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Neurogenin3 is Sufficient for *in vivo* Transdetermination of Hepatic Progenitor Cells into Islet-like cells but not Transdifferentiation of Hepatocytes

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Summary

The transcription factor Neurogenin3 (Ngn3) is required for islet-cell type specification. Here, we show that hepatic gene transfer of Ngn3 transiently induces insulin in terminally-differentiated hepatocytes but fails to transdifferentiate them, i.e. switch their lineage into islet cells. However, Ngn3 leads to long-term diabetes reversal in mice due to the emergence of periportal islet-like cell clusters. These neo-islets display glycemia-regulated insulin, β-cell-specific transcripts, and an islet-specific transcription cascade, and produce all four major islet hormones. They appear to arise from hepatic progenitor cells, most likely endoderm-derived oval cells. Thus, transfer of a single lineage-defining transcription factor, Ngn3, is sufficient to induce cell-lineage switching from hepatic to an islet lineage in these progenitor cells, a process consistent with transdetermination, i.e, lineage switching in lineage-determined, but not terminally-differentiated cells. This paradigm of induced transdetermination of receptive progenitor cells *in vivo* may be generally applicable to therapeutic organogenesis for multiple diseases including diabetes.

Introduction

Pancreatic organogenesis during development entails cell replication coupled with cell-type specification and subsequent differentiation orchestrated, in the gut endoderm, by mulitple transcription factors [reviewed in (Collombat et al., 2006)]. Once the ventral and dorsal pancreatic buds develop, the endocrine cell-specification is initiated in them by the basic helix-loop-helix transcription factor Ngn3 within the domain of Pdx1-positive cells (Gu et al., 2002; Apelqvist et al., 1999). This results in the formation of the islets of Langerhans that produce vital hormones including insulin from β-cells, the deficiency of which leads to diabetes. Islet replacement via either regeneration or neogenesis, hence, holds the key to curing diabetes. However, islet, and specifically β-cell, renewal is normally a rare event that occurs predominantly from pre-existing β-cells (Dor et al., 2004).

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The necessary role for Ngn3 in islet development is indicated by the complete absence of endocrine islets in the pancreas of Ngn3-null mice (Gradwohl et al., 2000). However, whether Ngn3 is sufficient for islet formation has not been addressed (Heremans et al., 2002; Schwitzgebel et al., 2000; Grapin-Botton et al., 2001). Ngn3 is expressed only during embryonic development (Lee et al., 2001) and forced or ectopic expression of Ngn3 has been reported to lead to low level insulin production by cells of undetermined origin (Kaneto et al., 2005; Wang et al., 2007; Song et al., 2007). Previous studies have used various upstream [Pdx1, (Ferber et al., 2000; Kojima et al., 2003)] and downstream transcription factors [Neurod1, MafA, (Kojima et al., 2003; Song et al., 2007)] to induce insulin production in the liver; however, many of them were limited by relatively weak hormone production, and deleterious exocrine enzyme production or co-expression of insulin with multiple hormones in the same cells. Importantly, there was no identification of receptive lineages or underlying mechanisms for purported 'transdifferentiation'. We hypothesized that in receptive lineages, Ngn3, with its islet-lineage defining function would be sufficient not only to induce islet-cell specification, but also to induce the full islet-differentiation cascade mirroring what occurs during embryonic islet development.

We asked whether Ngn3 is sufficient to induce islet organogenesis *de novo*, and if so, which cell type would be competent to undergo endocrine pancreas specification by this approach. Using helper-dependant adenoviral vectors (HDAd) that have negligible toxicity in mice, we demonstrate that Ngn3 is sufficient to induce, in the liver, endocrine pancreas organogenesis. Although Ngn3 rapidly induces insulin production in hepatocytes *in vivo*, the latter are not competent to undergo transdifferentiation into islet cell as they exhibit an aborted islet transcription cascade, and gradually lose their capacity to produce insulin. However, euglycemia is maintained because of the emergence of non-hepatocyte-derived periportal neo-islets containing β-cell-like cells that produce insulin in a glucose-responsive manner, completely reversing the diabetes. We identify hepatic oval cells as the apparent receptive lineage by marker expression, location and morphology, and show that they acquire the function of islet cells, producing the four major islet hormone-producing cell types and display a complete islet-specific transcription program. Our data suggest that the Ngn3-induced islet neogenesis in the liver is the result, not of transdifferentiation, but of transdetermination, i.e., by the switching of progenitor (oval) cells from one determined state to another closely related one (Hadorn, 1965). Thus, not only is Ngn3 necessary, it is also sufficient, in inducing islet formation in mice *in vivo* and oval cells may comprise an attractive receptive lineage for islet neogenesis.

Results

Gene transfer of neurogenin3 and betacellulin reverses hyperglycemia and restores glucose-stimulated insulin secretion in insulin-deficient diabetic mice

Diabetic mice with stable hyperglycemia for at least a week were administered a single IV injection of HDAd-Ngn3, which resulted in a rapid reversal of hyperglycemia within a week and the significantly reduced blood glucose was sustained for about two months, beyond which hyperglycemia recurred (Figure 1A). Betacellulin (Btc), an islet growth factor, was added to HDAd-Ngn3 to amplify the Ngn3 effects (Kojima et al., 2003). HDAd-Ngn3 combined with HDAd-Btc led to a rapid reversal of hyperglycemia within a week, which, in contrast to Ngn3 alone, was sustained for at least 6 months (Figure 1A). Empty vector and Btc alone had no effect on the hyperglycemia (Figure 1A). Ngn3-Btc-treated mice also gained weight (Figure 1B) and displayed little hepatotoxicity, as evidenced by normal serum transaminases (Figure 1C).

Fasting plasma insulin (Figure 1D) was normalized in a sustained fashion by Ngn3-Btc gene transfer at a level comparable to nondiabetic controls. In contrast, empty vector-treated diabetic

mice remained hyperglycemic with barely detectable plasma insulin levels. Insulin tolerance test indicated that a difference in insulin sensitivity did not account for the euglycemia (Supplemental Figure 1).

An IP glucose tolerance test (GTT) showed that Ngn3-Btc-treated mice had a normal glucose disposal, indistinguishable from that of nondiabetic controls (Figure 1E), with the GTT-stimulated plasma insulin excursions also being similar between the two groups (Figure 1F). In contrast, empty vector-treated diabetic mice were profoundly glucose intolerant with no change in plasma insulin in response to the glucose load. Thus, Ngn3-Btc treatment restored glucose-stimulated insulin secretion (GSIS) *in vivo*. We also excluded endogenous islet regeneration as a significant source of the insulin in these animals (Figure 1G).

It is important for β-cells to sense low blood glucose and shut off insulin secretion to forestall severe hypoglycemia. Blood glucose levels during a 72 hour fast in Ngn3-Btc treated diabetic and in nondiabetic control mice (Figure 1H) were indistinguishable between the two groups, indicating that insulin secretion in the Ngn3-Btc treated diabetic mice was appropriately turned off with fasting. Similar results were obtained in nondiabetic mice, with intact pancreatic islets, treated with Ngn3-Btc (Supplemental Figure 2A).

Ngn3 and betacellulin treatment induces insulin transcript and protein in the liver

Both *Ins1* and *Ins2* mRNA were expressed, by q-PCR, in the liver of Ngn3-Btc-treated mice, both in the early and late time points, and also in the liver of Ngn3-only treated mice in the absence of Btc co-treatment (Figure 2A). Determination of the total insulin content of the liver by acid-ethanol extraction revealed that insulin was readily detectable after Ngn3-Btc, its level reaching a plateau at 12-16 weeks after treatment, at which time the insulin content/mg total protein was ~ 20 % that of the nondiabetic pancreas (Figure 2C, top panel). We estimated the total liver insulin content 12 weeks after treatment to be similar to that of normal pancreas (Figure 2C, middle panel) since the liver is 4-7 times the size of the pancreas, and also proportionate to the total body weight, similar to that seen with nondiabetic pancreas (Figure 2C, bottom panel). Similar results were obtained for total liver C-peptide content (Supplemental Figure 3). The presence of insulin mRNA and C-peptide in the Ngn3-Btc liver confirmed that the insulin measured in the liver of the treated mice was actually synthesized in the liver as opposed to that taken up from the plasma. Thus, Ngn3-Btc treatment restores a full complement of insulin to the liver comparable to that in normal pancreas.

Ngn3 and betacellulin treatment induces glucose-stimulated insulin secretion (GSIS) from the liver

To determine if the liver is the source of the GSIS, we performed an *in situ* liver perfusion experiment (Figure 2D-E) wherein we cannulated the portal vein and infused solutions containing different concentrations of glucose and measured insulin and glucose in the liver effusate (Figure 2E). In contrast to empty vector treatment, Ngn3-Btc treatment led to GSIS as evidenced by a stepwise increase in insulin secretion in response to a stepwise increase in glucose concentration in the infusate from 2.8 mM to 11.1 mM and 25 mM (Figure 2D, bottom panel). Though this is lower than that seen with pancreas perfusion or islet perifusion studies, this magnitude of response is similar to that observed in perfusion studies on livers containing transplanted islets (Lau et al., 2007; Mattsson et al., 2004). Furthermore, the Ngn3-Btc-treated liver responded to a 0 mM glucose infusion by rapidly decreasing its insulin production, indicating that hepatic insulin production is tightly controlled by the glucose concentration of the infusate. Addition of glibenclamide (10 μM, abbreviated SU for sulfonylurea in Figure 2D, middle panel), a secretagogue belonging to the sulfonylurea class of drugs used to treat diabetic patients, to the infusate markedly stimulated insulin production only from the liver of Ngn3-Btc-treated mice (Figure 2D, bottom panel).

