Elevated Hedgehog/Gli signaling causes β-cell dedifferentiation in mice

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Although Hedgehog (Hh) signaling regulates cell differentiation during pancreas organogenesis, the consequences of pathway up-regulation in adult β-cells in vivo have not been investigated. Here, we elevate Hh signaling in β-cells by expressing an active version of the GLI2 transcription factor, a mediator of the Hh pathway, in β-cells that are also devoid of primary cilia, a critical regulator of Hh activity. We show that increased Hh signaling leads to impaired β-cell function and insulin secretion, resulting in glucose intolerance in transgenic mice. This phenotype was accompanied by reduced expression of both genes critical for β-cell function and transcription factors associated with their mature phenotype. Increased Hh signaling further correlated with increased expression of the precursor cell markers Hes1 and Sox9, both direct Hh targets that are normally excluded from β-cells. Over time, the majority of β-cells down-regulated GLI2 levels, thereby regaining the full differentiation state and restoring normoglycemia in transgenic mice. However, sustained high Hh levels in some insulin-producing cells further eroded the β-cell identity and eventually led to the development of undifferentiated pancreatic tumors. Summarily, our results indicate that deregulation of the Hh pathway impairs β-cell function by interfering with the mature β-cell differentiation state.

embryogenesis | signaling pathways | progenitor | glucose stimulated insulin secretion

Embryonic signaling pathways, including Notch and Wnt signaling, have been implicated in regulating adult β-cell expansion and function (1–3). Although manipulation of Hedgehog (Hh) signaling has profound effects on the developing endocrine cells within the pancreas (4–6), the role of this pathway in adult β-cells has not been analyzed. Ascertaining the role of Hh signaling in adult β-cells is of interest because increasing evidence suggest that the mature, differentiated state of these cells, defined by the ability to secrete insulin in response to glucose in an tightly controlled manner, is not permanent but can change under conditions of stress or disease (7–10). Loss of the mature β-cell phenotype was associated with impaired β-cell function in diabetic patients (11), defects that were attributed to changes in expression of critical transcription factors and various components of the insulin sensitivity and glucose secretion machinery (12). Here, we studied whether deregulated Hh signaling affects the differentiation and functional state of mature β-cells.

Activation of the Hh signaling pathway occurs upon binding of secreted Hh ligands to the transmembrane receptor Patched1 (Ptch1) on receiving cells. Signal transduction further requires modification of Gli transcription factors into their active form and their translocation into the nucleus (13). A growing body of evidence implicates the primary cilium, a cellular appendage presents on numerous cell types, as a central node for Gli protein processing and, thus, for Hh signaling modulation (6, 14–17).

Our prior work has demonstrated that regulated levels of Hh signaling activity are required for proper development of both endocrine and exocrine pancreas (4–6, 18). The Hh pathway is active at low levels in adult β-cells, and pathway inhibition throughout pancreas development results in impaired β-cell function during adulthood (5). Ectopic expression of Sonic Hh (Shh) in cultured β-cell lines has beneficial effects on insulin production and cell survival (19, 20). These effects are in part mediated through the elevated expression of Pancreatic and duodenal homebox 1 (Pdx1) (21), a transcription factor essential in maintaining the mature β-cell state (22). Notably, whether elevated Hh signaling has similar effects in pancreatic β-cells in vivo has not yet been investigated.

Here, we present in vivo evidence that increased Hh signaling in adult β-cells, induced by simultaneous expression of an activated form of the GLI2 transcription factor and the elimination of primary cilia in compound transgenic animals, results in glucose intolerance. This defect is caused by impaired glucose-stimulated insulin secretion (GSIS), likely resulting from a combined reduction in the expression of genes required for glucose sensing, insulin production and insulin secretion. In addition, overt Hh signaling inhibits the expression of transcription factors essential for the mature β-cell phenotype. Interestingly, deregulated Hh signaling also promotes the reactivation of non-β-cell transcription factors normally associated with a progenitor state. With time, transgenic mice regained glucose sensitivity, at least in part due to down-regulation of GLI2 expression by the majority of transgenic β-cells. However, sustained Hh activity in some β-cells results in neoplastic transformation and the development of insulin-negative, undifferentiated pancreatic tumors. Thus, our results demonstrate a need for restricting Hh levels below a certain threshold to maintain mature β-cell function in vivo.

Results

Increased Hh Activation in Adult β-Cells Results in Glucose Intolerance in Vivo. To study the role of Hh signaling in adult β-cells, we generated compound transgenic mice in which we could activate the pathway in a cell autonomous manner. First, we activated the pathway by ectopically expressing an active form of human GLI2, a transcriptional mediator of Hh signaling, by using *CLEG2* mice (18). In the absence of Cre activity, the constitutive active CAG promoter drives the expression of GFP. After Cre-mediated excision of the GFP coding sequence and its downstream "stop" sequence, the CAG promoter directs the expression of the Myc/GLI2 fusion protein consisting of a short N-terminal Myc-tag and a truncated form of the human GLI2 lacking the N-terminal repressor region (Δ*N*-GLI2 (18); illustrated in Fig. S1*A*). Expression of this activated version of the Myc/GLI2 transgene in β-cells was achieved by using *Pdx1-Cre*^{ER} mice in which tamoxifen (TAM) administration at 8- to 10-wk of age leads to Cre activation predominately in mature β-cells (23) (Fig. S1 *B* and *C*).

