Reversal of autoimmunity by mixed chimerism enables reactivation of β cells and transdifferentiation of α cells in diabetic NOD mice

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Type 1 diabetes (T1D) results from the autoimmune destruction of β cells, so cure of firmly established T1D requires both reversal of autoimmunity and restoration of β cells. It is known that β cell regeneration in nonautoimmune diabetic mice can come from differentiation of progenitors and/or transdifferentiation of α cells. However, the source of β cell regeneration in autoimmune nonobese diabetic (NOD) mice remains unclear. Here, we show that, after reversal of autoimmunity by induction of haploidentical mixed chimerism, administration of gastrin plus epidermal growth factor augments β cell regeneration and normalizes blood glucose in the firmly established diabetic NOD mice. Using transgenic NOD mice with inducible lineage-tracing markers for insulin-producing β cells, Sox9$^+$ ductal progenitors, Nestin$^+$ mesenchymal stem cells, and glucagon-producing α cells, we have found that both reactivation of dysfunctional low-level insulin expression (insulin^{lo}) β cells and neogenesis contribute to the regeneration, with the latter predominantly coming from transdifferentiation of α cells. These results indicate that, after reversal of autoimmunity, reactivation of β cells and transdifferentiation of α cells can provide sufficient new functional β cells to reach euglycemia in firmly established T1D.

beta cell regeneration | beta cell differentiation | autoimmune diabetes | gastrin | epidermal growth factor

Type 1 diabetes (T1D) results from the autoimmune destruction or dysfunction of insulin-producing β cells, leading to insufficient insulin production and hyperglycemia (1, 2). Cure of autoimmunity can prevent or reverse new-onset T1D. Many regimens have been reported to prevent T1D in mice, including anti-CD3 treatment and induction of major histocompatibility complex (MHC)-mismatched mixed chimerism (3–6). Dozens of regimens have also been reported to reverse new-onset T1D in nonobese diabetic (NOD) mice (7, 8). Currently, however, no regimen can cure autoimmunity and prevent T1D in humans, even though anti-CD3 treatment delayed T1D onset in patients with autoantibodies (9, 10); immunological modulation by LFA-3Ig (Alefacept) ameliorated new-onset T1D (11, 12); and autologous hematopoietic cell transplantation (HCT) ameliorated new-onset T1D in patients with low-level autoimmunity but not in patients with a high level of autoimmunity (13–15). Induction of haploidentical mixed chimerism (Haplo-MC) represents a curative approach for autoimmunity in firmly established diabetic T1D mice (16). Haploidentical HCT (Haplo-HCT) has been widely used for treating nonmalignant hematological disorders (17), and induction of Haplo-MC prevents the side effect of graft versus host disease (GVHD) (4, 8, 16, 18–20). GVHD is often associated with complete chimerism in classical HCT for treating malignant hematological diseases (21).

Reversal of autoimmunity and replacement or regeneration of insulin-producing β cells is required for cure of firmly established T1D (22). So far, however, only combination therapy of induction of MHC-mismatched mixed chimerism and administration of gastrin and epidermal growth factor (EGF) and T1D-relevant pMHC class II-coated nanoparticles (NPs) have been reported to cure firmly established T1D in the NOD mouse model (18, 23). Although islet transplantation under combined immunosuppressant treatment renders a portion of patients insulin independent, the effective time is limited to 3 y to 5 y due to chronic rejection mediated by alloimmunity and autoimmunity (24). In addition, one patient requires islets from two to three donors to reach insulin independence, leading to severe shortage of islet donors (25). Production of β cells from induced pluripotent stem cells (iPSCs) may become an unlimited source of β cells (26). However, the existing autoimmunity in T1D patients remains a hurdle, even with modified β cell expression of HLA that can avoid allorejection (27).

Significance

Cure of autoimmune type 1 diabetes (T1D) requires both reversal of autoimmunity and regeneration or resupply of insulin-producing β cells. We have observed that combination therapy with induction of haploidentical mixed chimerism and administration of gastrin and epidermal growth factor (EGF) cures firmly established T1D. The predominant source of β cell regeneration in mice comes from reactivation of dysfunctional insulin^{lo} β cells and transdifferentiation of α cells. These studies have provided insights into β cell regeneration mechanisms in firmly established autoimmune T1D, in particular, reactivation of the insulin^{lo} β cells after reversal of autoimmunity by induction of haploidentical mixed chimerism. These studies also provide a preclinical scientific basis for the feasibility of cure of long-standing T1D in humans.

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The authors declare no competing interest.

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The loss of insulin production in T1D may result from destruction, dysfunction, and dedifferentiation of β cells mediated by autoimmunity (28, 29). β cell regeneration in nonautoimmune mice can come from differentiation of $Sox9^+$ pancreatic ductal progenitors (30) and $Nestin^+$ mesenchymal stem cells (31) as well as transdifferentiation of α cells, δ cells, and acinar cells (32–42). The transdifferentiation of pancreatic ductal cells can be induced by transfection of Ngn3, Pdx1, and MafA (43); transdifferentiation of α cells can be induced by transfection of Pdx1 and MafA (36); and transdifferentiation of acinar cells can be induced by transfection of Ngn3, Pdx1, and MafA (40). β cell dedifferentiation is now thought to be an important mechanism of β cell functional failure (28). Recent reports show that firmly established T1D patients have different types of residual β cells, in particular, dedifferentiated or dysfunctional "sleeping" low-level insulin expression ($insulin^{lo}$) β cells (44–46). The dedifferentiation process may be a self-protection mechanism in response to autoimmune attack against insulin-producing β cells (29). However, the role of differentiation of progenitors, transdifferentiation of α cells, and redifferentiation or reactivation of β cells in β cell regeneration in autoimmune mouse and patients remains unclear.

Gastrin is a peptide hormone produced by G cells in the pyloric antrum of the stomach, duodenum, and pancreas. Gastrin is also expressed by α and β cells in the islets of diabetic rodents and humans (47). Gastrin in combination with other factors such as transforming growth factor alpha, EGF, or glucagon-like peptide-1 is capable of increasing β cell mass and lowering blood glucose levels in adult diabetic mice (48). Gastrin exerts its effect on transcription, at least in part, through gastrin receptor cholecystokinin B receptor (CCKBR) (48, 49). CCKBR is expressed in both somatostatin-expressing δ cells and glucagon-producing α cells in islets (48, 50, 51).

EGF is a ligand of EGF receptor and acts as a growth factor for embryonic pancreatic epithelial cells. EGF can activate the proliferation of immature epithelial cells, thus allowing an increase in the pool of precursor cells (52, 53). Although in vivo administration of gastrin or EGF alone was not able to effectively reverse new-onset T1D in NOD mice, combination of gastrin and EGF (GE) reversed new-onset T1D by reducing insulitis and increased pancreatic β cell mass (54). However, augmenting endogenous production of gastrin by administration of dipeptidyl peptidase-4 inhibitors in combination with proton pump inhibitors was not able to increase production of C peptide in firmly established T1D patients, possibly due to preexisting autoimmunity (55). Consistently, we observed that administration of GE together has augmented differentiation of $Sox9^+$ pancreatic ductal progenitor into insulin-producing β cells in streptozotocin (STZ)-induced diabetic nonautoimmune mice with blood glucose between 300 mg/dL and 450 mg/dL (30). Combination therapy of induction of MHC-mismatched mixed chimerism and administration of GE augmented β cell regeneration in NOD mice with firmly established T1D (18). However, the source of β cell regeneration in those firmly established autoimmune mice remains unknown.

We have developed a radiation-free conditioning regimen for induction of Haplo-MC in mouse models, in which recipients are first conditioned with antithymocyte globulin (ATG) and a low-dose of cyclophosphamide (CY) and pentostatin (PT), and then transplanted with $CD4^+$ T cell-depleted hematopoietic graft (16, 20). This regimen shows minimal toxicity in mice and is now under phase I safety clinical trial enrolling patients with sickle cell disease (56). In the current study, with this clinically applicable conditioning regimen for induction of Haplo-MC, we have observed that 1) combination therapy of induction of Haplo-MC and administration of GE cures firmly established T1D with augmentation of β cell regeneration, 2) reactivation of β cells and transdifferentiation of α cells contribute predominantly to the β cell regeneration, and 3) GE appears to be required for triggering reactivation of β cells but not for triggering transdifferentiation of α cells.

Results

Combination Therapy of Induction of Haplo-MC and Administration of Gastrin and EGF Cures Firmly Established T1D. New onset was defined as blood glucose above 300 mg/dL for consecutive 3 d. Firmly established diabetic NOD mice were 3 wk after onset, and all had blood glucose above 500 mg/dL. Firmly established diabetic NOD mice with blood glucose higher than 500 mg/dL were treated by induction of Haplo-MC and daily intraperitoneal injection (i.p. injection) of gastrin (3 μg/kg) and EGF (1 μg/kg) for 60 d, as described in our previous publication (18) and depicted in Fig. 1*A*.