We also performed an *in vitro* GSIS assay to complement the *in situ* study. We repeated the gene delivery experiment in transgenic mip-GFP mice, which express eGFP driven by the mouse insulin promoter (Hara et al., 2003). The same Ngn3-Btc regimen also reversed diabetes in STZ-diabetic mip-GFP mice. GFP-positive but not GFP-negative cells from the liver of these mice, isolated by flow cytometry, displayed a significant increase in insulin secretion when the concentration of glucose in the medium was increased from 2.8 to 25mM (Figure 2F). Incubation of untreated hepatocytes failed to display GSIS into the medium.

Ngn3 treatment induces an islet-like transcription profile in the liver

We asked if Ngn3 treatment induced the complete set of islet hormones in the liver, in addition to insulin. By q-PCR, both Ngn3-Btc-treated and Ngn3-only-treated, but not Btc- or empty vector-treated, liver produced readily detectable levels of transcripts of the other major islet hormones, viz., glucagon (Gcg), pancreatic polypeptide (PP), and somatostatin (Sst) (Figure 2A).

This suggests that Ngn3 is capable of activating a cascade of transcription factors that collectively specify the different islet cell lineages, a prediction that is corroborated by direct quantification of the transcripts for multiple transcription factors, including Pdx1, Neurod1, Isl1, Pax4, Pax6, Nkx2.2 and Nkx6.1 by q-PCR in both the Ngn3-Btc- and Ngn3-only treated liver (Figure 2B). All these factors are expressed during fetal endocrine pancreas development, and all of them except Pax4 (Sosa-Pineda et al., 1997) are known to be expressed in the adult pancreas as well. Interestingly, unlike normal adult pancreatic islets, Ngn3-Btc- and Ngn3-only treated liver continued to express Pax4. We note that induced expression of Ngn3 in concert with HNF-1α can directly activate Pax4 expression (Smith et al., 2003) and that Btc itself has been shown to induce Pax4 (Brun et al., 2008). Pdx1, a pancreas-defining transcription factor upstream of Ngn3, was also found to be expressed with Ngn3-Btc and Ngn3-only treatment. This is not unexpected as Pdx1 is normally expressed in mature β-cells and is required for normal insulin secretion (Holland et al., 2005). Interestingly, ectopic Pdx1 expression by gene transfer (Kojima et al., 2003; Ber et al., 2003) or in transgenic mice (Miyatsuka et al., 2003) was shown to lead to pancreatic exocrine enzyme expression in the liver, but the appearance of Pdx1 in the Ngn3-induced neo-islets did not lead to exocrine enzyme expression (Supplemental Figure 4).

Other β-cell specific transcripts essential for normal β-cell function, such as sulfonylurea receptor-1 (*Abcc8*), Kir6.2 (*Kcnj11*) and the prohormone convertases 1/3 (*Pcsk1*) and 2 (*Pcsk2*) were expressed in the Ngn3-Btc- and Ngn3-only treated liver (Figure 2A, Supplemental Table 1). Therefore, Ngn3, with or without Btc, induces in the liver the developmental cascade of transcription factors that are normally seen in developing islets, along with other molecules characteristic of mature pancreatic islets.

Ngn3 induces two waves of insulin production – First wave occurs in parenchymal hepatocytes

Ngn3-Btc gene transfer led to the presence of immunoreactive insulin by 3 weeks in parenchymal hepatocytes (Figure 3A). However, by 6 weeks most of the insulin immunofluorescence had disappeared (Figure 3B) and by 12 weeks, insulin was no longer detectable in parenchymal hepatocytes. This transient induction of insulin production in hepatocytes was sufficient to suppress the elevated PEPCK expression (Supplemental Figure 5), a key gluconeogenic gene, in diabetic mice back to that of a nondiabetic control.

Second wave of insulin production occurs in neo-islets in the periportal region

Ngn3-Btc-induced reversal of hyperglycemia occurred within days, and was associated with the production of immunoreactive insulin early in the hepatocytes. However, the normalization

of blood glucose, plasma insulin and insulin content in the liver was sustained beyond 6 months after Ngn3-Btc even after insulin immunofluorescence had disappeared from the hepatocytes (Figures 1A, D and 2C). Further examination revealed that as the insulin expression was waning in parenchymal hepatocytes (size ~20-40 μM), there was the emergence of clusters of cells that were significantly smaller than hepatocytes in the periportal region that stained strongly positive for immunoreactive insulin 6 weeks after Ngn3-Btc (Figure 3C). Over time, there was a gradual increase in the size, number and density of these periportal insulin-positive cell clusters (Figures 3D, 3K, and 3L). As in Ngn3-Btc-treated mice, in Ngn3-only animals without Btc, the disappearance of insulin-positive hepatocytes coincided with the appearance of periportal insulin-positive cell clusters (Figures 3E and 3M), indicating that Btc was not required for the induction of these cells, though the addition of Btc did augment the response. Neither the first nor the second wave of insulin production was observed in any of the empty vector-treated livers (Figures 3F and 3N).

On higher magnification, the periportal insulin-positive cells in Ngn3-Btc-treated mice have a granular cytoplasm that stains positive with anti-insulin antibody (Figures 3G and H; and Figures 3K and 3L). Another notable feature of these periportal islet-like clusters of insulin-positive cells is their size (7-10 μm) and high nuclear-cytoplasmic ratio, a morphological characteristic of hepatic oval cells. Most of the portal triads appeared to harbor some insulin-positive clusters, though the size in terms of the number of cells per cluster varied (Supplemental Figure 6). Significantly, very few of these types of cells occurred in the portal region in the empty vector-treated livers, none of which stained positive for insulin (Figures 3F and 3N). We also detected immunoreactive proinsulin only in the liver of Ngn3-Btc- (Figures 3Q and 3R) and Ngn3-only-treated mice (Figure 3S) that was present in islet-like periportal cell clusters. The presence of proinsulin in the periportal cells is consistent with the insulin being produced, and not taken up, by these cells in response to Ngn3 treatment.

The Ngn3-Btc-induced first and second wave of insulin production in different cell types in the liver was highly reproducible, not only in multiple experiments in wild-type STZ-diabetic mice, but also in STZ-diabetic mip-GFP mice, using GFP as a surrogate marker for insulin production (see Supplemental Text and Supplemental Figure 7).

Neo-islets express multiple islet hormones

The first wave of insulin expression in hepatocytes in mice treated with Ngn3, with or without Btc, was not associated with detectable amounts of non-insulin islet hormones either by immunostaining or by RT-PCR of RNA obtained by LCM (see later). On the other hand, these other islet hormones were easily detected in the periportal neo-islets; Gcg, PP and Sst-expressing cells appeared along with or just before the appearance of insulin positive cells in the periportal region of both Ngn3-Btc (Figures 4A-C) and Ngn3-only (Supplemental Figure 8D-L) treated mice. Indeed, a unique observation is the occurrence of individual hormone-expressing cells in these neo-islets suggesting that the Ngn3, with or without Btc, initiates a complete islet development program in the periportal cells in the liver. We note, however, that some of the insulin-positive cell clusters weakly co-express other hormones, a feature also seen in regenerating pancreatic islets (Fernandes et al., 1997; Guz et al., 2001). In contrast to the spatial organization of normal rodent islets, which present with a central core of β-cells surrounded by a peripheral rim of α-cells and scattered PP and δ-cells, the periportal neo-islets are loosely clustered with intervening nonhormone-producing cells that have not committed to the islet cell lineage (Figures 3D, 4A-B and Supplemental Figures 8F, 8I and 8L). None of the islet hormones were detected in the livers of empty vector treated diabetic mice (Supplemental Figure 8A-C) or in the non-diabetic control mice (data not shown), in keeping with the mRNA data (Figure 2A). Therefore, in response to Ngn3 gene transfer, the neo-islets

that have arisen from periportal cells express insulin as well as other major islet hormones. We next examined the lineage origin of these cells.

Bone marrow-derived cells are not the source of hepatic neo-islets

To determine whether the insulin-positive cell clusters arose from bone marrow-derived cells, we performed lineage tracing with bone marrow transfer experiments (see Supplemental Text), which excluded both bone marrow-derived fusion cells and other bone marrow-derived cells in the liver (Supplemental Figure 9), including circulating blood cells, Kupfer cells and stellate cells, as the source of the insulin-producing cells.

Lineage tracing establishes that oval cells express albumin in their lineage history

We designed lineage tracing experiments to determine whether parenchymal hepatocytes and oval cells, which are not derived from bone marrow cells, are involved in the two waves of insulin production that occur after Ngn3-Btc treatment. Lineage tracing of hepatocytes is well established. However, to date, there is no known transgenic reporter mouse that can be used for tracing the origin of oval cells. The timing of albumin expression during mouse embryonic development suggests that albumin promoter-driven reporter gene expression could be used as a lineage marker not only for hepatocytes, but also for oval cells. Embryologically, the liver is formed from the developing foregut. It is noteworthy that the foregut endoderm expresses albumin in the foregut precursor cells as early as e9.5 days of mouse embryo development, prior to cell aggregation and liver formation (Cascio and Zaret, 1991). Hence, the early albumin-expressing foregut endoderm encompasses precursor cells that develop into multiple cell types in the liver, except for those derived from bone marrow. While albumin expression has been used for tracking parenchymal hepatocytes, whether transgenic albumin-promoter driven reporter gene expression can be used for lineage tracing of oval cells has not been explored.