Pdx1-Cre^{ER}*;CLEG2* male mice were analyzed for potential defects in β-cell function by performing glucose tolerance tests, whereas TAM-treated Cre-negative littermate males served as controls. Untreated *Pdx1*-Cre^{ER};*CLEG2* mice and those analyzed 1 wk after TAM treatment behaved similar to non-transgenic controls (Fig. 1 *A* and *B*). However, 4 wk after TAM injection, we noticed that transgenic animals can be divided into two groups based on their glucose response, named "A" (four

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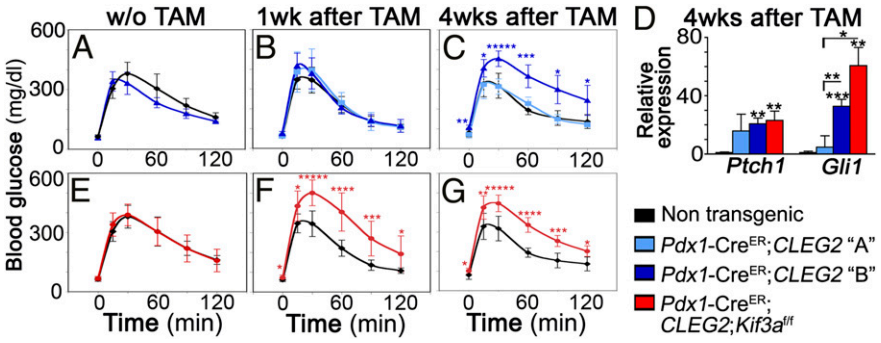

Fig. 1. Glucose intolerance correlates with increased Hh signaling in adult β-cells. *Pdx1-Cre^{ER};CLEG2* (*A–D*, blue lines; $n \geq 4$) and *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* (*D–G*, red lines; $n \geq 7$) transgenic (tg) and nontransgenic (non tg; black lines; $n \geq 8$) males were i.p. injected with TAM (2 mg per mouse on 5 consecutive days at 8–10 wk of age). Glucose tolerance test were performed before TAM injection (*A* and *E*) and 1 wk (*B* and *F*) or 4 wk (*C* and *G*) after injection. (*A–C* and *E–G*) After overnight fasting, mice were i.p. injected with dextrose (2 mg/g body weight) and their blood glucose levels were measured at indicated times. (*D*) Islets from control and transgenic mice were isolated 3–5 wk after TAM injection, their RNA was extracted, and *Ptch1* and *Gli1* expression levels were analyzed by qPCR ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$, and *****$P < 0.0005$ (Student's *t* test). Data represent mean ± SD.

mice of nine) and "B" (five mice of nine). Group "B" showed significant higher blood glucose levels upon challenge (Fig. 1*C*, dark blue line), whereas the rest of the cohort (group A; Fig. 1*C*, light blue line) displayed normal response to glucose stimuli. To understand whether the physiological differences in response to glucose challenge of the TAM-treated *Pdx1*-Cre^{ER};*CLEG2* mice might be explained by differences in Hh signaling activity, we analyzed the expression levels in isolated islets of two Hh target genes, *Ptch1* and *Gli1*. Whereas mice in group A with normal glucose control displayed only a trend toward Hh activation, a statistically significant increase in Hh pathway activity was observed in *Pdx1*-Cre^{ER};*CLEG2* group B mice that exhibited the glucose intolerance phenotype (Fig. 1*D*). Notably, the increase in *Gli1* expression in glucose-intolerant *Pdx1*-Cre^{ER};*CLEG2* animals of group B was also significantly higher compared with mice in group A (Fig. 1*D*), thus providing a correlation between elevated Hh levels and impaired β-cell function.

Primary cilia are cellular structures known to regulate Hh signaling (17). They are found on many mammalian cells, including pancreatic β-cells (24). Primary cilium can be eliminated by depletion of *Kif3a*, a gene coding for a component of the kinesin-2 complex, which is required for cilia formation and maintenance (25) (illustrated in Fig. S1 *D–F*). Elimination of cilia from either the developing pancreas or adult β-cells does not have a measurable effect either on Hh activity or glucose tolerance (6, 24) (Fig. S2 *A–D*). However, synergistic activities between Gli2 activation and cilia loss have been reported in various cell types (6, 16, 26). Our previous work has demonstrated that simultaneous elimination of primary cilia and ectopic expression of the active GLI2 transgene increases the level of Hh signaling in developing and early postnatal epithelial cells in the pancreas (6), but the consequences of these manipulations have not been examined in the mature pancreas.