Haplo-MC was induced through a clinically applicable conditioning regimen of ATG + CY + PT and infusion of $CD4^+$ T cell-depleted spleen ($CD4^-$-SPL) and bone marrow (BM) from haploidentical ($H\text{-}2^{g7/s}$) F1 donors, as described in our previous publications (16, 20). The firmly established diabetic NOD mice developed stable Haplo-MC with both donor- and host-type T, B, and myeloid cells in the peripheral blood, spleen, BM, and thymus (*SI Appendix*, Fig. S1 *A* and *B*). The Haplo-MC NOD mice showed no signs of GVHD as indicated by steady body weight curve (*SI Appendix*, Fig. S1*C*), and normal percentage of donor-type $CD4^+CD8^+$ thymocytes, although with reduction of host-type $CD4^+CD8^+$ thymocytes (*SI Appendix*, Fig. S1*B*), as well as little infiltration in the GVHD target tissues liver and lung (*SI Appendix*, Fig. S1*D*). Due to donor cell-mediated deletion of host-type cells (16), reduction of sialitis in the salivary gland was seen, as compared to mice given conditioning alone (*SI Appendix*, Fig. S1*D*). Interestingly, there was an expansion of donor-type but not host-type $Foxp3^+$ regulatory T cells that expressed amphiregulin ($Areg^+$) in the Haplo-MC NOD mice (*SI Appendix*, Fig. S2). $Areg^+$ Treg cells were previously reported to augment tissue regeneration (57, 58).

Concerned about the negative impact of blood glucose on the efficacy of the treatment, we divided the firmly established diabetic mice into two groups: one with starting blood glucose 500 mg/dL to 600 mg/dL and the other >600 mg/dL. Treatment with conditioning + GE or induction of Haplo-MC alone was not able to reverse hyperglycemia at all, regardless of the initial blood glucose levels (Fig. 1*B*). However, with combination therapy, 10/14 of mice with initial blood glucose 500 mg/dL to 600 mg/dL became normoglycemic, although only 2/13 of mice with initial blood glucose >600 mg/dL showed normoglycemia (Fig. 1*B*). The euglycemic mice after the combination therapy were able to normalize blood glucose within 120 min after injection of glucose during fasting i.p. glucose tolerance test (IPGTT), with a threefold spike in insulin production 10 min after glucose administration (Fig. 1 *C* and *D*). Although these mice had delayed recovery compared to 6-wk-old NOD mice, their results were remarkably better than that of mice from the other groups, including mice with no reversal of hyperglycemia after the combination therapy (Fig. 1 *C* and *D*). None of the mice given conditioning + GE alone and mice given induction of mixed chimerism alone were able to normalize blood glucose at 120 min, and they had minimal increase in serum insulin. Of note, however, is that mice with no reversal of hyperglycemia after the combination therapy had a statistically significant increase in serum insulin, although they failed to normalize their blood glucose (Fig. 1 *C* and *D*).

Reversal of hyperglycemia in firmly established diabetic NOD mice after the combination therapy was associated with the presence of intact islets and marked increase in insulin-producing β cells as well as elimination of insulitis (Fig. 1 *E* and *F*). These results indicate that both reversal of autoimmunity by induction of Haplo-MC and β cell regeneration by stimulation of GE are

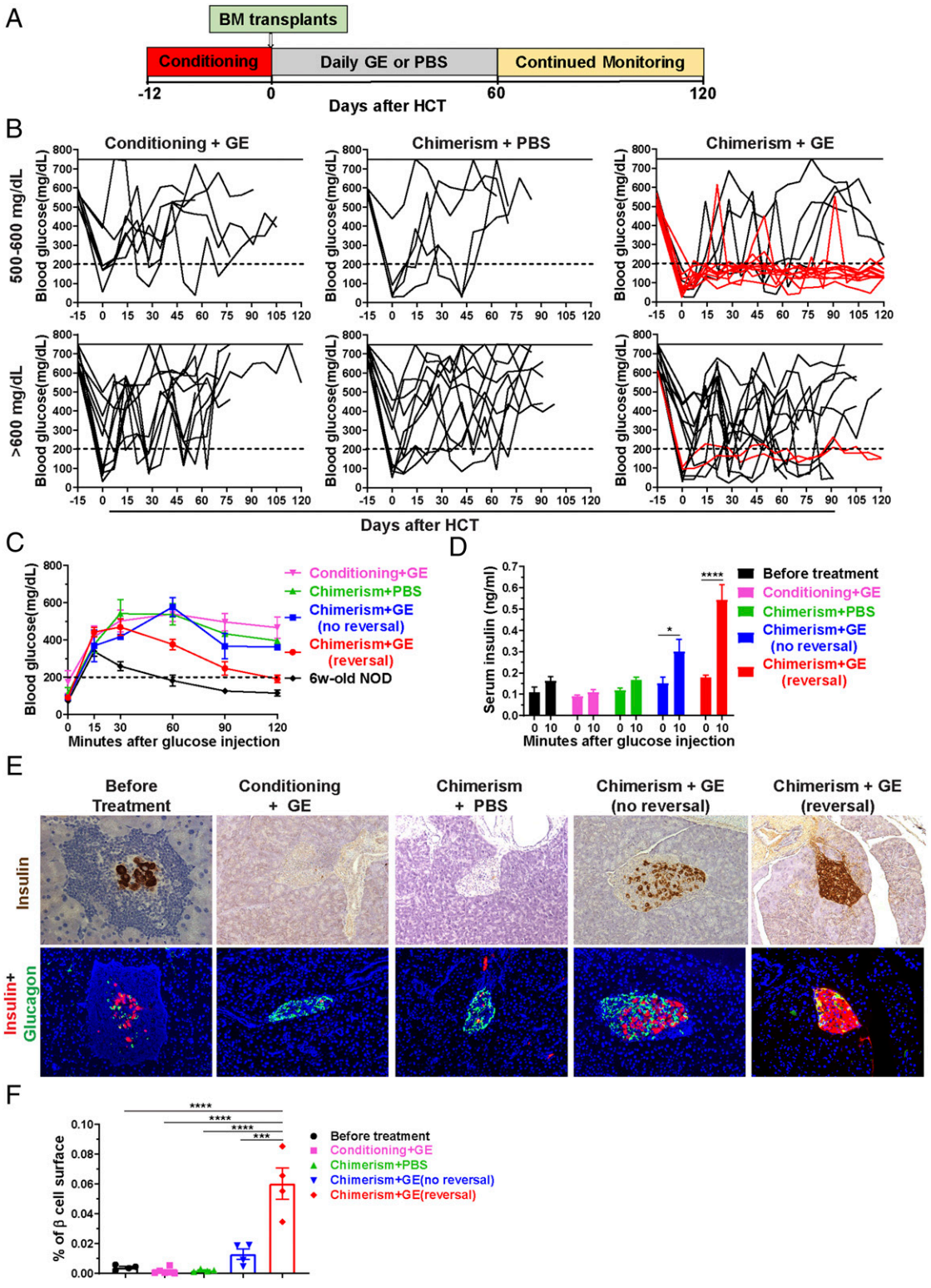

Fig. 1. Combination therapy with induction of Haplo-MC and administration of GE results in reversal of firmly established T1D. (*A*) Experimental scheme. Firmly established diabetic (3 wk after onset) NOD mice (blood glucose level of >500 mg/dL) were given conditioning for 12 d and HCT on day 0 for induction of Haplo-MC. One day after HCT, mice were given daily i.p. injection of gastrin (3 mg/kg) and EGF (1 mg/kg) for 60 d. Insulin pellet or insulin (1 U) was given to help control blood glucose to be below 500 mg/dL. (*B*) (*Upper*) Curves of blood glucose levels of mice with initial blood glucose of 500 mg/dL to 600 mg/dL of different groups, including conditioning + GE ($n = 6$), chimerism+ phosphate-buffered saline (PBS) ($n = 5$), and chimerism + GE ($n = 14$) groups. (*Lower*) Curves of blood glucose levels of mice with initial blood glucose greater than 600 mg/dL of different groups, including conditioning + GE ($n = 10$), chimerism+ PBS ($n = 10$), and chimerism + GE ($n = 13$) groups. Red lines: reversal recipients. (*C* and *D*) At 120 d after treatment, mice were given IPGTT. Curves of blood glucose levels during IPGTT ($n = 4$ to 6) and serum insulin levels ($n = 5$) were measured and shown as mean ± SEM. (*E*) Representative immunohistochemistry (IHC) staining patterns for insulin (brown) and immunofluorescent (IF) staining patterns for insulin (red)/glucagon (green). One representative photomicrograph (original 20×) is shown of four to six replicate experiments. (*F*) Mean ± SEM of percentage of β cell surface among total pancreatic tissue surface ($n = 4$ to 6). ***$P < 0.001$, ****$P < 0.0001$.