To determine if such a strategy would work, we used bigenic mice resulting from a cross between a ROSA-Stop-Lox-eGFP reporter mouse and an albumin promoter-Cre mouse. In these animals, all cells that expressed albumin anytime in their lineage history also express eGFP. As there are very few oval cells in untreated mouse liver, we utilized the model of D-galactosamine-induced oval cell proliferation (Lemire et al., 1991; Hsiao et al., 2000) in which oval cells appear in the periportal region within days of treatment. We sacrificed the bigenic mice 10 days after D-galactosamine treatment and identified oval cells in the periportal region by their typical morphology. These oval cells all express eGFP, indicating that hepatic oval cells are derived from cells that expressed albumin in their lineage history (Supplemental Figure 10) and that these mice are indeed a useful reporter model to trace not only the hepatocyte lineage (Magnuson, 1992), but also the oval cell lineage, a finding that has not been appreciated in the past.

Cells expressing the first wave of insulin come from hepatocytes and the second wave from periportal cells

Having established that, like parenchymal hepatocytes, oval cells express albumin in their lineage history, we used ROSA-Stop-Lox-eGFP/albumin-Cre bigenic mice to trace the origin of both parenchymal hepatocytes and oval cells. We rendered these mice diabetic by STZ and treated them with either an empty vector or Ngn3-Btc. The diabetic bigenic mice displayed Ngn3-Btc-induced diabetes reversal as in wild-type diabetic mice. We sacrificed the mice at various times afterwards to assess if the insulin-positive cell clusters were eGFP positive. All parenchymal hepatocytes were eGFP positive (Figures 5A-C), including hepatocytes that were insulin-positive within 3 weeks of Ngn3-Btc treatment (Figure 5B). At later time points when insulin immunostaining had disappeared from the parenchymal hepatocytes, like D-

galactosamine-induced oval cells, all periportal insulin-positive oval cell clusters were also eGFP positive (Figure 5C).

Use of bigenic mice indicates that both the first wave and second wave of insulin-positive cells express albumin in their lineage history. However, the experiment did not reveal whether albumin was currently being produced by these cells. We therefore performed a parallel gene transfer experiment in which we assessed liver sections for immunoreactive albumin and insulin expression by immunofluorescence. We found that while immunoreactive albumin was indeed present in the typical parenchymal hepatocytes that also produced insulin 3 weeks after Ngn3-Btc treatment (Figure 5D and Supplemental Figure 11), it was absent from the insulin-positive periportal small cells that had the typical morphology of oval cells (Figure 5E). These complementary experiments further support the conclusion that parenchymal hepatocytes constitute the initial wave (at 3 weeks) of insulin-producing cells. Importantly, they indicate that the periportal insulin-positive cells originate *not* from hepatocytes, but from periportal cells that appear to be oval cells, based not only on their location and morphology, but also by the absence of immunoreactive albumin.

Ngn3-induced periportal neo-islets appear to originate from oval cells

To further investigate the origin of the insulin-producing cells induced by Ngn3 with or without Btc, we next examined the liver of diabetic mice at different times after treatment by immunohistochemistry. We found little change in morphology within a week of Ngn3-Btc treatment (data not shown). At 3 weeks, we observed the appearance of small cells (~7-10 μm in diameter) with a high nuclear-cytoplasmic ratio in the periportal areas and radiating outwards (Figures 6A-D), which became more restricted to only the periportal regions by 6 weeks and beyond (Figures 6E-T and Supplemental Figures 12A-B). The classic morphological characteristics of periportal location, size, and high nuclear-cytoplasmic ratio, identify these newly appearing cells as oval cells. Once again, there are more of these cells in the liver of Ngn3-Btc-treated as compared to Ngn3-only-treated mice. These cells appeared in abundance only in the Ngn3-treated (Supplemental Figures 12A-B) and Ngn3-Btc-treated livers (Figures 6A-T), and rarely in the empty vector-treated (Figures 6D and Figure 6U-X) or nondiabetic livers (not shown). They displayed immunoreactivity toward an oval cell-specific monoclonal antibody A6. The pattern of all insulin-producing cells being A6-positive leads us to conclude that the neo-islets are derived from oval cells. At 3 weeks, there was little insulin immunostaining in these oval cell clusters (Figures 6E-H). By 6 weeks and later, however, groups of strongly insulin-positive cells appeared among the oval cell clusters, co-expressing A6 reactivity in both Ngn3-Btc (Figures 6E-T and Supplemental Figure 13) and Ngn3-only-treated (Supplemental Figure 14) mice.

Oval cells express other antigens, e.g., CK-19 and Thy1, which are also expressed in other cell types such as biliary cells (Crosby et al., 1998) and hematopoietic progenitors (Miller and Lipton, 1983), respectively. However, the concomitant expression of CK-19 and Thy1 has been reported only in hepatic oval cells (Petersen et al., 1998). Indeed, we detected both CK-19 (Figure 6E-T, Supplemental Figure 13 and 14) and Thy1 (Supplemental Figure 12C-F) immunoreactivity in all insulin-producing periportal cells at all time points. While this unique staining pattern further establishes the oval cell origin of the newly formed islets, we note that as transgenic reporter mouse models that genetically track oval cell lineage exclusively are not currently available, we cannot rule out the possibility that other albumin lineage positive cells, not originating from the bone marrow, could have de-differentiated and acquired an oval cell morphology and expression pattern after Ngn3 treatment. It is interesting that CK-19 has also been observed in insulin-positive cells during islet neogenesis (Gao et al., 2005) and in islet progenitors in the pancreas during islet regeneration (Xu et al., 2008). Therefore, the Ngn3-

induced neo-islets are more similar to neo-islets in the pancreas than they are to mature pancreatic islets.

We analyzed the global expression profile of LCM-captured Ngn3-Btc-induced neo-islets and mature pancreatic islets, as well as hepatocytes and non-transduced oval cells by cDNA microarray. Correspondence analysis, an unbiased method whereby both genes expressed and individual samples can be projected into the same multi-dimensional space and analyzed simultaneously (Fellenberg et al., 2001), reveals that induced periportal neo-islets cluster much more closely with pancreatic islets than either one with oval cells or hepatocytes (Figure 7A, also see Supplemental Methods).

Ngn3 induces a full-fledged β-cell transcription program in proliferating periportal cells but an aborted transcription cascade in hepatocytes

To address the mechanism that underlies the differential response of parenchymal hepatocytes vs. periportal cells to Ngn3 expression, we analyzed the expression of different transcription factors that characterize normal β-cells. We performed LCM to obtain RNA specifically from small periportal cells and from larger parenchymal hepatocytes at different times after Ngn3-only or Ngn3-Btc gene transfer, using LCM-RNA from normal pancreatic islets as controls. By RT-PCR, we readily detected Ins1 mRNA in parenchymal hepatocytes at 3 weeks, but the transcript almost completely disappeared by 6 weeks (Figure 7B) with both Ngn3-only and Ngn3-Btc treatment. Ins2 mRNA was barely detectable and only at the 6 week time point in these hepatocytes with Ngn3-Btc treatment. The time course of insulin mRNA expression correlates with that of immunoreactive insulin staining presented in Figures 3 and 5. We failed to detect Ins1 or Ins2 expression at 12 weeks in hepatocytes, despite the continued expression of vector-driven Ngn3. We also did not detect any of the other islet hormone transcripts in the parenchymal hepatocytes at any of the time points. None of the islet-defining transcription factors are expressed in the hepatocytes at 3 weeks. By 6 weeks, the major early β-cell transcription factors (Pdx1, Neurod1 and Isl1) can be detected in the hepatocytes of both Ngn3-Btc and Ngn3-only treatment, while Pax6 could be detected in the hepatocytes with Ngn3-Btc and not with Ngn3-only treatment. However, the transcriptional cascade in hepatocytes appears to be aborted at this stage with failure of expression of the downstream transcription factors Pax4, Nkx2.2, or Nkx6.1 (Figure 7B).

In contrast to hepatocytes, the small periportal cells express transcripts for both Ins1 and Ins2 robustly along with the other islet hormones, Gcg, PP and Sst, whether or not Btc was included in the regimen (Figure 7B). Thus, the transcript expression profile agrees with the immunostaining results (Figure 4 and Supplemental Figure 8). Importantly, there is also a robust expression of the entire transcription cascade of developing islet-cells, including Pdx1, Neurod1, Isl1, Pax6, Nkx2.2 and Nkx6.1 with both Ngn3-Btc- and Ngn3-only treatment, though Pax4 was expressed only with Ngn3-Btc treatment. It has been reported that Btc by itself may increase Pax4 expression (Brun et al., 2008). Interestingly, the endogenous Ngn3 transcript is not detected, as would be predicted from the fact that Ngn3 represses its own expression (Smith et al., 2004), since the vector-derived Ngn3 was expressed in all the time points in both hepatocytes and oval cells. Furthermore, the level of expression of the major transcription factors is comparable to that seen in normal pancreatic islets (Figure 7B). All the transcription factors along with Ins1 and Ins2 continue to be expressed at 12 weeks. The two waves of Ngn3-Btc-induced cellular insulin expression, from hepatocytes at 3-6 weeks and oval cell-derived periportal cells at 6 weeks and later, and their relation to the blood glucose of the treated mice are depicted in Figure 7C. Therefore, the appearance of insulin transcripts and protein at the 6 week time point appears to coincide with a switch of cell fate of the oval cells to a β-cell specific transcription signature, normally found only in pancreatic β-cells. We note that transcripts for the β-cell-specific molecules, the potassium channel *Kcnj11* and the

prohormone convertase *Pcsk1*, like some of the transcription factors downstream to Isl1 and Pax6, were also expressed in the neo-islets but not in the hepatocytes (Figure 7B). Also of note was that Thy1 was expressed only in the neo-islets, consistent with these cells being derived from oval cells (Petersen et al., 1998). Interestingly, albumin, which was expressed at high level in hepatocytes, was weakly positive in some of the oval cell-derived RNA samples. We cannot exclude the possibility of contamination from adjoining hepatocytes, as we did not detect albumin in oval cells by immunostaining (Figure 5E); albumin protein is generally thought to be absent in oval cells, however, very low levels of albumin transcript have been described (Nagy et al., 1994). We also note that not all oval cells picked by LCM, especially early on, had switched their cell-fate, as revealed by insulin and A6 immunostaining in Figures 6E-T and Supplemental Figures 13 and 14.

