913–18918 (2008).

**Tamoxifen injection.** For lineage tracing, adult mice older than 8 weeks of age (approximately 25–30 g in body weight) were injected intraperitoneally with tamoxifen (Sigma T-5648) at 0.2 mg/g body weight. To get higher recombination efficiency, we injected tamoxifen at 0.2 mg/g body weight 4 times every other day.

**Tissue preparation for X-gal staining and paraffin section.** X-gal staining and paraffin section were performed as previously described<sup>14</sup>.

**Immunofluorescence.** The antigen retrieval procedure was performed with Target Retrieval Solution pH 6.0 (Dako), Target Retrieval Solution High pH 9.0 (Dako) or Proteinase K (Dako) in accordance with the manufacturer's instructions, followed by overnight incubation at 4°C with primary antibodies (Supplementary Table S5). The sections were washed in PBS and incubated for 60 min with secondary antibodies (Supplementary Table S6), followed by counterstaining with 4, 6-diamidino-2-phenylindole (DAPI). Immunostaining for Hes1 and Ptfla required the use of the TSA amplification Kit (Perkin Elmer). Images were visualized using HS All-in-one Fluorescence Microscopy BZ-9000E (Keyence).

**Cell counting.** We chose 20 fields at random and counted X-gal-positive acinar cells in each mouse. For short-term chase in the adult pancreas, we examined 20 fields per mouse to count the X-gal-positive acinar cells and averaged them. Cell numbers were counted by ×200 magnification in 40 fields (at adult stage) and 6 fields (at P1) using three sections per mouse and the ratio of Sox9<sup>+</sup> cells among all epithelial cells was calculated. To determine the ratio of Hes1<sup>+</sup> cells among CK19<sup>+</sup> duct cells, cell numbers were counted by ×400 magnification in 6 fields per mouse. All values are shown as mean ± s.e.m. All error bars represent s.e.m. P values were calculated using an unpaired Student’s t test (two-tailed); P < 0.05 was considered significant.

**Western blotting.** Whole pancreatic tissues were lysed in RIPA buffer (Thermo Scientific Pierce) in the presence of protease inhibitor (Roche). Obtained samples were electrophoresed in 10% SDS-PAGE gels and transferred to PVDF membrane (Bio-Rad). Membranes were blocked with 5% skim milk in TBS-T and incubated with anti-SOX9 antibody (Millipore 1:500) or anti-β-actin antibody (MBL 1:1000) overnight at 4°C. After washing, membranes were reacted with HRP-conjugated secondary antibodies (DAKO) at 1:2000 dilution. Chemiluminescence was detected with Immobilon Western horseradish peroxidase substrate (Thermo Scientific Pierce) and visualized with a charge-coupled device camera (EZ-capture). The intensity of the bands was quantified with imaging analysis software (Image J) using β-actin as an internal control. P values were calculated using an unpaired Student’s t test (two-tailed); P < 0.05 was considered significant.

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Author contributions
Y.K. and S.H. designed the study, analyzed the data and prepared the manuscript. S.H. performed the experiments. H.A. and R.K. generated the mice. K.F., M.H., Y.A., K.T., M.S., T.G., K.H., Y.N. and W.T. provided technical support and discussion. S.U. supervised the project.

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