To test the effects of activation of the Hh pathway in absence of primary cilia in adult β-cells, we generated *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice and treated adult animals with TAM (Fig. S1 *B, C, E,* and *F*). As shown in Fig. 1*D*, the expression levels of the Hh target genes *Gli1* and *Ptch1* were significantly higher in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* islets compared with nontransgenic controls. Although combined elimination of cilia and ectopic expression of active GLI2 did not increase levels of *Ptch1* compared with mice only expressing GLI2, the expression level of *Gli1* was significantly elevated in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice (Fig. 1*D*). Therefore, as was shown in other cell types (6, 16, 26), elimination of primary cilia enhanced Hh signaling activation in β-cells expressing an activated form of GLI2.

Next, we analyzed whether increased Hh signaling in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice affects the function of pancreatic β-cell by performing glucose tolerance tests. As expected, in the absence of TAM, *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* transgenic mice showed a glucose response comparable with nontransgenic control mice (Fig. 1*E*). However, as early as 1 wk after TAM injection, all *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice presented with significantly higher glucose levels upon challenge compared with nontransgenic controls (Fig. 1 *F* and *G*). Thus, active GLI2 expression in the absence of primary cilia accelerated the glucose intolerance phenotype detected in a subset of *Pdx1*-Cre^{ER};*CLEG2* mice (cohort B), further suggesting a negative correlation between levels of Hh signaling and β-cell function. Given the more robust activation of the Hh signaling pathway and severe glucose intolerance phenotype observed in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice, we have focused our analysis on these animals.

It should be noted that additional experiments with a cohort of mice in which transgene expression is also active in developing β-cells, confirmed the glucose intolerance phenotype upon Hh elevation (*Ins1-Cre;CLEG2;Kif3a$^{f/f}$* mice; Fig. S3*A*). Summarily, these data indicate that deregulation of Hh signaling in β-cells impairs glucose homeostasis in vivo.

Increased Hh Signaling Compromises Adult β-Cell Function. To confirm that the glucose intolerance phenotype observed in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice was due to a defect in pancreatic β-cells, we next tested for changes in secreted insulin levels. Measurements of serum insulin in response to glucose stimuli revealed significantly reduced levels in blood of TAM-treated *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice compared with control mice (Fig. 2*A*), suggesting impaired GSIS in transgenic mice 4 wk after TAM injection. In support of β-cell–specific defects rather than peripheral insulin resistance in transgenic mice, *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice maintained insulin sensitivity after TAM injection (Fig. S4*A*).

Notably, transgenic islets presented with normal gross morphology (Fig. S4 *B* and *C*), and our analysis did not reveal changes in β-cell proliferation or apoptosis (Fig. S4 *D–I*). However, as expected from previous work showing that elevated Hh signaling protects cultured β-cells from apoptosis (20), we could detect a significant increase in the expression level of the anti-apoptotic gene Bcl2 (Fig. S4*J*).

Because decreased serum insulin levels can result from impaired β-cell function, we analyzed isolated *Pdx1*-Cre^{ER};*CLEG2*;*Kif3a$^{f/f}$* islets 4 wk after TAM injection for their ability to respond to glucose in culture. As shown in Fig. 2*B*, transgenic islets secreted significantly less insulin upon glucose challenge compared with control. This finding implicates β-cell malfunction as the cause of glucose intolerance in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice.

Further analysis indicated a reduction in the intracellular insulin protein content in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* islets as measured by ELISA (Fig. 2*C*), an observation confirmed by immunofluorescence analysis (Fig. 2*D*). Quantitative PCR (qPCR) analysis revealed a twofold decrease in both *Insulin1* and *Insulin2* gene expression levels in transgenic islets compared with control, whereas expression of *Glucagon* was unaffected (Fig. 2*E*). Thus, *Pdx1*-Cre^{ER};*CLEG2*;*Kif3a$^{f/f}$* β-cells are marked by reduced insulin gene and protein expression.

Next, we analyzed the expression level of *Kir6.2* and *Sur1*, components of β-cell K(ATP) channels required for insulin se-

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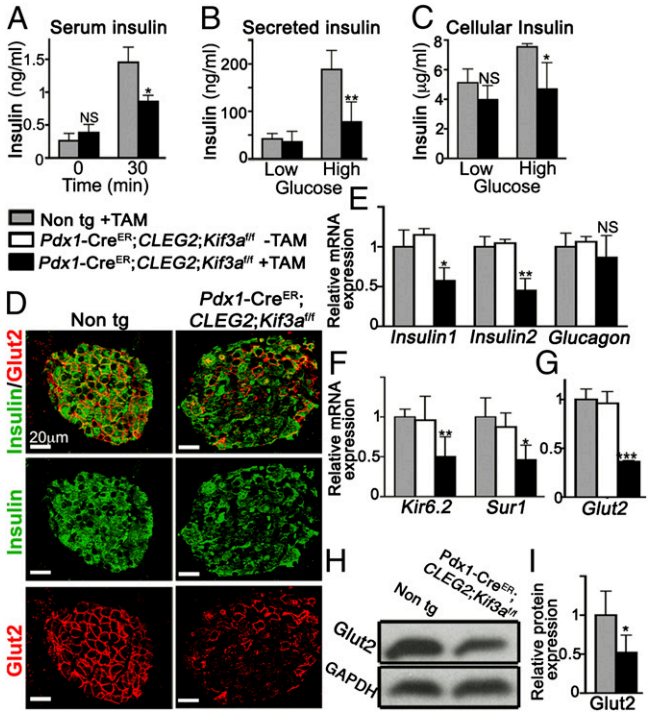