Taken together, our results indicate that the hepatic oval cells appear to have differentiated into the endocrine pancreas, a lineage that is different from their original determination, viz., biliary cells or hepatocytes, a process that is consistent with transdetermination (McClure and Schubiger, 2007). Since transdetermination tends to occur in actively proliferating cells, we assayed the proliferation activity of the periportal oval cell clusters. The strong signals for both BrdU labeling and PCNA staining at 3 weeks in the periportal oval cells indicate that many are in the S-phase of the cell cycle (Figures 7D and 7E, and Supplemental Figure 15), consistent with active proliferation. Interestingly, the proliferation rate is twice as high in the oval cells in the Ngn3+Btc regimen as compared with the Ngn3-only regimen. In contrast, BrdU and PCNA-positive cells were rarely identified among the neighboring parenchymal hepatocytes with either treatment. The absence of cell proliferation and an aborted transcription cascade in the hepatocytes go along with their failure to transdifferentiate into β-cells, even though they can be induced to produce insulin transiently. On the other hand, our results indicate that oval cells have undergone a transdetermination process (Figure 8); in response to induced Ngn3 expression, they display a complete β-cell transcription profile, express islet-specific molecules that lead to reversal of diabetes.

Discussion

Regeneration, which often recapitulates ontogeny, is constrained by the limited availability of competent multipotent progenitor cells in tissues such as pancreatic islets, heart and the nervous system. Furthermore, recent evidence suggests that most somatic cell-initiated transdifferentiation phenomena are the result of cell fusion rather than true transdifferentiation (Wagers and Weissman, 2004), while investigators have continued to test different protocols to attempt to effectuate the transdifferentiation of different types of cells into insulin and islet cell lineages as a means to treat diabetes [reviewed in (Gangaram-Panday et al., 2007)] with few documented success (Zhou et al., 2008).

Ngn3 induces insulin production in the liver via two distinct cell type-specific responses

We examined if the endocrine pancreas lineage-defining transcription factor, Ngn3, would coax cells in the liver, many of which are of endodermal origin, to recapitulate pancreatic islet development *in situ* and observed that both Ngn3-only and Ngn3-Btc (but not Btc-only) gene transfer in diabetic mice led to a rapid restoration of euglycemia within a week. Further analysis revealed that Ngn3 induced hepatic insulin production and the cells responsible of this first wave of insulin are parenchymal hepatocytes. Vector-derived Ngn3 expression led to the expression of *Neurod1*, *Isl1* and *Pax6*, which are downstream of Ngn3, in the parenchymal hepatocytes. However, the β-cell specific transcription cascade was aborted at this stage; moreover, the curtailed transcription program was short-lived, lasting about 6 weeks, despite the continued expression of Ngn3 in these hepatocytes.

Interestingly, as insulin production by parenchymal hepatocytes was waning, morphologically distinct insulin-positive cells started to emerge in the periportal region, which established a sustained second wave of insulin production that maintained euglycemia for the duration of the experiment (6 months). We characterize these cells as oval cells based on their typical morphology, location and marker expression. A detailed time course analysis reveals that oval cells appear 3 weeks after treatment with Ngn3-Btc but at that time they do not express any insulin either by immunostaining (Figure 6) or by RT-PCR (Figure 7); subsequently, by 6 weeks after treatment many of the oval cells start producing insulin (Figure 6). Therefore, a combination of multiple experiments indicates that there are two waves of insulin production, first wave transiently by hepatocytes (which continue to express albumin) and a second, sustained wave, apparently by oval cells. It excludes transdifferentiation of hepatocytes and supports the model of transdetermination of oval cells being the mechanism that underlies Ngn3-induced islet neogenesis in the liver. Oval cells are a multipotential progenitor cell population that is committed to becoming hepatocytes or biliary cells (Zheng and Taniguchi, 2003). Under the influence of Ngn3, they have differentiated into endocrine pancreatic hormone-producing cells, a distinct endoderm-derived lineage. In adult rodents Ngn3-positive facultative endocrine progenitors located in the ductal lining can differentiate into insulin-producing β cells after partial pancreatic duct ligation (Xu et al., 2008). It has been suggested that, like the ductal-derived cells in the pancreas, oval cells in the liver can act as “facultative stem cells” that regenerate the liver under specific types of insults (Dor and Melton, 2008). Herein, we showed that induced Ngn3 expression can guide oval cells to differentiate into glycemia-responsive insulin-producing cells.

Other studies have suggested that oval cells may have the potential to differentiate into β-like cells (Yang et al., 2002). De-repression of Ngn3 in *Hes-1* null mice led to development of insulin-positive islet-like clusters in the biliary tree (Sumazaki et al., 2004), where oval cells normally reside. Indeed, the co-expression of oval cell markers, such as CK-19, has also been observed in insulin-positive cells during islet neogenesis (Gao et al., 2005) and in islet progenitors in the pancreas during islet regeneration (Xu et al., 2008). Our experiments indicate that induced expression of Ngn3 in oval cells *in vivo* was sufficient to induce the formation of pancreatic islet-like cells, which express insulin as well as other β-cell markers, similar to islets undergoing regeneration or neogenesis.

Transdetermination versus transdifferentiation

An important question that arises is the nature and the mechanism underlying the differential response of differentiated parenchymal hepatocytes and hepatic progenitor cells to the induced Ngn3 expression. Did the hepatocytes transdifferentiate into islet-cell type, albeit transiently? Transdifferentiation, defined as a switch from one differentiated state to another, usually without an intermediate stage of de-differentiation to a more embryonic-like state (Wagers and Weissman, 2004), would entail that the insulin-producing parenchymal hepatocytes lose their hepatocyte phenotype, both morphologically and functionally, e.g., lose the capacity to express albumin, a hallmark of differentiated hepatocyte function. We demonstrate that the hepatocytes never lose their differentiated phenotype (continued robust albumin expression and unchanged morphology) and, other than a short-lived insulin expression, they do not attain any consequential islet phenotype even in the face of continued Ngn3 expression. Hence, Ngn3 gene transfer in parenchymal hepatocytes induces transient insulin production, but fails to effectuate true transdifferentiation. We conclude that hepatocytes are not a competent cell lineage for Ngn3-induced transdifferentiation into pancreatic islets.

In contrast, in response to Ngn3 a periportal cell population that we conclude corresponds to oval cells attains a sustained and robust islet-lineage phenotype that is manifest at the level of cell morphology and gene expression, a complete islet-specific transcription profile and

multiple islet-specific hormone and cell markers at the mRNA and protein levels. We propose that transdetermination underlies this Ngn3-induced cell fate switching.

Transdetermination is the switching of lineage commitment in progenitors to closely related cell-lineages in metazoa. It has been extensively studied in *Drosophila* imaginal discs, the primordia of the adult fly appendages that maintain their cell fate during larval development even after they are cultured for prolonged periods (Hadorn, 1968; Maves and Schubiger, 1998; Sustar and Schubiger, 2005). Transdetermination is said to occur when disc cells at specific locations known as 'weak points' switch their cell fate, e.g., from leg to wing, in a rare event that tends to occur in certain replicative stages due to the unmasking of differentiation programs (Gehring, 1987; Lee et al., 2005).

Transdetermination has not been studied extensively in mammals, though some well documented examples exist. Transgenic mice with hyperactive Wnt signaling in the lung exhibit a switch of lung progenitor cells to intestinal cell types (Okubo and Hogan, 2004). Another example involves detection of lung specific transcription factors (TTF-1) and ciliated intestine-specific transcription factor (HFH-4) and the concomitant occurrence of gastric broncho-pulmonary cells in the gastric mucosa of human atrophic gastritis (Rau et al., 2005). In both these studies there seemed to be a switch from one endodermal-derived lineage to another. Herein, we showed that delivery of a lineage-defining transcription factor can induce in adult animals the transdetermination of hepatic oval cells *in vivo*.

We conclude that the Ngn3-induced islet neogenesis from periportal hepatic progenitor cells is a transdetermination event and these cells, which have many of the characteristics of oval cells, represent a 'weak point' in the portal triads. This conclusion is supported by the following: 1. Hepatic progenitor cells and islet cells have a very close embryological origin – both are derived from the foregut endoderm. 2. The islet cell fate induction in these periportal hepatic progenitor cells, which are not differentiated but determined normally to hepatic or biliary cell fates, occurs only in the presence of Ngn3 and in the face of regenerative proliferation in the cells involved. 3. Unlike hepatocytes that exhibit an aborted transcriptional cascade, these periportal small cells appear to differentiate into islet cell types with no detectable intermediate cell types. These are consistent with criteria proposed for transdetermination events (Wei et al., 2000; McClure and Schubiger, 2007).