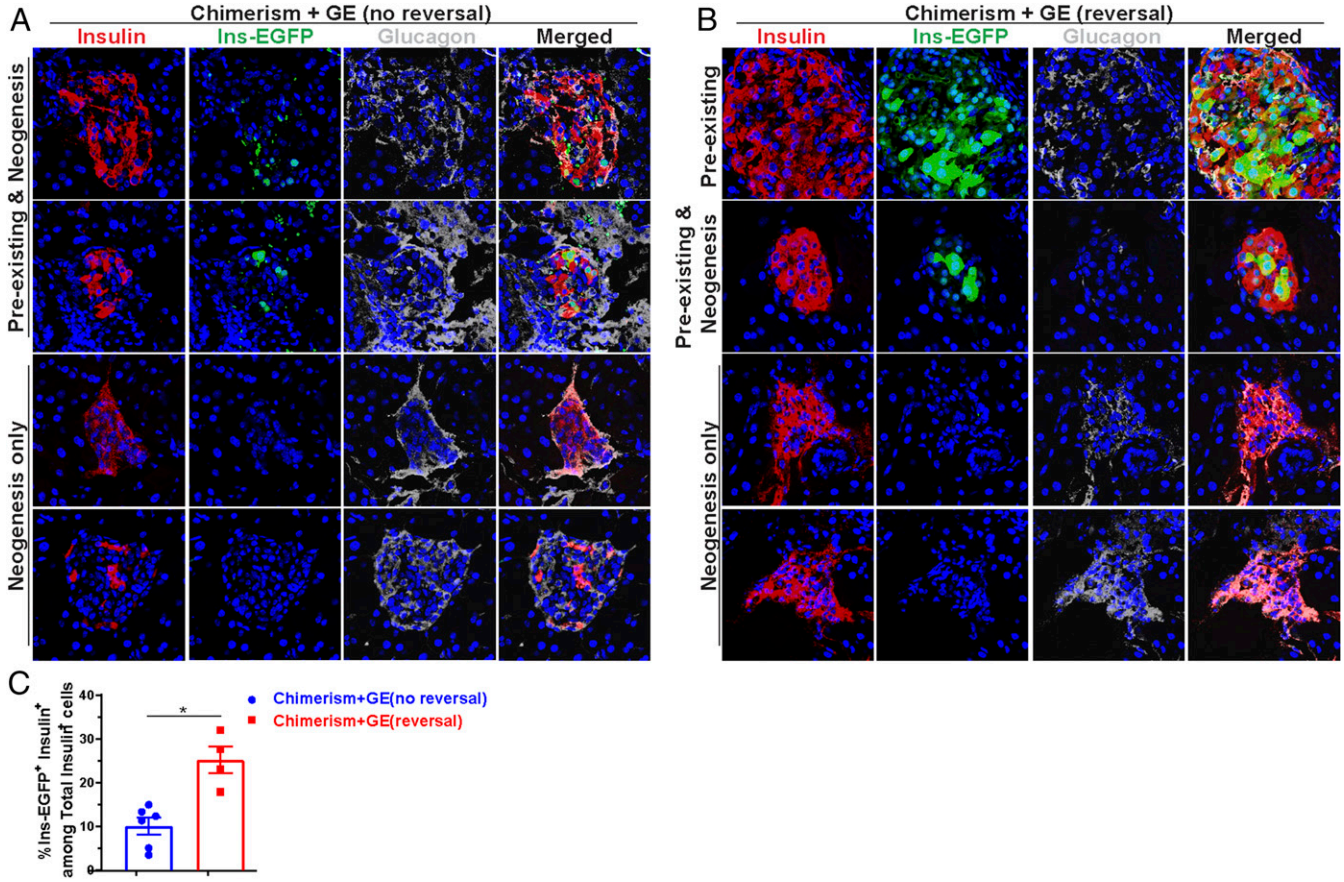

Fig. 2. Administration of GE augments both β cell neogenesis and expansion of preexisting β cells in chimeric firmly established diabetic Ins-EGFP NOD mice. The 10-wk-old RIP$^{Cre-ERT}$R26^{EGFP} NOD mice were injected with tamoxifen (20 mg per mouse) to label preexisting β cells. Haplo-MC was induced in the firmly established diabetic (3 wk after onset) RIP$^{Cre-ERT}$R26^{EGFP} NOD mice in combination with GE administration. Pancreata were harvested at terminal collection and stained for insulin (red), Ins-EGFP (green), glucagon (white), and DAPI (blue). Representative islet staining patterns (original magnification 400×) from (*A*) no reversal recipients and (*B*) reversal recipients are shown. (*C*) Mean ± SEM percentage of Ins-EGFP$^+$Insulin$^+$ cells among total insulin$^+$ cells (n = 4 to 6). *$P < 0.05$.

required for cure of firmly established diabetes in autoimmune NOD mice.

Both Preexisting and De Novo-Generated β Cells Contribute to β Cell Regeneration in Firmly Established Diabetic NOD Mice after Combination Therapy. We used lineage-tracing NOD mice with Insulin–Cre–ERT–EGFP to distinguish the origin of β cells in cured firmly established diabetic NOD mice after the combination therapy. Prediabetic (~10 wk old) NOD mice were used to check the labeling efficacy by i.p. injection of tamoxifen (20 mg per mouse) to label preexisting β cells. Greater than 95% of preexisting β cells were labeled with EGFP (*SI Appendix*, Fig. S3 *A* and *B*). Next, firmly established diabetic NOD mice with EGFP-labeled β cells were given the combination therapy as described in Fig. 1*A*. The pancreata of mice with or without normalization of blood glucose after the combination therapy were examined. There were many islets with insulin$^+$ β cells in mice treated with the combination therapy (Fig. 2). Among them, mice without reversal of hyperglycemia had only a few Insulin–EGFP$^+$ Insulin$^+$ preexisting β cells in the islets (Fig. 2*A*); however, mice with reversal of hyperglycemia not only had islets containing Insulin–EGFP$^+$ Insulin$^+$ preexisting β cells, but also had islets consisting of predominantly de novo-generated Insulin–EGFP$^-$ Insulin$^+$ β cells (Fig. 2*B*). The percentage of Insulin–EGFP$^+$ Insulin$^+$ β cells among total insulin$^+$ β cells was ~10% in mice without reversal of hyperglycemia and ~25% in mice with reversal of hyperglycemia (Fig. 2*C*). These results indicate that both reactivation of preexisting β cells and neogenesis of β cells contribute to regeneration of β cells in firmly established diabetic NOD mice treated with the combination therapy of induction of Haplo-MC and administration of GE.

Dedifferentiation/Dysfunction of Insulin^{lo} β Cells Progresses in NOD Mice with Age and T1D Progression. Dedifferentiation of β cells to cells expressing little or low levels of insulin (insulin^{lo} β cells) is now known to be an important mechanism of β cell dysfunction (28). Dedifferentiated dysfunctional insulin^{lo} β cells exist in prediabetic NOD mice (29) and in T1D patients (45). Although it is difficult to find insulin-producing β cells in 3-wk firmly established diabetic NOD mice, about 1/4 of the insulin-producing β cells in mice with reversal of T1D after the combination therapy were from preexisting β cells (Fig. 2), suggesting they may have arisen from insulin^{lo} β cells. Accordingly, we compared insulin^{lo} β cells in 6-wk-old prediabetic, 12-wk-old prediabetic, new-onset, and 3-wk firmly established diabetic NOD mice, using prolonged exposure time during immunofluorescence microscopy, as described by Kushner and coworkers (45) with human islets. We used glucagon staining to identify islets and observed that there were mainly insulin^{hi} β cells in prediabetic NOD mice, a mixture of insulin^{hi} β cells and insulin^{lo} β cells in the new-onset mice, and only insulin^{lo} cells in the 3-wk firmly established diabetic NOD mice (Fig. 3*A*). The insulin^{lo} β cells from firmly established diabetic NOD mice expressed β cell lineage nuclear factors Nkx6.1 and Pdx1, but not β cell maturation marker Ucn3 (Fig. 3*B*). These results suggest that