Fig. 2. Impaired glucose-stimulated insulin secretion by transgenic β-cells. *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice were presented with defects in β-cell function 4 wk after TAM injection. (*A*) Reduced serum insulin levels in transgenic mice (black) compared with nontransgenic control (gray). Blood was collected from tail veins before and 30 min after glucose injection (3 mg/g body weight), and serum insulin levels were determined by ELISA (n = 3). (*B*) Transgenic islets have impaired glucose-stimulated insulin secretion. Isolated islets from TAM-treated transgenic (black) and nontransgenic (gray) mice were incubated with either low (30 mg/dL) or high (300 mg/dL) glucose, and supernatant was collected and analyzed by ELISA (n = 3). (*C*) Protein extract of isolated islets treated as described in *B* was analyzed for insulin levels by ELISA. Transgenic islets show significantly reduced insulin levels (n = 3). (*D*) Pancreatic tissues from TAM-injected transgenic (*Right*) and control (*Left*) mice were immunostained for Insulin (green) and Glut2 (red). (*E*) RNA was extracted from transgenic islets (black) and nontransgenic (gray) mice 3–5 wk after TAM injection. Islets from untreated transgenic mice served as additional controls (white bars). The expression of *Insulin1*, *Insulin2,* and *Glucagon* genes was analyzed by qPCR (n = 4). (*F* and *G*) Reduced expression of mature β-cell genes in transgenic islets. RNA was extracted from isolated islets (see above in *E*), and expression of indicated genes was analyzed by qPCR (n = 4). (*H* and *I*) Reduced Glut2 protein levels in transgenic islets. Western blot analysis for Glut2 was performed on isolated islets 4 wk after TAM. A representative blot is shown (*H*), and quantification of data from four mice in each group is provided (*I*). Protein levels were normalized to GAPDH, and average levels in nontransgenic controls was set to "1". For statistical analysis, unpaired two-tailed Student's t test was performed. All data are in comparison with TAM-treated nontransgenic controls. P values: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, NS, nonsignificant. Data represent the mean ± SD.

cretion (27). Transcript levels of both genes were significantly reduced in TAM-treated transgenic islets compared with control, suggesting potential secretion defects (Fig. 2*F*). Accurate sensing of blood glucose levels is essential for β-cell function, a process in which the Glucose Transporter 2 (Glut2) plays a key role (28). Analysis of *Glut2* transcript and protein levels revealed a significant reduction in its expression levels in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* transgenic mice compared with control (Fig. 2 *G–I*). Immunohistochemical analysis further demonstrated dramatically perturbed Glut2 protein distribution in transgenic islets (Fig. 2*D*), implicating defects in glucose sensing. Thus, our results demonstrate that increased Hh signaling in β-cells of *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* transgenic mice leads to impaired GSIS through defects in insulin production and secretion as well as glucose sensing.

β-Cell Transcription Factor Expression Profile Is Altered upon Increased Hh Signaling. Numerous studies have delineated the transcription factor network that guides β-cell development and ensures proper β-cell function by maintaining the fully differentiated state of the cells (12, 29). To study whether the impaired GSIS observed in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice was a result of the loss of the mature β-cell phenotype, we analyzed the expression levels of such factors. Transgenic islets expressed lower levels of Pdx1, as shown by significant reduction of both transcript and protein levels compared with nontransgenic control (Fig. 3 *A–C*). Our analysis further revealed significant reduction in the expression of *MafA*, *NeuroD1, Nkx6.1*, and *Neurogenin3* (*Ngn3*) in transgenic islets 4 wk after TAM treatment compared with control (Fig. 3*A*). Thus, elevated Hh signaling causes reduction of critical β-cell markers, suggesting a loss of the mature β-cell phenotype.

To further address the possibility of β-cell dedifferentiation in response to increased Hh signaling, we asked whether transcription factors expressed in embryonic pancreas progenitor cells are elevated in transgenic islets. Sox9, whose function is required in pancreas progenitor cells, is normally excluded from mature β-cells (30–33). Interestingly, *Sox9* transcript and protein levels were in-