In summary, the transfer of a single lineage-defining transcription factor, viz., Ngn3, into an endoderm-derived lineage, is sufficient to effectuate cell lineage switching and induce the formation of neo-islets by a process consistent with transdetermination. The newly formed islets display a complete islet-specific transcription program and produce insulin in a glucose-responsive manner, stably reversing diabetes. These findings also shed light on the fundamental processes of cell fate determination and reprogramming, and the role of transdetermination vs. transdifferentiation as a paradigm for *de novo* organogenesis; they provide the first evidence for the hypothesis that periportal hepatic oval cells are "facultative stem cells" amenable to *in vivo* reprogramming (Dor and Melton, 2008; Oertel and Shafritz, 2008). This model of induced islet neogenesis (Figure 8) offers a useful tool to decipher the interacting network of transcription factors involved in islet cell-fate decisions and underlying developmental programs. Moreover, the feasibility of inducing organogenesis by a single transcription factor, using induced transdetermination as a blueprint, suggests the possibility of harnessing the power of lineage-defining transcription factors to exploit other receptive progenitor cell lineages for regenerative medicine in the treatment of disease.

Experimental procedures

Animals

Diabetes was induced by IP streptozotocin 125 mg/kg on two consecutive days. HDAds were injected intravenously via tail vein after 1 week of stable diabetes. GTT was performed, 6 weeks after treatment, by injecting 1.5 gm/kg of D-Glucose IP. D-galactosamine (Sigma) was injected IP at 0.9 gm/kg body weight for oval cell induction in lineage tracing experiments. To study cell proliferation, BrdU was injected 15μl/gm IP 2 hours prior to sacrifice.

LCM

Liver frozen sections were nuclear-stained in a RNase free environment and LCM was performed using the Veritas microdissection system. Cells with a high nuclear-cytoplasmic ratio present in clusters in the periportal region and large parenchymal hepatocytes were collected separately. Total RNA, extracted using the Pico Pure RNA isolation kits and amplified twice with the RiboAmp kits, DNAseI treated, quantitated, reverse transcribed and used in PCR reactions to assess gene expression.

Gene Expression

RNA, obtained by Trizol extraction from tissues, was DNAseI treated, reverse transcribed and subjected to a quantitative real-time PCR using specific primers (Supplemental Table 2) and SYBR-Green mix and normalized to the expression of two housekeeping genes (GAPDH and eEF1γ).

Immunostaining

Paraffin embedded tissue sections were deparaffinized, hydrated with graded ethanol, permeabilized and antigen retrieval was performed. Primary antibody was incubated overnight at 4C and the secondary antibody for 1 hour at RT. Avidin-biotin horseradish peroxidase (Vectastain Elite ABC reagent, Vector Laboratories) and NovaRed substrate kits for peroxidase (Vector Laboratories) were used to visualize for immunohistochemistry while fluorophore conjugated secondary antibodies were used for immunofluorescence. Imaging microscopy was performed using an Axiovert (Zeiss) microscope with Axiovision imaging software. All the antibodies and their dilutions used are listed online in the Supplemental methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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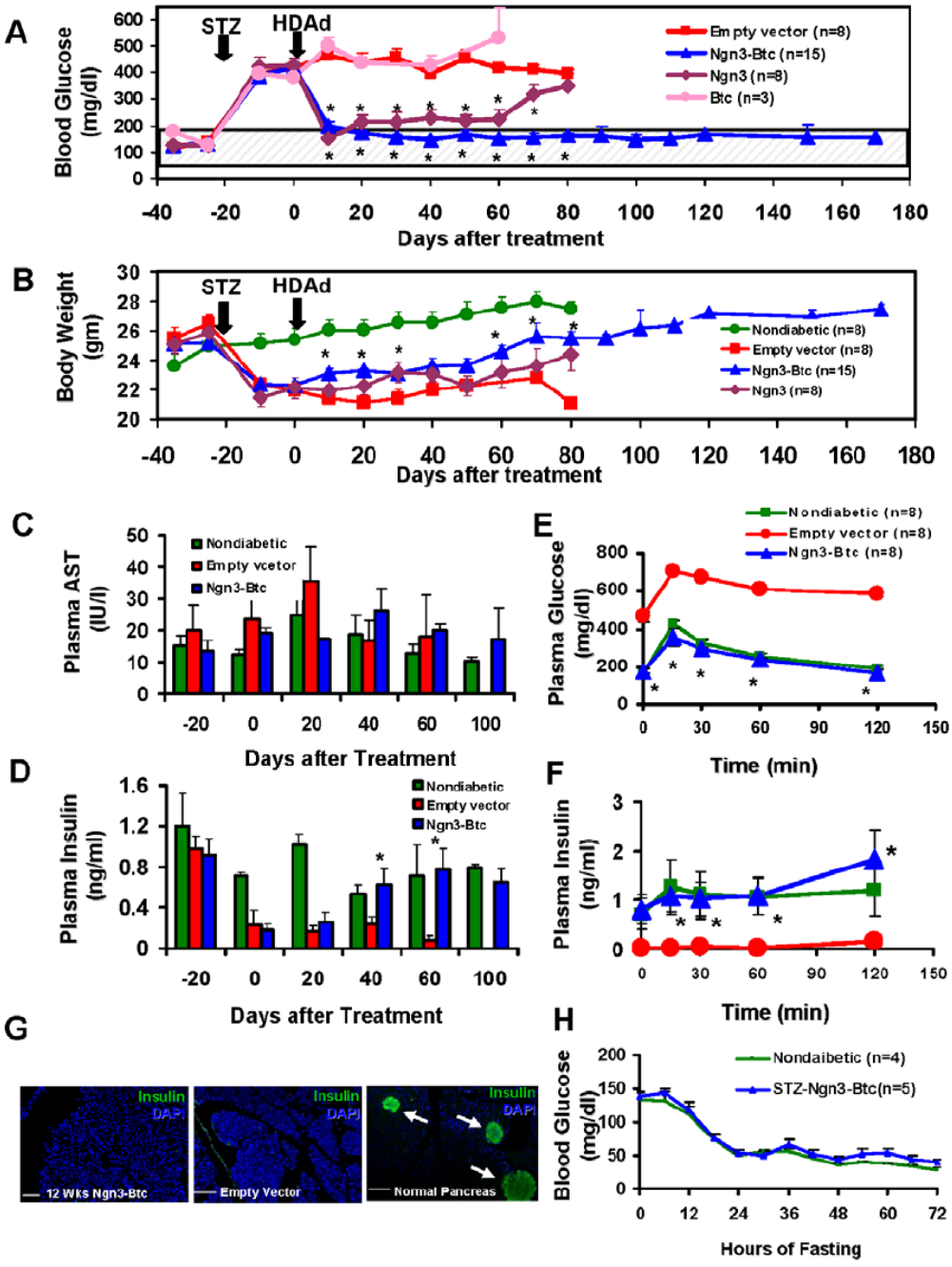

Figure 1. Ngn3-Btc reverses diabetes in insulin-deficient mice

A-B: Fasting blood glucose and body weight in STZ-diabetic mice. The hatched box covers the normal glucose range (mean ± 2 SD of 8 nondiabetic mice).

C-D: Plasma aspartate aminotransferase (AST) and fasting insulin at indicated time points (n=4-5).

E-F: Plasma glucose and insulin during an IP-GTT at 6 weeks after treatment.

G: Representative pancreas sections of STZ-diabetic mice treated with either Ngn3-Btc or empty vector are shown along with nondiabetic control by insulin IF (green) and by DAPI nuclear stain (blue). Scale bars represent 100μm.

H: Blood glucose during a 72 h fast in Ngn3-Btc-treated diabetic and nondiabetic control mice.