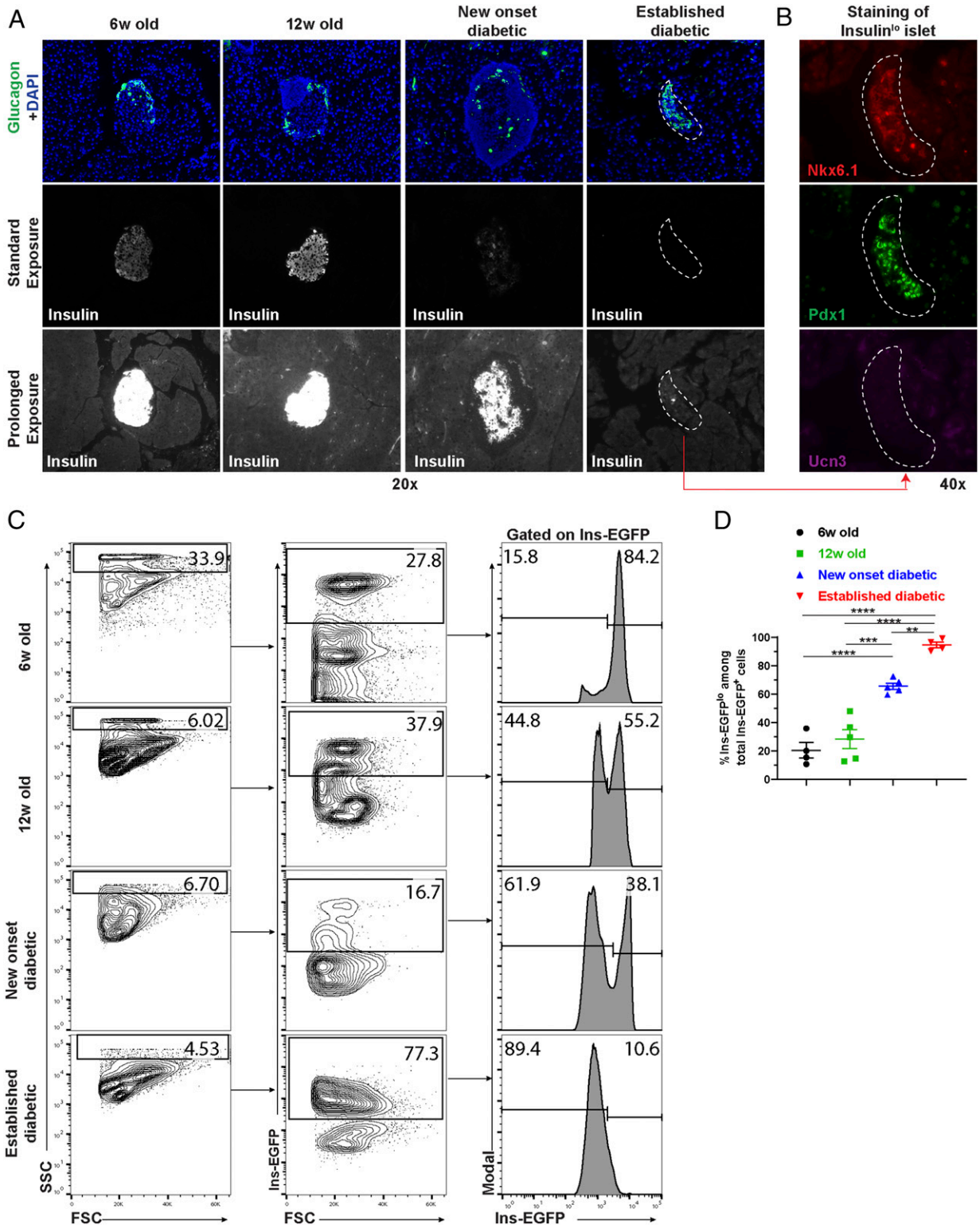

Fig. 3. Insulin^{lo} β cells increase as T1D progress in NOD mice. The pancreata from 6-wk-old, 12-wk-old, new-onset, and firmly established diabetic (3 wk after onset) NOD mice were harvested and stained for insulin (white), glucagon (green), and DAPI (blue). Insulin^{lo} cells were defined as having distinct insulin expression under prolonged exposure (1,000 ms) but not standard (50 ms) exposure. (*A*) One representative islet for each group is shown. (*B*) Insulin^{lo} cells were stained for insulin (white), Nkx6.1 (red), Pdx1 (green), and Ucn3 (purple), and one representative staining pattern is shown for three replicate experiments. (*C*) Islets from 6-wk-old, 12-wk-old, new-onset, and firmly established diabetic (3 wk after onset) RIP$^{Cre-ERT}$R26^{EGFP} NOD mice were analyzed by flow cytometry and shown in forward scatter (FSC) versus side scatter (SSC). SSC^{hi} cells were then gated and shown in FSC versus Ins-EGFP. The Ins-EGFP^{+} cells were identified as conditionally labeled β cells. Ins-EGFP^{+} expression was further displayed as a histogram and subdivided into two distinct populations. Percentage of either Ins-EGFP^{hi} or Ins-EGFP^{lo} β cells among total Ins-EGFP^{+} β cells is displayed. Representative FACS profiles are shown for one of four to five replicate experiments. (*D*) Quantification of the percentage of Ins-EGFP^{lo} β cells among total Ins-EGFP^{+} β cells. Mean ± SEM is shown ($n = 4$ to 5 mice per group). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

there are dedifferentiated insulin^{lo} β cells in firmly established diabetic NOD mice.

We also used the transgenic Insulin–EGFP NOD mice to directly analyze the percentage of Insulin^{hi} and Insulin^{lo} β cells. With EGFP as the indicator for insulin, we could quantify the percentage of Insulin–EGFP^{lo} and Insulin–EGFP^{hi} β cells in the islets of prediabetic, new-onset, and firmly established diabetic NOD mice using flow cytometry analysis. From 6- and 12-wk-old prediabetic NOD mice to new-onset and 3-wk firmly established diabetic mice, there was a gradual reduction in Insulin–EGFP^{hi} β cells from ∼80% to 72%, 32.5%, and 5%; in contrast, there was a gradual increase of Insulin–EGFP^{lo} β cells from 20% to 28%, 67.5%, and 95% (Fig. 3 *C* and *D*).

A previous report showed that immature insulin^{lo} and mature insulin^{hi} β cells could be separated by forward scatter versus side scatter plotting in flow cytometry due to differences in cell size and granularity. Insulin^{hi} cells had high side scatter, whereas insulin^{lo} cells had low side scatter (29). We found that, while this observation held true for prediabetic mice, it was no longer the case in firmly established diabetic mice in which both side scatter high and low populations contained only insulin^{lo} cells (*SI Appendix*, Fig. S4). Additionally, in STZ-induced diabetic nonautoimmune C57BL/6 mice, we observed few insulin^{lo} β cells (*SI Appendix*, Fig. S5). These results validate that there is a progressive dedifferentiation of β cells in diabetic NOD mice as T1D progresses, and almost all residual β cells in firmly established diabetic NOD mice have become dedifferentiated insulin^{lo} β cells.

Reactivation of Insulin^{lo} into Insulin^{hi} β Cells Contributes to β Cell Regeneration in Firmly Established Diabetic NOD Mice after Combination Therapy. We tested whether combination therapy of induction of Haplo-MC and administration of GE could augment redifferentiation of dedifferentiated insulin^{lo} β cells. We also used glucagon^{+} cells to locate islets, followed by standard and prolonged exposure for insulin expression. We observed a near absence of insulin^{hi} β cells in the islets of 3-wk firmly established diabetic NOD mice before treatment and mice given induction of Haplo-MC alone. However, we did observe an increase of frequency of insulin^{hi} β cells in the mice given combination therapy, in particular, in the mice with reversal of hyperglycemia (Fig. 4 *A* and *B*).

We also used transgenic Insulin–EGFP NOD mice to quantify the percentage of Insulin–EGFP^{hi} and Insulin–EGFP^{lo} β cells via flow cytometry. As compared to before treatment, induction of Haplo-MC alone increased Insulin–EGFP levels and increased the percentage of Insulin–EGFP^{hi} cells among the residual β cells (Fig. 4 *C*–*E*). As compared to Haplo-MC alone, the combination therapy of Haplo-MC + GE further up-regulated Insulin–EGFP levels and increased the percentage of Insulin–EGFP^{hi} cells, particularly in the recipients with reversal of hyperglycemia (Fig. 4 *C*–*E*). On the other hand, as compared to before treatment, Haplo-MC alone reduced and GE treatment further reduced the percentage of Insulin–EGFP^{lo} cells (Fig. 4 *C*–*E*). These results indicate that reversal of autoimmunity by Haplo-MC and administration of GE synergistically augment reactivation of dysfunctional β cells in the firmly established diabetic NOD mice.

Differentiation of Sox9^{+} Ductal Progenitors into β Cells Makes Minimal Contribution to β Cell Neogenesis in Firmly Established Diabetic NOD Mice after Combination Therapy, due to Loss of the Sox9^{+} Progenitors. As preexisting β cells only accounted for ∼1/4 of the islet β cells in cured firmly established diabetic NOD mice (Fig. 2*C*), 3/4 of the islet β cells in these mice should have arisen from neogenesis. We previously reported that STZ-induced nonautoimmune diabetic mice showed β cell neogenesis from Sox9^{+} pancreatic ductal progenitor cells after treatment with GE (30). Thus, we tested whether Sox9^{+} ductal progenitors gave rise to mature insulin-producing β cells in firmly established diabetic NOD mice after combination therapy, using inducible Sox9-EGFP lineage-tracing NOD mice. The EGFP-labeling efficiency of Sox9-EGFP^{+} cells was over 90% (*SI Appendix*, Fig. S3 *C* and *D*). We found that there were only scattered immature Insulin^{+}Glucagon^{+} β cells in the pancreatic islets of firmly established diabetic NOD mice treated with induction of Haplo-MC only, and <1% of the insulin^{+} cells were Sox9-EGFP^{+}(Fig. 5 *A* and *D*). After the combination therapy, there were clusters of insulin^{+} β cells in mice without reversal of hyperglycemia (Fig. 5 *B* and *D*) and intact islets of insulin^{+} β cells in mice with reversal of hyperglycemia (Fig. 5 *C* and *D*); however, there were still few Sox9-EGFP^{+} cells among the Insulin^{+} β cells, only 2 to 3%. These results indicate that, although GE treatment appears to augment differentiation of Sox9^{+} progenitors into β cells, those β cells account for a very small portion of the β cell neogenesis in firmly established diabetic NOD mice after combination therapy.