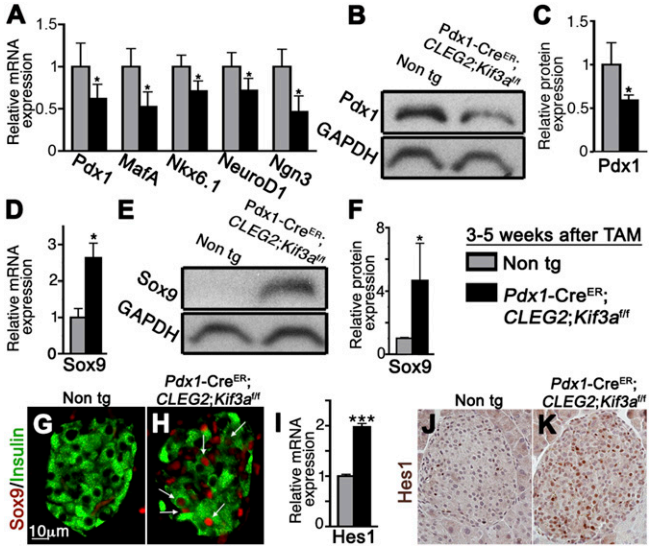

Fig. 3. Change in transcription factors expression in transgenic β-cells. (*A–C*) Reduced expression of β-cell transcription factors in transgenic islets 3–5 wk after TAM. (*A*) RNA was extracted from *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* (black) and nontransgenic (non tg, gray) isolated islets and analyzed by qPCR for mRNA expression levels of indicated genes (n = 4). (*B* and *C*) Western blot analysis shows reduced levels of Pdx1 protein in transgenic islets (black bars) 4 wk after TAM (compared with nontransgenic control; gray bars). (*C*) Quantification for Western blot. Data represent protein levels normalized to GAPDH. Mean levels in nontransgenic control was set to "1" (n = 5) (*D–H*) Sox9 expression in TAM-treated transgenic β-cells. (*G*) RNA was isolated from control (gray) and transgenic (black) islets, and qPCR analysis was performed (n = 4). (*E* and *F*) Western blot analysis of Sox9 protein expression. A representative blot is shown (*E*), and quantification of data from four mice in each group is provided (*F*). Data represent protein levels normalized to GAPDH. Mean levels in nontransgenic control was set to "1". (*G* and *H*) Pancreatic tissues from transgenic (*H*) and control (*G*) mice were immunostained for Sox9 (red) and Insulin (green). Arrows point to Insulin and Sox9 double-positive cells. (*I–K*) Hes1 is expressed in TAM-treated transgenic islets. (*I*) qPCR analysis for *Hes1* expression was performed on islets isolated from transgenic islets (black bars) and nontransgenic controls 4 wk after TAM (n = 4). (*J* and *K*) Immunohistochemistry for Hes1 (brown) and Hematoxilin (blue) of transgenic (*K*) and control (*J*) tissues. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ (Student's t test). Bar diagrams represent mean ± SD.

creased twofold and fourfold, respectively, in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* transgenic islets compared with controls (Fig. 3 *D–F*). Immunofluorescence analysis confirmed this data and further revealed that in contrast to control tissues, insulin and Sox9 double-positive cells were detected in the transgenic pancreas (Fig. 3 *G* and *H,* marked with arrows). Hes1, a transcription factor activated by the Notch signaling pathway, is expressed in embryonic and adult pancreata but excluded from β-cells (34). *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* islets showed a twofold increase in *Hes1* transcript levels compared with controls (Fig. 3*I*), accompanied by a profound up-regulation of the Hes1 protein (Fig. 3 *J* and *K*).

Summarily, increased Hh levels in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* transgenic islets are associated with reduced expression of mature β-cell transcription factors, and inappropriate activation of progenitor markers that likely accentuate the observed impairment of maintenance of mature β-cell differentiation and function.

Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$ Transgenic Mice Regain Glucose Sensitivity Over Time. To test whether glucose-intolerant *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice become diabetic over time, we measured their blood glucose levels under fed and fasted conditions in the weeks after TAM injection. As expected from their impaired response to glucose (Fig. 1*G*), transgenic mice showed significantly increased blood glucose levels 4 wk after transgene induction (Fig. 4 *A* and *B*). However, although transgenic mice presented with slightly higher fed blood glucose levels in the following weeks (Fig. 4*B*), they returned to normoglycemia under fasting conditions within 6–10 wk (Fig. 4*A*).

To study whether the glucose response of *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice changes over time, we performed glucose tolerance tests 10 wk after TAM injection. Surprisingly, not only did transgenic mice present with normal response to glucose at this time point (Fig. 4*C*), the expression of *Insulin*, *Glut2,* and β-cell related transcription factors had returned to normal levels as well (Fig. 4*D* and Fig. S5*A*). In support of these gene expression data, immunofluorescence analysis showed that the majority of *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* β-cells presented with normal expression pattern of Glut2 and insulin proteins (Fig. 4*E*). However, a minority of cells remained negative for both markers (Fig. 4*E*, marked with asterisks). Therefore, restoration of the mature phenotype in the majority of *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* β-cells 10 wk after TAM likely allowed for the reestablishment of glucose sensitivity in these animals. This conclusion is supported by similar data derived from aging *Ins1-Cre;CLEG2;Kif3a$^{f/f}$* mice in which the glucose intolerance observed early on (2-mo-old mice; Fig. S3*A*) is compensated for at later stages (6-mo-old mice; Fig. S3*B*).