All values are mean±SEM; * $p \leq 0.05$

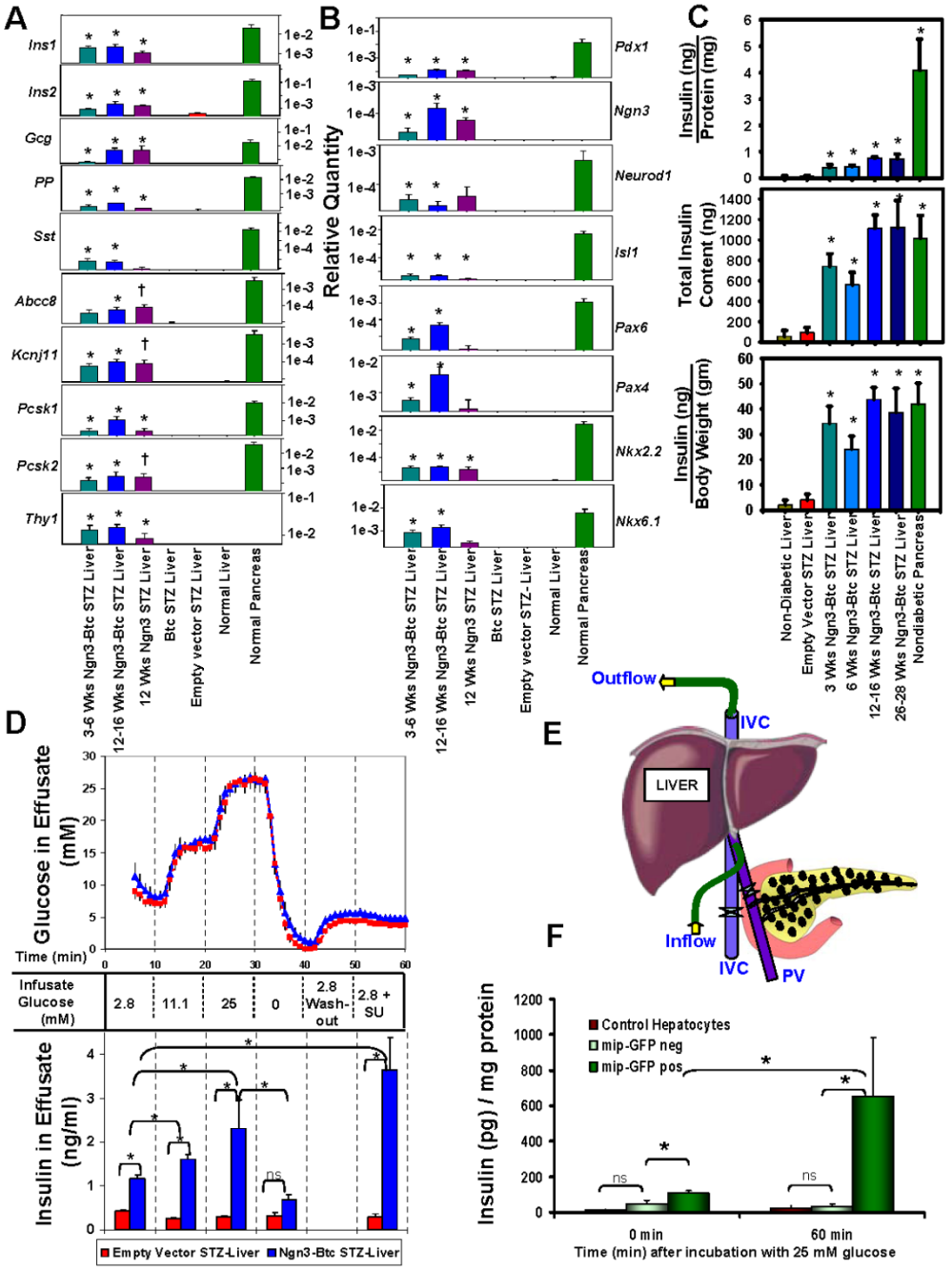

Figure 2. Ngn3-Btc induces regulated insulin production from the liver

A-B: Quantitative RT-PCR for islet hormones, proteins (A), and transcription factors involved in islet development (B) from the livers of treated mice (n=3-5). Values are expressed after normalization relative to GAPDH and eEF1γ.

C: Liver insulin content (ng) per mg protein (upper panel), normalized to the whole organ weight (middle panel) and to the body weight (lower panel) (n=3-4).

D-E: Glucose-stimulated insulin secretion (GSIS) from the livers of Ngn3-Btc-treated and empty vector treated diabetic mice (n=3-4), 6-8 weeks after treatment, by *in situ* liver perfusion (Schematic drawing in E). Glucose (upper panel) and insulin (lower panel) in effusate are shown at indicated infused glucose concentrations. SU-sulfonylurea (10nM Glibenclamide).

F: GSIS from GFP-negative and GFP-positive cells from Ngn3-Btc-treated mip-GFP diabetic mouse livers, 6-8 weeks after treatment and control hepatocytes are from nondiabetic mouse livers (n=3 each)

All values are mean±SEM; * $p \leq 0.05$; † $p = 0.057$; ns-not significant

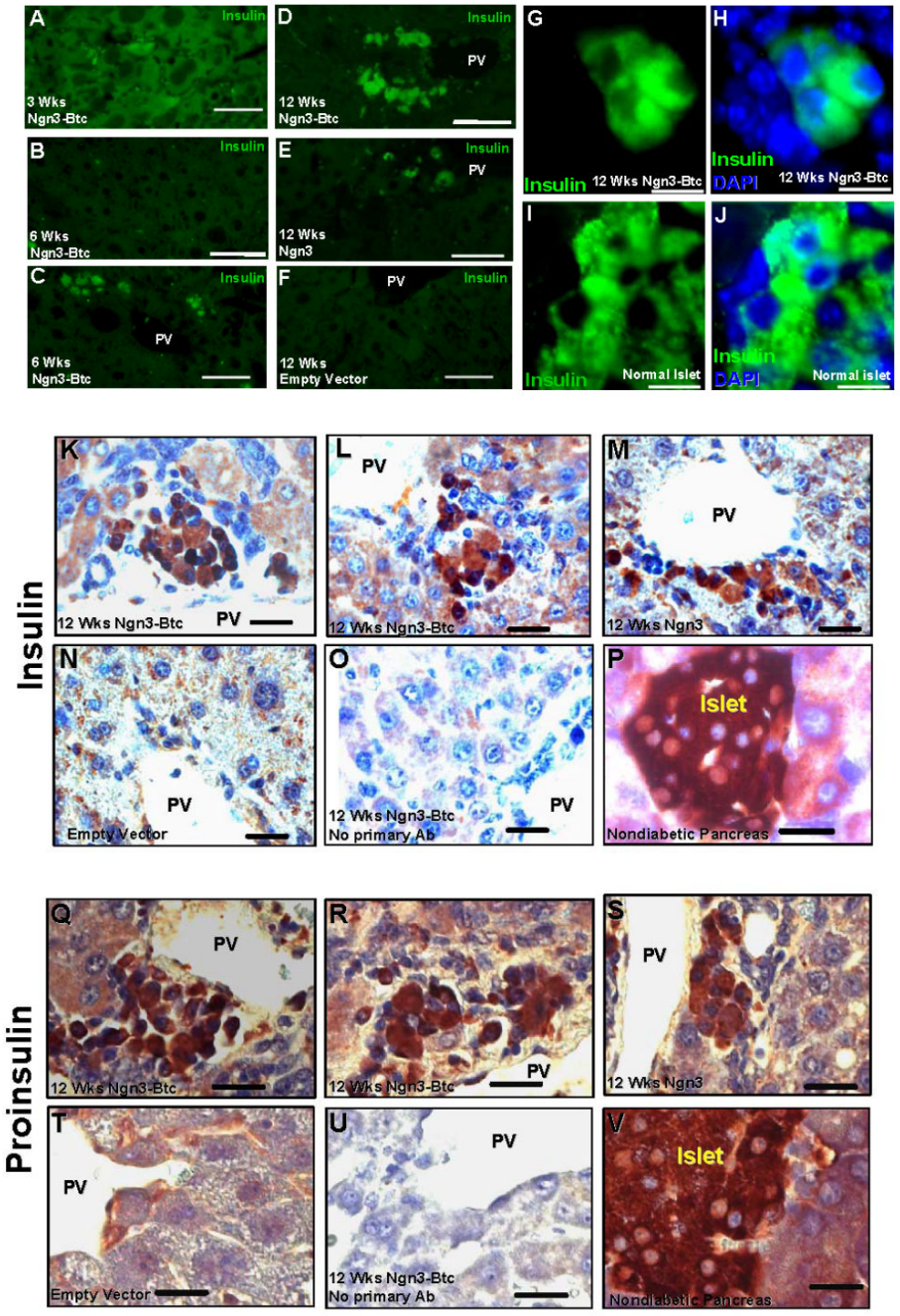

Figure 3. Ngn3-Btc induces two waves of insulin expression – first in parenchymal hepatocytes and later in periportal neo-islets

A-F: Representative sections of STZ-diabetic mouse liver stained for insulin (green) by immunofluorescence (IF). Ngn3-Btc induces insulin expression in the parenchymal hepatocytes at 3 weeks (A) which fades by 6 weeks (B), at which time periportal clusters of strongly insulin-positive cells appear (C) which increase in number and intensity by 12 weeks (D). Ngn3-only treatment is sufficient to induce periportal insulin positive clusters (E), though fewer in number as compared to treatment with Ngn3+Btc (D). No insulin IF is seen in the empty vector STZ-diabetic control (F).

G-J: Higher magnification of the periportal insulin-positive cluster (G-H); an islet from a nondiabetic control pancreas (I-J) is shown for comparison. G&I are stained for insulin (green) and H&J are merged with DAPI (blue).
K-P: Periportal clusters of insulin-positive cells in Ngn3-Btc (K, L) and Ngn3-only (M) treated diabetic livers; none are seen with empty vector treatment (N). No primary antibody control (O) and an islet from a nondiabetic mouse pancreas (P) are shown for reference.
Q-V: Periportal clusters of proinsulin-positive cells in Ngn3-Btc (Q, R) and Ngn3-only (S) treated diabetic livers; none are seen with empty vector treatment (T). No primary antibody control (U) and an islet from a nondiabetic mouse pancreas (V) are shown for reference. Scale bar represents 20μm. PV, Portal vein