The Sox9^{+}CD133^{+}CD71^{+} cell population from the pancreas contain ductal progenitors, with ∼30% of these cells being actual pancreatic colony-forming units or progenitor cells (59). We tested whether the lack of β cell neogenesis from Sox9^{+} ductal progenitors in firmly established diabetic NOD mice was associated with a lack of Sox9^{+}CD133^{+}CD71^{+} cell population. We compared the percentage and yield of Sox9^{+}CD133^{+}CD71^{+} cell population in 12-wk-old prediabetic, new-onset, and 3-wk firmly established diabetic NOD mice with blood glucose 500 mg/dL to 600 mg/dL or >600 mg/dL. Although we did not observe a statistically significant difference in percentage of Sox9^{+}CD133^{+}CD71^{+} cells, the yield was significantly increased in new-onset diabetic NOD mice as compared with prediabetic mice. However, there was a marked reduction in both percentage and yield of Sox9^{+}CD133^{+}CD71^{+} cells in 3-wk firmly established diabetic NOD mice as compared to new-onset diabetic mice (*SI Appendix*, Fig. S6). Additionally, the percentage and yield of progenitors in the mice with blood glucose of >600 mg/dL trended lower than that of mice with blood glucose of 500 mg/dL to 600 mg/dL. These results indicate that short-term and medium levels of hyperglycemia stimulate expansion, but prolonged high levels (>500 mg/dL) of hyperglycemia cause loss of Sox9^{+} pancreatic ductal progenitors, and that pancreatic progenitors have limited capacity to differentiate into mature β cells in firmly established diabetic NOD mice with blood glucose above 500 mg/dL. This is consistent with our prior publication showing that GE treatment reversed medium hyperglycemia (300 mg/dL to 450 mg/dL) with augmentation of Sox9^{+} progenitors proliferations and differentiation into β cells (30).

Little Differentiation of Nestin^{+} Mesenchymal Stem Cells into β Cells Is Observed in Firmly Established Diabetic NOD Mice after Combination Therapy. It has been reported that Nestin^{+} mesenchymal stem cells isolated from adult pancreatic islets can differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes, and these Nestin^{+} islet-derived progenitor cells are a distinct population of cells that reside within pancreatic islets and may participate in the neogenesis of islet endocrine cells (31). It has also been reported that β cells can be derived from mesenchymal stem cells in diabetic mice, although this remains controversial (60, 61). Using Nestin-EGFP lineage-tracing mice with >90% labeling efficiency (*SI Appendix*, Fig. S3 *E* and *F*), we observed that, although cured firmly established diabetic NOD mice had islets of insulin^{+} β cells at different developmental stages, none of the insulin^{+} β cells appeared to be Nestin-EGFP^{+} (*SI Appendix*, Fig. S7). These results indicate that Nestin^{+} mesenchymal stem cells are not able to give rise to insulin-producing β cells in firmly established diabetic mice after combination therapy.

Transdifferentiation of α Cells into β Cells Is the Major Source of β Cell Neogenesis in Firmly Established Diabetic NOD Mice after Combination Therapy. Firmly established diabetic NOD mice have lost nearly all of their β cells, with islets containing mostly α cells (*SI Appendix*, Fig. S8). It is known that α cells can transdifferentiate into β cells

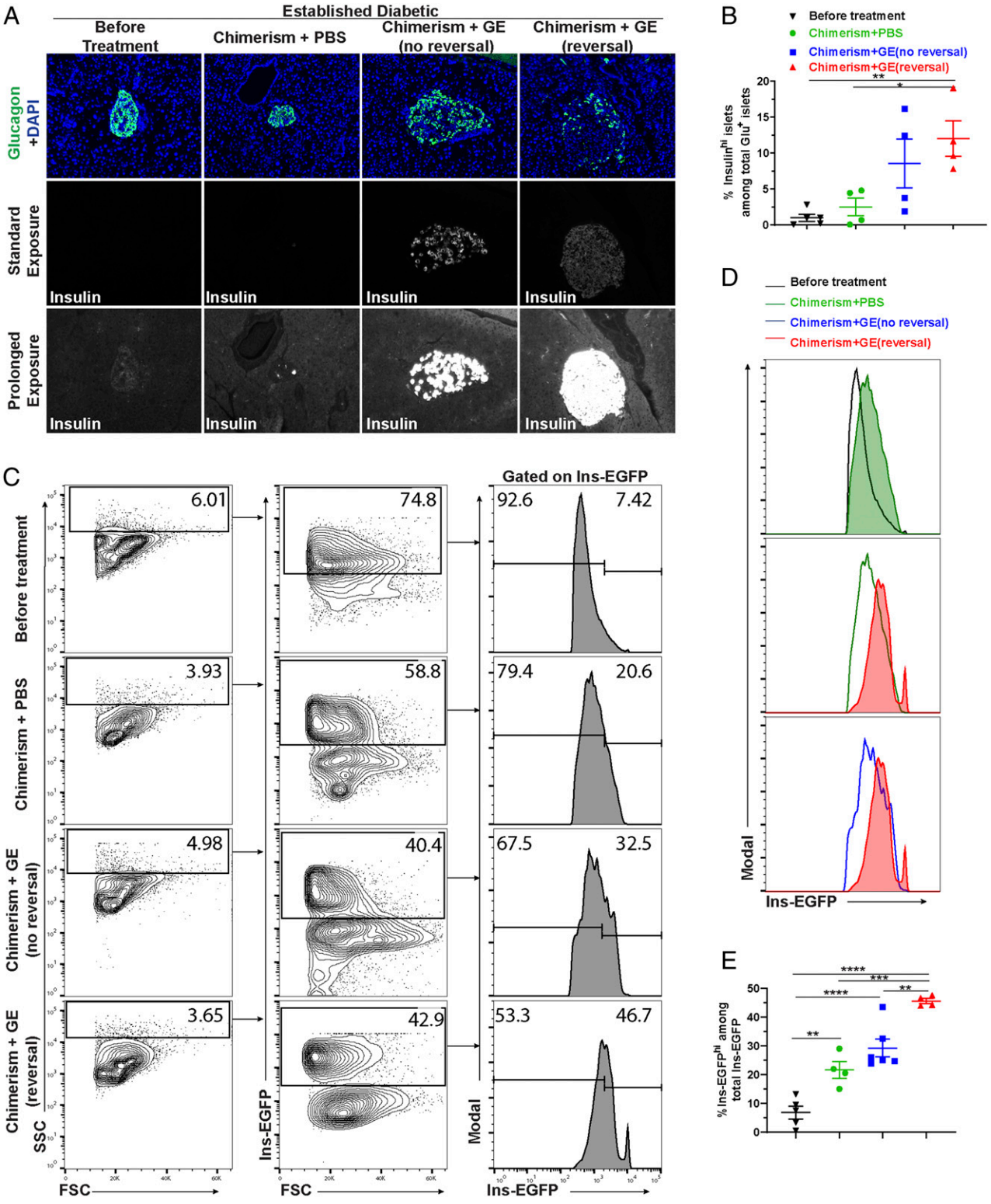

Fig. 4. Redifferentiation of insulin^{lo} β cells to insulin^{hi} after the combination therapy. The pancreata from firmly established diabetic (3 wk after onset) NOD before treatment and mice with treatment of Haplo-MC + phosphate-buffered saline (PBS), mice with treatment of Haplo-MC + GE and no reversal of hyperglycemia, and mice with treatment of Haplo-MC + GE and reversal of hyperglycemia were harvested for analyzing percentage of Insulin^{hi} and Insulin^{lo} β cells. (*A* and *B*) Pancreatic tissues were stained for insulin (white), glucagon (green), and DAPI (blue). Insulin^{lo} and Insulin^{hi} cells were defined as having distinct insulin expression under prolonged exposure (1,000 ms) but not standard exposure (50 ms). (*A*) One representative photomicrograph (original 20×) is shown for four replicate experiments as described in Fig. 3. (*B*) Quantification (mean ± SEM) of the islets containing insulin^{hi} β cells among total glucagon^{+} islets; $n = 4$. (*C*–*E*) Islets from firmly established diabetic (3 wk after onset) RIP$^{Cre-ERT}$R26^{EGFP} NOD for each treatment group were further analyzed by flow cytometry as described in Fig. 3. The conditionally labeled preexisting β cells were identified via Ins-EGFP^{+} expression. Ins-EGFP^{+} expression was further displayed as histogram and subdivided into two distinct populations. (*C*) Representative FACS patterns are shown for one of four to six replicate experiments. (*D*) Representative overlay histograms of before treatment versus Haplo-MC + PBS and Haplo-MC + GE with reversal versus Haplo-MC + PBS or Haplo-MC + GE with no reversal. (*E*) Quantification (mean ± SEM) of the percentage of Ins-EGFP^{hi} β cells among total Ins-EGFP^{+} β cells; $n = 4$ to 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