A possible explanation for the regeneration of the mature β-cell phenotype would be the reduction of Myc/GLI2 transgene expression and, thus, Hh signaling, 10 wk after TAM injection. Immunofluorescence analysis confirmed this hypothesis as the majority of insulin- and Glut2-expressing cells in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* islets did not express detectable Myc/GLI2 protein levels (Fig. 4*F* and Fig. S5*B*). Ten weeks after TAM injection, only 16% of insulin-positive cells in transgenic islets were also Myc/GLI2-positive, a dramatic decrease from the ≈50% double-positive cells found 4 wk after TAM (Fig. 4*F*). Thus, reduction of Myc/GLI2 transgene expression over time allowed for the normalization of glucose responsiveness in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice.

The loss of Myc/GLI2 transgene expression over time could be explained in several ways. First, some β-cells that have escaped Cre-activation might have expanded more than those in which the Myc/GLI2 transgene is expressed. To address this question, we looked at the extent and location of GFP-positive cells within the transgenic pancreas. The *CLEG2* transgenic locus is designed to constantly drive the expression of a GFP reporter in the absence of Cre-recombinase (18) (Fig. S1*A*). Ten weeks after TAM injection, the vast majority of insulin-expressing cells in *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* islets were negative for GFP (Fig. S5*C*), indicating efficient Cre-mediated recombination of the *CLEG2* transgenic locus in most Myc/GLI2-negative β-cells. Second, it is possible that the transcriptional expression of the transgene becomes silenced in TAM-treated *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* β-cells over time. This scenario is unlikely because mRNA levels of human *GLI2*, expressed by the *CLEG2* transgene, remained stable between 4 and 10 wk after TAM treatment (Fig. S5*D*). Thus, our analysis indicates that *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* β-cells regain their mature, functional differentiated state by reducing the level of active GLI2 protein via a posttranscriptional regulation process.

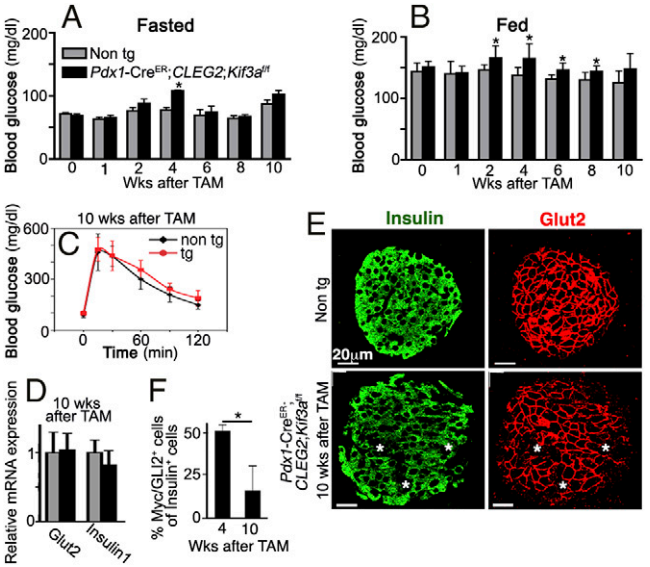

Fig. 4. Restoration of glucose sensitivity and mature β-cell gene expression in aged *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice. (*A* and *B*) Blood glucose levels of transgenic (black bars) and control (gray bars) mice after overnight fast (*A*) or upon normal fed conditions (*B*) were analyzed at different time points after TAM injection (n = 5). (*C*) Normal glucose response of transgenic mice 10 wk after TAM injection (red line) compared with control (black line). Mice were treated as described in Fig. 1 (n = 4). (*D* and *E*) Restoration of Insulin and Glut2 expression in transgenic islets 10 wk after TAM injection. qPCR analysis (*D*) shows normal expression levels (n = 4) and immunofluorescence (*E*) confirms normal expression pattern of Insulin (green) and Glut2 (red) in the majority of islet cells. Asterisks mark cells negative for both markers. (*F*) Reduced number of Myc/GLI2-expressing β-cells 10 wk after TAM injection. Transgenic tissues 4 and 10 wk after TAM injection were stained with antibodies against the Myc-tag (which detects the Myc/GLI2 transgenic fusion protein) and Insulin, and the percentages of Myc/GLI2$^+$ (identified as Myc$^+$Insulin$^+$ double-positive cells) out of total β-cell population (identified as Insulin$^+$ cells) were counted. Images were exposed for extended period to ensure detection of all insulin-expressing cells. (n = 3). *P < 0.05 (Student's t test). Data represent the mean ± SD.

Prolonged Elevation of Hh Signaling in β-Cells Results in Development of Undifferentiated Pancreatic Tumors. Although the vast majority of β-cells reduce Myc/GLI2 levels over time, some cells failed to do so efficiently (Fig. 4 *F* and Fig. S5*B*), presenting us with the opportunity to determine the consequences of prolonged Hh activation in these cells over time. Starting at 10 wk after TAM injection, we could detect proliferative lesions containing Insulin$^-$Myc/GLI2$^+$ cells in pancreata of *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mice (Fig. S6 *A* and *B*), accompanied with development of pancreatic tumors at later time points. To study whether increased Hh signaling specifically in β-cells can lead to pancreatic tumor formation and to exclude potential exocrine contribution, we further analyzed *Ins1-Cre;CLEG2;Kif3a$^{f/f}$* mice for tumor formation. All transgenic mice analyzed developed massive pancreatic tumors with undifferentiated histology and succumbed to the disease at the latest by 13 mo of age (Fig. S6 *C*, *D*, and *G*). Tumors were highly proliferative and expressed the Myc/GLI2 transgene, accompanied by high levels of Hh signaling (Fig. 5 *B* and *C*). Although they originated from *Ins1*-Cre