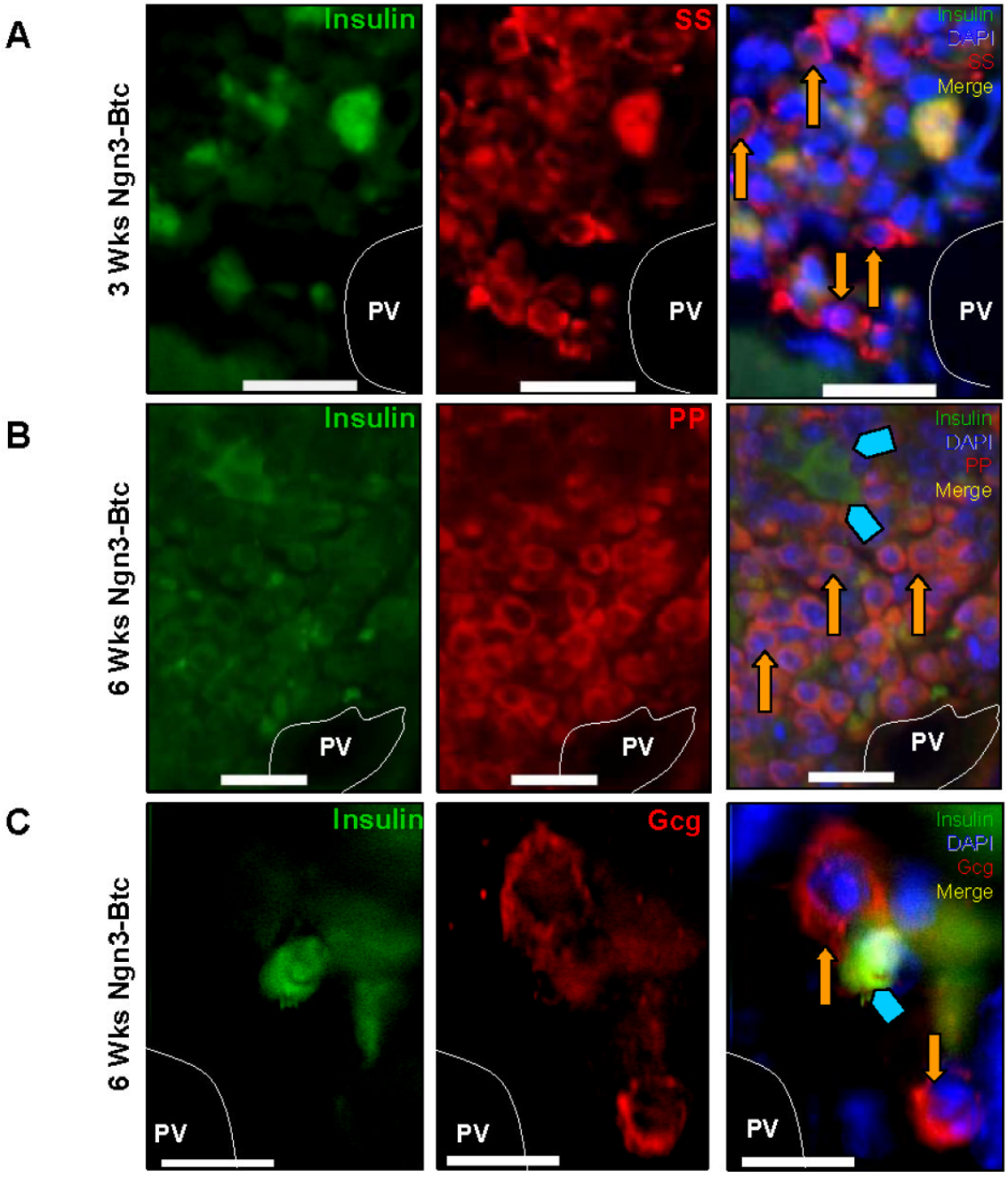

Figure 4. Ngn3-Btc-induced neo-islets express individual islet hormones

A-C: Representative sections of Ngn3-Btc treated STZ-diabetic mouse liver stained by IF for insulin – left panels; pancreatic polypeptide (PP), somatostatin (SS) or glucagon (Gcg) - middle panels; or merged images - right panels. Individual hormone-producing cells can be seen for each of the hormones (blue arrows for insulin and orange arrows for others). Scale bar represents 20μm in A-B and 10μm in C.

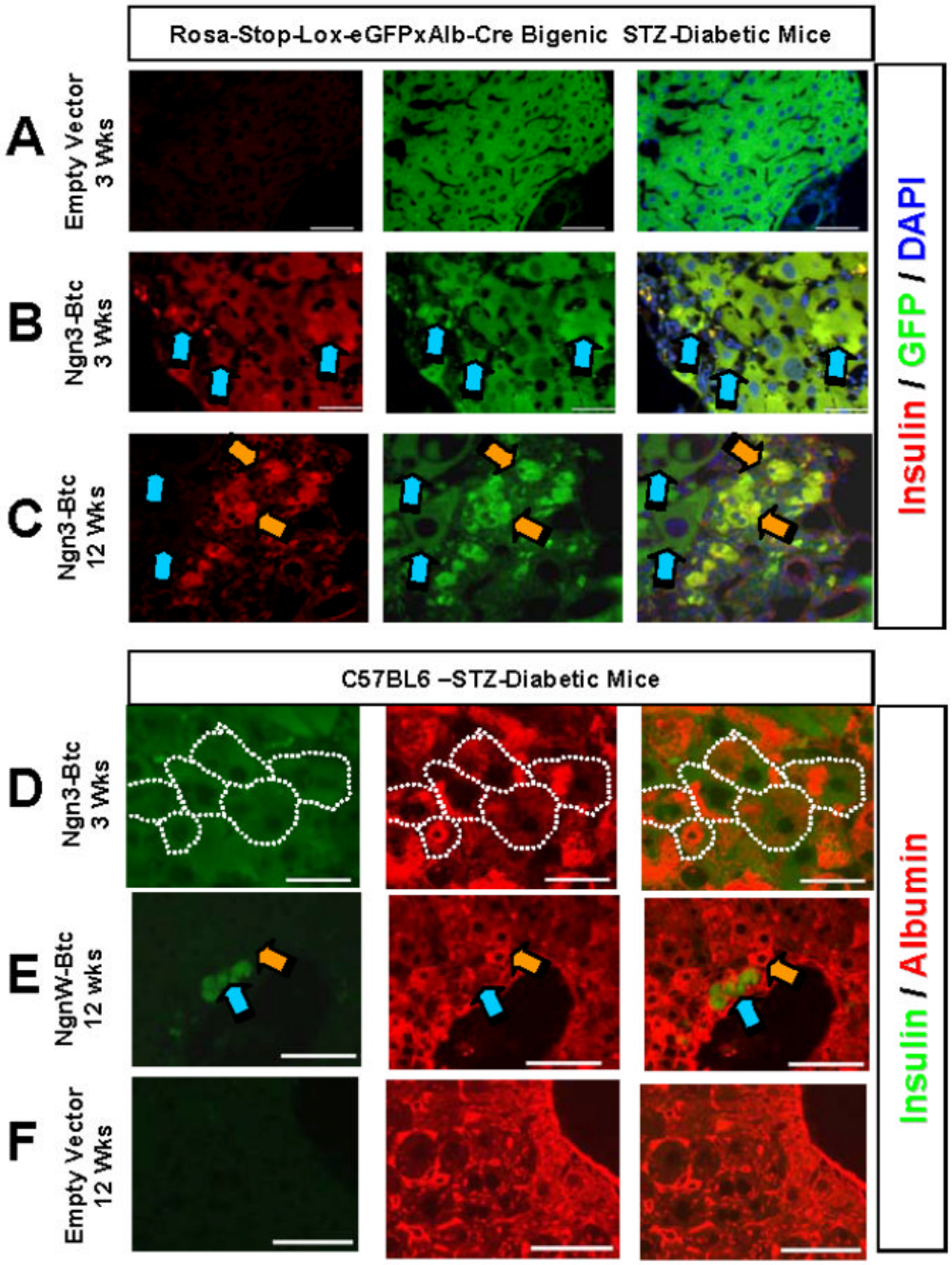

Figure 5. Lineage tracing demonstrates that cells expressing the first wave of insulin come from hepatocytes and the second wave from periportal oval cells

A-C: Representative sections of STZ-diabetic bi-genic (Rosa26-Stop-Lox-eGFP × Albumin-Cre) mouse liver stained for insulin (left panel) and GFP (middle panel) or merged with DAPI (right panel). Green GFP staining indicates albumin-expressing lineage cells. Of note, the parenchymal hepatocytes co-express insulin and GFP at 3 weeks (B, blue arrows), but no insulin is seen in these GFP-positive hepatocytes at 12 weeks (C, blue arrows). The neo-islets (C, orange arrows) co-express insulin and GFP.

D-F: Representative sections of STZ-diabetic wild type C57/BL6 mouse liver immunostained for insulin and albumin treated with Ngn3-Btc or empty vector. All parenchymal hepatocyte

expressing insulin in panel D (left) also co-express immunoreactive albumin (middle) in the same cell though to varying degrees as seen in the outlined cells, while the insulin-expressing neo-islets in panel E do not. Orange arrow points to albumin-only expressing cell, while the blue arrow points to an insulin-only producing cell. Scale bar represents 50μm.