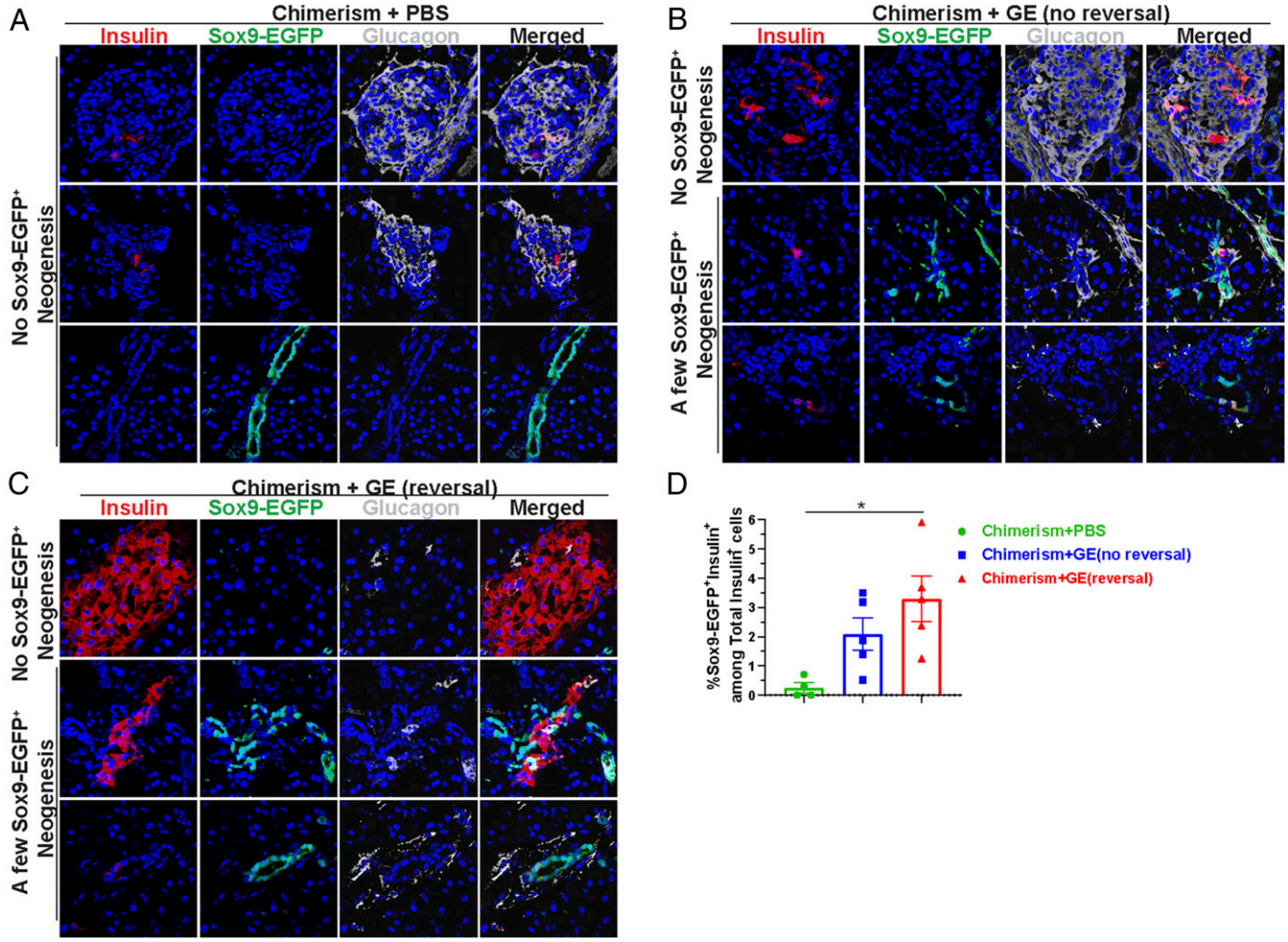

Fig. 5. Little differentiation of pancreatic Sox9$^+$ ductal cells into β cells in firmly established diabetic NOD mice after the combination therapy. The Sox9$^{Cre-ERT}$R26^{EGFP} NOD mice were injected with tamoxifen (20 mg per mouse) to label preexisting Sox9$^+$ ductal progenitor cells. The labeled firmly established (3 wk after onset) diabetic Sox9$^{Cre-ERT}$R26^{EGFP} NOD mice were given the combination therapy of induction of Haplo-MC and administration of GE. Pancreata were harvested at terminal collection and stained for insulin (red), Sox9-EGFP (green), glucagon (white), and DAPI (blue). One representative staining pattern (original magnification 400×) is shown for islets or ducts from four to five mice of each group, including (*A*) mice given induction of Haplo-MC alone, (*B*) mice given Haplo-MC and GE without reversal of hyperglycemia, and (*C*) mice given induction of Haplo-MC and GE with reversal of hyperglycemia. (*D*) Mean ± SEM percentage of Sox9-EGFP$^+$Insulin$^+$ cells among total Insulin$^+$ β cells; $n = 4$ to 5. *$P < 0.05$.

in nonautoimmune mice in response to hyperglycemia (32). We tested whether α cells could transdifferentiate into β cells in firmly established diabetic NOD mice, using Glucagon-RFP (red fluorescence protein) lineage-tracing mice with >85% labeling efficacy (*SI Appendix*, Fig. S9). It was reported that β cells can become Insulin$^+$Glucagon$^+$ immature cells under chronic stress (28). Using Insulin–EGFP lineage-tracing mice, we found that some insulin$^+$ β cells also became Glucagon$^+$, and the Glucagon$^+$ β cells were 5.9% in 10-wk-old prediabetic and 6.4% in new-onset diabetic NOD mice, with no statistically significant difference between the two groups (*SI Appendix*, Fig. S10). Thus, we labeled the α cells in Glucagon-RFP NOD mice at the onset of T1D. We found mostly scattered immature Insulin$^+$Glucagon$^+$ β cells in the pancreatic islets of firmly established diabetic NOD mice treated with induction of Haplo-MC only, and ∼13% of the Insulin$^+$ cells were transdifferentiated from Glucagon-RFP$^+$ cells, that is, Glucagon-RFP$^+$Insulin$^+$ (Fig. 6 *A* and *E*). In firmly established diabetic mice that failed to establish normoglycemia after combination therapy, there were clusters of insulin$^+$ β cells consisting of both mature Insulin$^+$Glucagon$^-$ and immature Insulin$^+$Glucagon$^+$ β cells, with ∼56% of Insulin$^+$ cells derived from Glucagon-RFP$^+$ cells (Fig. 6 *B* and *E*).

There were several kinds of islets in cured firmly established diabetic mice with normoglycemia: big islets consisting of β cells with mature Insulin$^+$Glucagon$^-$ phenotype, big islets consisting of both mature Insulin$^+$Glucagon$^-$ and immature Insulin$^+$Glucagon$^+$ β cells, and islets consisting of scattered immature β cells only. Among Insulin$^+$ cells, ∼50% were Glucagon-RFP$^+$Insulin$^+$ from α cell transdifferentiation (Fig. 6 *C* and *E*). Glucagon-RFP$^+$Insulin$^+$ β cells expressed β cell nuclear factors Nkx6.1 and Pdx1 (Fig. 6*D*). The percentage of Glucagon-RFP$^+$Insulin$^+$ β cells in mice treated with combination therapy was close to four-fold higher than those treated with induction of Haplo-MC only, although no significant difference was found between mice with or without normoglycemia after combination therapy (Fig. 6*E*). However, among Insulin$^+$ β cells from Glucagon-RFP$^+$ cells, ∼80% were Insulin$^+$Glucagon$^-$ mature β cells in mice with reversal of hyperglycemia but only ∼30% were Insulin$^+$Glucagon$^-$ mature β cells in mice without reversal of hyperglycemia (Fig. 6*F*). In addition, there was no significant difference in percent α cell surface area in the pancreas of those mice (*SI Appendix*, Fig. S11). These results indicate that 1) reversal of autoimmunity by induction of Haplo-MC alone allows for α cell transdifferentiation into insulin-producing β cells; 2) administration of GE may augment