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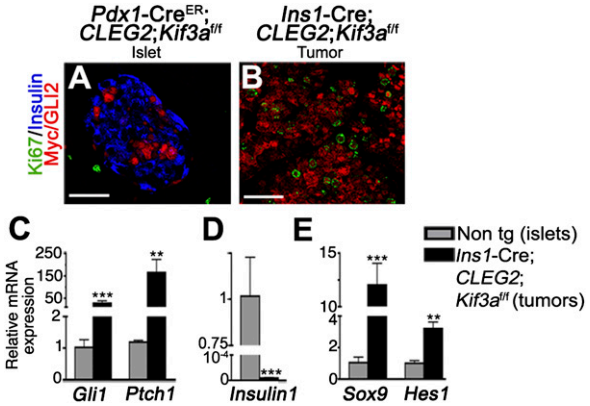

Fig. 5. Prolonged Hh activation in β-cells promotes tumor formation. (*A* and *B*) Pancreatic tumors express the Myc/GLI2 transgene, are proliferative, and lack insulin expression. Pancreatic tissue from TAM-treated *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* mouse (*A*) and an 8-mo-old *Ins1-Cre;CLEG2;Kif3a$^{f/f}$* mouse (*B*) were stained with antibodies against insulin (blue), the proliferation marker KI67 (green), and Myc-tag (red, identifying the Myc/GLI2 fusion protein). (*C*) Tumors display increased Hh signaling levels. RNA was extracted from islets isolated from nontransgenic mice (gray bars) and *Ins1-Cre;CLEG2;Kif3a$^{f/f}$* tumors (black bars). Expression of the Hh signaling target genes *Ptch1* and *Gli1* was analyzed by qPCR ($n = 4$). (*D*) Tumors lack *Insulin1* transcript expression. RNA was extracted as described in *C*, and *Insulin1* expression levels were analyzed by qPCR ($n = 4$). (*E*) Tumors express precursor cell genes. RNA was extracted from islets as described in *C*, and *Sox9* and *Hes1* expression levels were analyzed by qPCR ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ (Student's *t* test). Data represent the mean ± SD.

expressing cells (Fig. S6 *E* and *F*), tumor cells lacked insulin expression (Fig. 5 *B* and *D*) and disease progression was not accompanied by hypoglycemia (Fig. S6*H*). Interestingly, both *Sox9* and *Hes1* were expressed at high levels in *Ins1*-Cre;*CLEG2*;*Kif3a*$^{f/f}$ tumors (Fig. 5*E*), suggesting that tumors are derived from β-cells that underwent a process of dedifferentiation during neoplasia. Thus, our data indicate that prolonged deregulation of Hh signaling imparts an oncogenic effect on β-cells.

Discussion

Data presented here demonstrate that increased Hh signaling in adult β-cells in vivo leads to the development of glucose intolerance in transgenic mice. Transgenic β-cells display impaired GSIS correlated with a profound loss in the mature differentiation state of these cells. The loss of differentiation presented itself as a combination of a marked reduction in genes required for normal β-cell function accompanied by increased expression of factors usually found in progenitor cells. However, this loss of β-cell function and associated hyperglycemia was transient because the majority of transgenic cells regained their differentiated state by down-regulating Hh signaling over time. Transgenic cells that have failed to do so continued to express progenitor markers at increasing levels and eventually underwent neoplastic transformation. Thus, our study presents evidence for high-level of Hh signaling as a repressor of the mature β-cell phenotype (summarized in Fig. 6).

Mechanisms Underlying β-Cell Dedifferentiation. Increasing evidence indicates that the differentiation state of mature cells is not permanent, and adult β-cells can lose their identity and functionality in response to a variety of signals, including changes in the transcriptional profile normally present in these cells (7, 12, 35, 36). Our results demonstrate that transgenic β-cells with increased Hh signaling can transiently dedifferentiate toward a more progenitor-like state characterized by decreased Pdx1, Glut2, and insulin expression levels. Importantly, similar changes in gene expression have been noted in human patients as well as in mice suffering from impaired GSIS (37–39). Furthermore, upon elevation of Hh signaling levels, transgenic islets showed

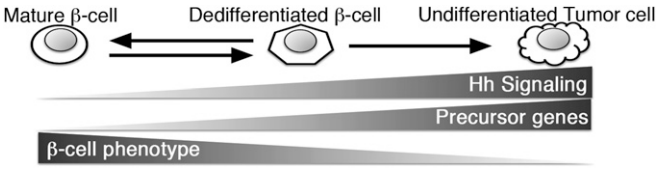

Fig. 6. A model for the effect of increased Hh signaling on β-cell differentiation state. Mature β-cells express low levels of Hh signaling (*Left*) (5). Increased Hh signaling leads to dedifferentiation of β-cells (*Center*), accompanied by reduced expression of β-cell marker genes, and increased precursor genes expression. β-cells dedifferentiation is reversible, and cells can reacquire their differentiation state upon down-regulation of Hh signaling. However, when high Hh signaling persists, cells further lose their β-cells identity (*Right*) and no longer express insulin. Precursor genes expression increases in those β-cell–derived undifferentiated cells.