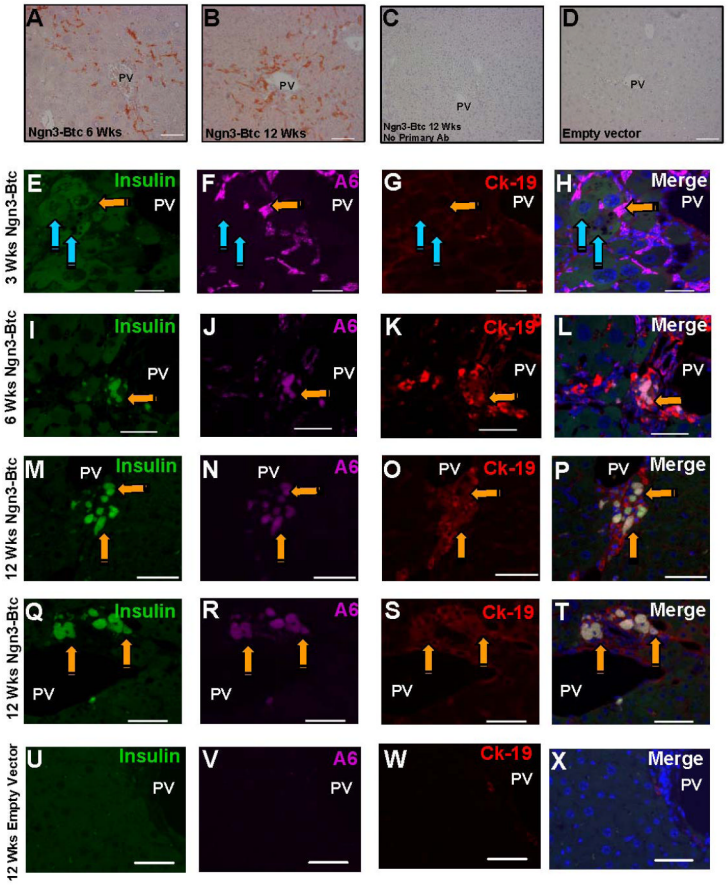

Figure 6. Ngn3-Btc-induced periportal neo-islets originate from oval cells

A-D: Periportal clusters of orange-red staining A6 positive oval cells by immunohistochemistry in Ngn3-Btc treated diabetic livers (A-B), but none with empty vector-treatment (D). No primary antibody control (C) is shown for reference. Scale bar represents 50μm. PV, Portal vein.

E-X: Representative sections of Ngn3-Btc (E-T) and empty vector (U-X) treated STZ-diabetic mouse livers - immunostained for insulin (green – left panels E, I, M, Q, U), oval-specific antigen A6 (pseudo-colored magenta – panels F, J, N, R, V), CK-19, an oval cell and biliary epithelial antigen (red – panels G, K, O, S, W) and the merged images with DAPI (right panels – H, L, P, T, X). Insulin positive hepatocytes do not co-express A6 or CK-19 (blue arrows). Oval cells do not co-express insulin at 3 weeks after Ngn3-Btc (E-H orange arrows), but do so at later time points (I-T, orange arrows). No insulin or A6 positive cell is seen with empty vector treatment, while CK-19 positive biliary cells are seen as expected. (U-X). Scale bar represents 50μm.

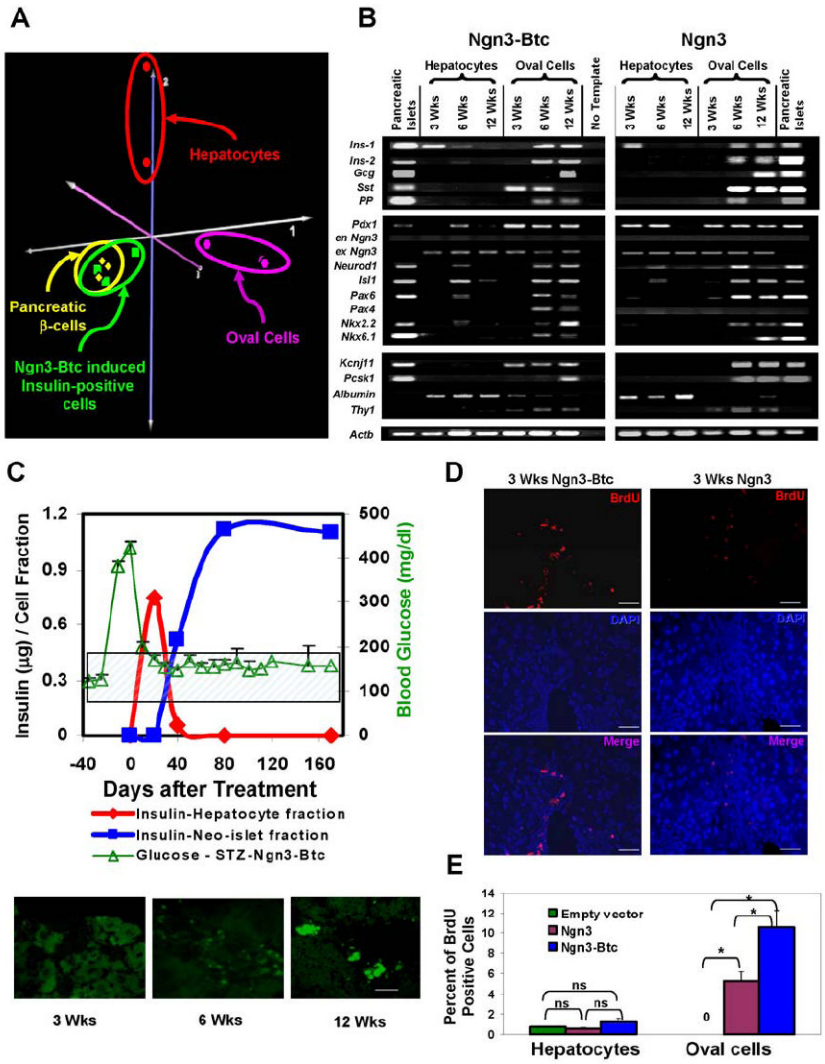

Figure 7. Ngn3 induces islet neogenesis via transdetermination of oval cells and not transdifferentiation of hepatocytes

A: Correspondence Analysis reveals that the Ngn3-Btc-induced insulin-positive cells cluster with mature pancreatic β-cells much more closely than with chemical diet-induced oval cells or normal hepatocytes (details in Supplemental Text).

B: RT-PCR of LCM- RNA from Ngn3-Btc (left) and Ngn3-only (right) treated STZ-diabetic mouse livers and from nondiabetic pancreatic islets. All are 40 cycles except for Ins1, Ins2, Albumin and Actb (32 cycles).

C: Estimated insulin content of the parenchymal hepatocyte and the neo-islet fractions (left Y-axis) and the blood glucose of the Ngn3-Btc-treated group (right Y-axis) and normal range (hatched area) (from Fig. 1A). Note the concordance of the estimated insulin content (upper panel) with the insulin immunostaining predominantly in the parenchymal hepatocytes at 3 weeks and in the periportal neo-islets at 12 weeks (lower panels).

D-E: BrdU staining (D) shows significant proliferative activity in the periportal oval cells as compared to the neighboring hepatocytes more so with Ngn3-Btc (left) than with Ngn3-only (right) 3 weeks after treatment. The quantitation of BrdU labeling is shown (E).

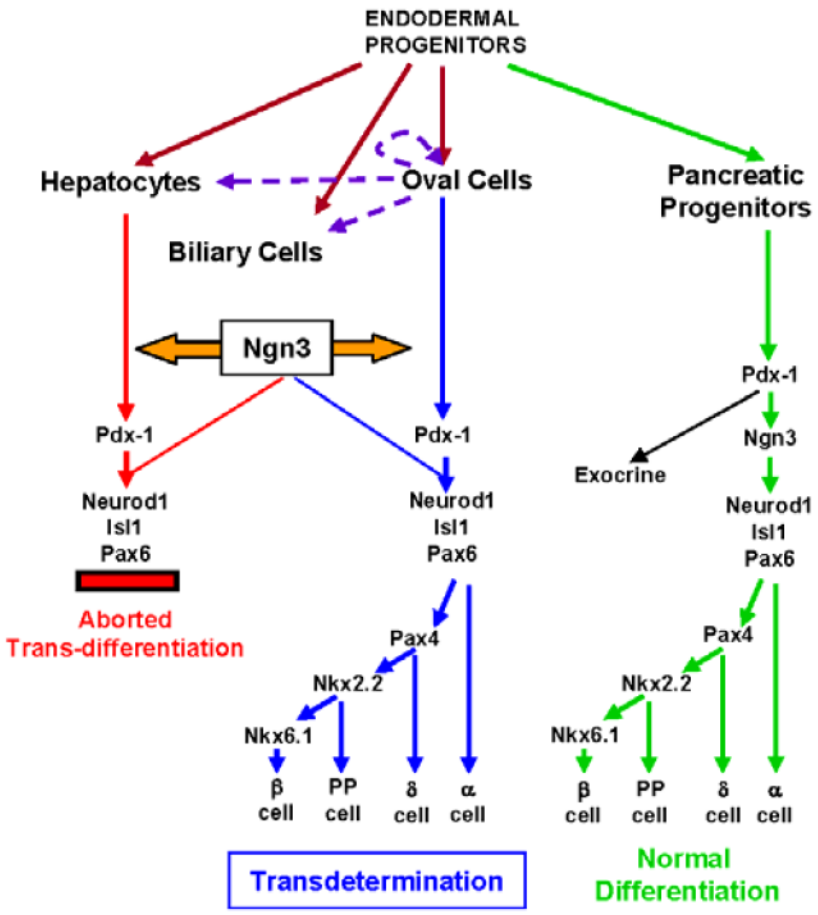

Figure 8. Model for Ngn3-induced transdetermination of hepatic oval cells

Ngn3 induces transdetermination in oval cells (in blue) and an aborted transdifferentiation in Ngn3-transduced parenchymal hepatocytes (in red). Normal islet differentiation cascade is also shown (in green). Normally, oval cells give rise to hepatocytes and biliary cells (interrupted lines). The origin of hepatocytes and biliary cells from the common endodermal progenitors is also shown (in brown).