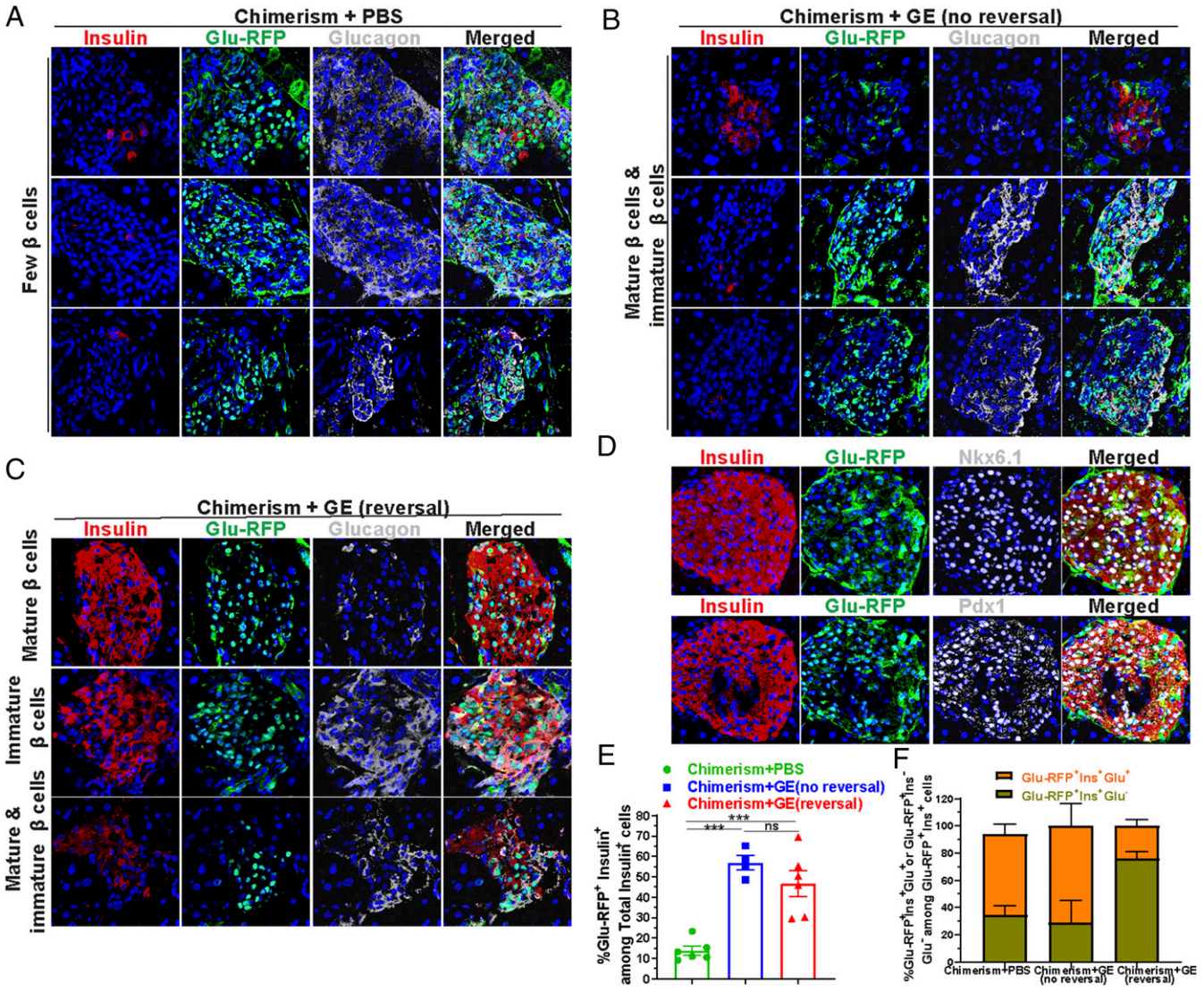

Fig. 6. Augmentation of α cell transdifferentiation into β cells in firmly established diabetic NOD mice after the combination therapy. The $Glu^{rtTA}TetO^{Cre}R26^{tdRFP}$ NOD mice were administered with doxycycline to label preexisting glucagon cells. The labeled firmly established (3 wk after onset) diabetic $Glu^{rtTA}TetO^{Cre}R26^{tdRFP}$ NOD mice were given the combination therapy of induction of Haplo-MC and administration of GE. Pancreata were harvested at terminal collection and stained for insulin (red), Glu-RFP (green), glucagon (white), and DAPI (blue). One representative staining pattern (original magnification 400×) is shown for islets from four to six mice of each group, including (*A*) mice given induction of Haplo-MC alone, (*B*) mice given Haplo-MC and GE without reversal of hyperglycemia, and (*C*) mice given induction of Haplo-MC and GE with reversal of hyperglycemia. (*D*) Pancreata from mice given induction of Haplo-MC and GE with reversal of hyperglycemia were stained for insulin (red), Glu-RFP (green), Nkx6.1 or Pdx1 (white), and DAPI (blue). Representative islet staining is shown of four replicate experiments. (*E*) Mean ± SEM of $Glu\text{-}RFP^+Insulin^+$ cells among total $Insulin^+$ β cells; $n = 4$ to 6. (*F*) $Glu\text{-}RFP^+Insulin^+$ were further categorized as either monohormonal ($Glu\text{-}RFP^+Insulin^+Glucagon^-$) or bihormonal ($Glu\text{-}RFP^+Insulin^+Glucagon^+$). Mean ± SEM; $n = 4$ to 6. $^{***}P < 0.001$; ns, nonsignificant.

early transdifferentiated β-like cell proliferation and expansion as well as augment their maturation; this is consistent with our previous report that elimination of insulitis by induction of mixed chimerism allows for expansion of β cells (8); and 3) β cells from α cell transdifferentiation are the major source of β cell neogenesis and contribute to reversal of hyperglycemia in firmly established diabetic NOD mice after combination therapy of induction of Haplo-MC and administration of GE.

Discussion

β cell regeneration in nonautoimmune mice can come from the differentiation of $Sox9^+$ pancreatic ductal progenitors, transdifferentiation of α cells, and other mesenchymal stem cells (30–36, 62). Although we have reported that combination therapy of induction of MHC-mismatched mixed chimerism and administration of GE results in β cell regeneration in firmly established diabetic NOD mice (18), the source of β cell regeneration remained unknown. In the current study, β cell regeneration in firmly established T1D mice cured by the combination therapy of induction of Haplo-MC and administration of GE is found to arise from both preexisting residual β cells and neogenesis (Fig. 2*B*). We find that close to 90% of residual β cells in firmly established T1D mice have dedifferentiated into dysfunctional $insulin^{lo}$ β cells. Induction of Haplo-MC alone significantly increases the insulin expression in the residual β cells, and GE treatment further increases the expression. We also find that transdifferentiation of α cells but not differentiation of $Sox9^+$ ductal progenitors or $Nestin^+$ mesenchymal stem cells contribute predominantly to the

neogenesis of β cells in the firmly established T1D mice, although $Sox9^+$ ductal progenitors may make a minor contribution. GE treatment may augment the expansion and maturation of early β-like cells transdifferentiated from α cells.

Our current studies provide several insights into the β cell regeneration in autoimmune NOD mice. First, reversal of autoimmunity by induction of Haplo-MC and administration of GE synergistically augments redifferentiation or reactivation of $insulin^{lo}$ β cells into $insulin^{hi}$ β cells in NOD mice with firmly established T1D. We observed that firmly established T1D mice had mostly $insulin^{lo}$ β cells, and induction of mixed chimerism alone significantly up-regulated their expression of insulin. GE treatment further up-regulated their expression of insulin and increased the numbers of $insulin^{hi}$ β cells. Conversely, we observed few $insulin^{lo}$ β cells in STZ-induced nonautoimmune diabetic mice, although $insulin^{lo}$ β cells occurred in NOD mice as early as 6 wk of age and increased as T1D autoimmunity progresses. Therefore, we theorize that autoimmunity triggers β cell dedifferentiation or dysfunction, and reversal of autoimmunity and administration of GE synergistically augment redifferentiation or reactivation of $insulin^{lo}$ β cells into $insulin^{hi}$ β cells. As long-standing T1D patients have $insulin^{lo}$ cells even decades after T1D onset (45), induction of Haplo-MC and administration of GE may wake up those "sleeping" dysfunctional β cells.

Second, induction of Haplo-MC alone allows transdifferentiation of α cells into β cells, and GE plays a role in augmentation. Consistent with previous reports that hyperglycemia induced transdifferentiation of α cells into insulin-producing β cells in nonautoimmune mice (32), reversal of autoimmunity by induction of Haplo-MC alone led to α cell transdifferentiation into β cells (~13%), with the majority being at a $Glucagon^+Insulin^+$ immature stage, and administration of GE increased the percentage to ~50%, with the majority being at a $Glucagon^-Insulin^+$ mature β cell stage, suggesting that GE is not required for the initiation of transdifferentiation of α cells, but augments the expansion and maturation of the early β cells transdifferentiated from the α cells. We should point out that bihormonal $Insulin^+Glucagon^+$ β cells can be immature β cells at a transitional stage (63) or dysfunctional (28). There was a much higher percentage of bihormonal cells in recipients without reversal of hyperglycemia as compared to those with reversal of hyperglycemia after the combination therapy. Whether they are immature or dysfunctional remains unclear, although we classify them as immature in the current report.