reduced expression of *MafA*, *NeuroD,* and *Ngn3*—shown to be essential for maintaining the mature β-cell phenotype and normal response to glucose (10, 40, 41). Therefore, we conclude that the impaired GSIS observed in *Pdx1-Cre^{ER};CLEG2;Kif3a*$^{f/f}$ mice likely resulted from reduced expression of multiple transcription factors required for maintenance of the β-cell phenotype due to overt Hh signaling activation.

In addition, our results show ectopic expression of Sox9 and Hes1 transcription factors upon increased Hh levels, both genes normally excluded from mature β-cells and endocrine precursors (33, 34, 42, 43). Furthermore, Hes1 was shown to inhibit endocrine fate during embryogenesis (44) and a similar role was suggested in adult β-cells, where it was shown to directly bind and suppress *Insulin* and *Ngn3* gene expression and to down-regulate *Pdx1* and *NeuroD1* levels (45, 46). Its activity was further correlated with human β-cell dedifferentiation in cell culture (47). Sox9 was shown to directly promote *Ngn3* expression in ductal cells (31), but its effect on other β-cell genes is yet to be determined. Interestingly, both *Sox9* and *Hes1* were shown to be direct targets of Gli transcription factors. Gli1 directly interacts with regulatory elements in the *Sox9* gene promoter (48), and *Hes1* expression in the retina is mediated by direct binding of the Gli2 transcription factor in a Notch-independent manner (49). Therefore, GLI2 transgene expression could directly result in ectopic Sox9 and Hes1 expression in *Pdx1-Cre^{ER};CLEG2;Kif3a*$^{f/f}$ islets, which might contribute to β-cell dedifferentiation observed in these mice.

Distinct Functions of Hh Signaling in β-Cells. The data presented here are somewhat in conflict with previous work showing increased insulin production by β-cell lines in response to elevated Hh signaling in vitro (19). Several explanations could account for the divergent findings. First, although β-cell lines maintain some insulin production and GSIS, they do not retain the fully differentiated state found in adult β-cells in vivo (50). Second, activation of the signaling pathway was achieved by different manners. Thomas et al. (19) ectopically expressed the Hh ligand Shh in cultured cells, whereas we activated a downstream transcriptional mediator in a cell autonomous manner. Given the complex regulation of Hh signaling at distinct levels of the pathway (13), the diverse approaches may result in different outcomes and precludes direct comparison. Finally, we have shown that pancreatic loss of Hh signaling affects β-cell differentiation and function (5). These observations suggest that a specific range of Hh signaling is required for optimal β-cell function. Thus, the positive results obtained from prior studies with regard to increased insulin production in response to expression of Hh ligands could be due to elevation of Hh levels within this beneficial range.

Primary Cilia as Cell-Autonomous Regulators of Hh Signaling Levels. Primary cilia have emerged as essential signaling nodes for the control of Hh signaling within cells. Interestingly, depending on cellular context, they are implicated in regulating both the formation of Gli-activator and Gli-repressor proteins. Our data

indicate that primary cilia regulate GLI2 activity in β-cells. However, over time, even in the absence of primary cilia, GLI2 protein levels were suppressed. This finding strongly implies the presence of additional, primary cilium independent mechanisms capable of maintaining low levels of Hh signaling in β-cells. Because *Pdx1-Cre^{ER};CLEG2;Kif3a$^{f/f}$* islets maintained high *CLEG2* transcript levels at this late time point, this regulatory mechanism likely acts at the posttranscription stage(s). Exploring the compensatory mechanisms underlying the resetting of Hh activity could reveal novel and more general insights into how β-cells cope with cellular stress.

In summary, our study strongly suggests that the ability to control Hh signaling levels is essential for maintaining β-cell function. Our findings also indicate that the control of Hh levels in β-cells is achieved at multiple layers of the signaling pathway. The lack of reports describing increased Hh signaling in type 2 diabetes might suggest that such mechanisms are efficient in preventing overt activation of the pathway in human β-cells. Curbing Hh activity below a certain threshold in β-cells is important as demonstrated by our findings in which cells with high Hh levels had impaired function and differentiation state and, in extreme cases, underwent neoplastic transformation, eventually giving rise to pancreatic tumors devoid of insulin expression.

Methods

Mice. *Pdx1-Cre^{ER}* (23), *CLEG2* (18), *Kif3a^{f}* (25), and *Insulin1-Cre* (51) transgenic mice were maintained according to protocols approved by the Committee on Animal Research at the University of California, San Francisco.

Reagents and procedures are described in detail in *SI Methods*, and a list of antibodies and probes can be found in Table S1 and Table S2, respectively.

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