Third, loss of $Sox9^+$ pancreatic ductal progenitor cells in firmly established T1D mice reduces the contribution of the progenitor differentiation in firmly established T1D mice. There were very few β cells derived from $Sox9^+$ ductal progenitor cells in firmly established T1D mice treated with the combination therapy, which correlates with loss of the $Sox9^+CD133^+CD71^+$ population that contains the ductal cell progenitors. The loss of the $Sox9^+$ progenitors in T1D mice was associated with the severity and duration of hyperglycemia. Although $Sox9^+CD133^+CD71^+$ cells were expanded at the onset of hyperglycemia with blood glucose of 300 mg/dL to 400 mg/dL, but they were markedly reduced when blood glucose reached 500 mg/dL to 600 mg/dL, and even near undetectable when blood glucose reached >600 mg/dL. This indicates that the loss of $Sox9^+$ progenitors is not due to autoimmunity but due to glucose toxicity. From this point of view, T1D patients may have better preservation of pancreatic progenitors because blood glucose in human is better controlled than in mouse. Thus, we cannot exclude the potential role of pancreatic ductal progenitors in human β cell regeneration (64). However, our finding that firmly established T1D mice can be cured despite minimal contribution from $Sox9^+$ progenitors suggests that $Sox9^+$ progenitors may be dispensable for β cell regeneration in firmly established T1D patients.

Fourth, control of hyperglycemia can augment β cell regeneration. Firmly established T1D mice with initial blood glucose of >600 mg/dL had a significantly reduced percentage of mice reaching euglycemia after combination therapy, as compared to mice with blood glucose at 500 mg/dL to 600 mg/dL (15% versus 71%). This indicates that, even though GE treatment may enhance β cell resistance to glucose toxicity, as previously reported (30), the protection cannot be beyond 600 mg/dL. It is very difficult to control blood glucose with insulin pellets or subcutaneous injection of insulin in firmly established T1D mice, as mice can easily die of hypoglycemia. However, controlling blood glucose in patients is no longer an issue with use of an advanced insulin pump (65). Thus, β cell regeneration can potentially be robust with the combination therapy of induction of Haplo-MC and administration of GE.

Fifth, induction of Haplo-MC may augment β cell regeneration. We previously reported that GE treatment enhanced β cell differentiation from $Sox9^+$ progenitors and reversed hyperglycemia in nonautoimmune alloxan-induced diabetic mice with blood glucose of 300 mg/dL to 450 mg/dL, but not in mice with blood glucose above 500 mg/dL (30). In contrast, induction of Haplo-MC and administration of GE was able to reverse hyperglycemia in the majority of diabetic mice with blood glucose at 500 mg/dL to 600 mg/dL, suggesting that Haplo-MC may be better than syngeneic HCT at augmenting β cell regeneration. Indeed, there was an expansion of donor-type $Areg^+$ $Foxp3^+$ Treg cells in the Haplo-MC, and it is known that $Areg^+$ Treg cells can augment tissue regeneration (57, 58).

Sixth, multiple sources of β cell regeneration are likely required for reaching euglycemia in firmly established T1D mice treated with combination therapy. Comparing the portion of mice treated with combination therapy that reach euglycemia and those that remained hyperglycemic, we found that, while both had similar percentages of β cells from α cell transdifferentiation, the former had a significantly greater percentage of $Glucagon^-Insulin^+$ mature β cells transdifferentiated from α cells as well as greater percentage of the reactivated β cells from $Insulin^{lo}$ β cells. This suggests that transdifferentiation of α cells alone may not be sufficient to generate enough β cells for reaching euglycemia. However, the observation may also be a consequence of disease severity; increased severity of T1D results in greater loss of potential alternative sources of β cells, including $Insulin^{lo}$ β cells and even $Sox9^+$ progenitors.

We should point out that the extent of regenerated β cells from β cell reactivation or from α cell transdifferentiation remains unclear. Although, based on β cell lineage tracing using NOD mice with an Insulin–EGFP transgene, ~25% of β cells were from preexisting β cells, those cells may not be all from reactivation, because they also could be from replication of β cells. Although, based on α cell lineage tracing using NOD mice with a Glucagon-RFP transgene, ~50% β cells in the "cured" mice were from α cell transdifferentiation, we do not know what percentage of them were from preexisting $Insulin^+Glucagon^+$ β cells before treatment. However, it is unlikely that those cells accounted for all of the β cells transdifferentiated from α cells. Thus, our data strongly suggest that α cell transdifferentiation is an important source for the "cured" diabetic mice. In addition, α cell transdifferentiation is unlikely to be from the ductal cells, either, although misexpression of Pax4 in adult α cells was reported to augment ductal lining cells to express glucagon and then transdifferentiate into $insulin^+$ β cells (41). If ductal lining cells gave rise to β cells in the diabetic NOD mice after the combination therapy, they should have been $Sox9\text{-}EGFP^+$, but most of the β cells from neogenesis were $Sox9\text{-}EGFP^-$.

Finally, induction of Haplo-MC and administration of GE represents a potentially practical curative therapy for firmly established T1D. Although immune modulation regimens such as LFA-3Ig could ameliorate new-onset T1D in patients with low

autoantibodies, and autologous HCT provides insulin independence for new-onset patients with low autoimmunity, neither showed an effect for new-onset T1D patients with high autoimmunity (11–15). Thus, reversal of autoimmunity in T1D patients is likely the key step for cure of the disease, and no regimen has yet been shown to work for firmly established T1D in humans. In mice, induction of Haplo-MC is currently the only curative approach for autoimmunity in firmly established T1D mice (16). Induction of Haplo-MC has strong clinical potential, because haploidentical HCT has been widely applied to treat nonmalignant hematological disorders (17), and induction of Haplo-MC does not cause GVHD in mice or humans (4, 8, 16, 18–20, 66, 67). Our regimen of induction of Haplo-MC is undergoing a phase 1 clinical trial for treatment of sickle cell disease, and initial results are promising, with much reduced toxicity (NCT03249831). This trial does not use GE, but GE has been used in patients (68, 69), and it has been shown that islets from human donors with higher but not lower hemoglobin A1c levels respond to gastrin treatment in vitro (48). We will further evaluate GE's effect on human islet cells using freshly isolated human islets or live pancreas slice assay as recently reported by Speier and coworkers (70).

It is important to understand that induction of mixed chimerism is different from classical autologous or allogeneic HCT, which are not fit for treating T1D patients, due to the low benefit/risk ratio. Autologous HCT for new-onset T1D patients reported by others required myeloablative conditioning in order to eliminate pathogenic memory T cells (13–15), and the myeloablative conditioning regimen is too toxic for T1D patients. Classical allogeneic HCT is even less acceptable, due to the severe side effect of GVHD in ~30% of patients (71). The current regimen of induction of mixed chimerism is markedly different from classical HCT. First, our nonmyeloablative conditioning regimen of ATG and low-dose of CY and PT has only mild toxicity. Second, the use of $CD4^+$ T-depleted hematopoietic graft to induce mixed chimerism not only prevents GVHD but also reduces cytokine storm early after donor cell infusion.

In addition, since induction of Haplo-MC can cure autoimmunity in firmly established T1D (16), it can also serve as a platform for testing other cellular therapies and for testing reagents that augment β cell regeneration in firmly established T1D patients, including β cells derived from human iPSCs that will not cause alloimmunity (26) and drugs that induce human β cell expansion (72).

Materials and Methods

Mice. Wild-type C57BL/6, NOD/LtJ, and $R26^{EGFP}$ NOD were purchased from the Jackson Laboratory. SJL/J and congenic H-2^{g7} C57BL/6 were purchased from National Cancer Institute animal production program. The RIP^{CreERT} mice were obtained from the Melton laboratory at Harvard University (18). $Sox9^{CreERT}$ mice were previously described (73) and provided by M. Sander's laboratory at University of California San Diego. $Glu^{rtTA}TetO^{Cre}R26^{tdRFP}$ mice were provided by P.S.'s laboratory at University of Calgary. Nes^{CreERT} NOD mice were backcrossed from Nes^{CreERT} B6 mice purchased from Jackson Laboratory. Lineage-tracing NOD mice were generated by crossing two strains of NOD mice at City of Hope Animal Research Facilities (COH ARC). Breeding strategies are described in *SI Appendix*. All experimental mice were housed in the specific pathogen-free rooms in the ARC. The animal use procedures were approved by the COH Institutional Animal Care and Use Committee.

Methods. Induction of Haplo-MC and administration of GE, induction of EGFP and RFP for lineage tracing, immunohistochemistry and immunofluorescence staining, morphometric analysis and cell counting, IPGTT, pancreas isolation and flow cytometry, and statistical analysis were described in our previous publications (16, 30, 74) and in *SI Appendix, Materials and Methods*.

Data Availability. All study data are included in the article and *SI Appendix*.

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