

Inactivating the permanent neonatal diabetes gene *Mnx1* switches insulin-producing β -cells to a δ -like fate and reveals a facultative proliferative capacity in aged β -cells

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ABSTRACT

Homozygous *Mnx1* mutation causes permanent neonatal diabetes in humans, but via unknown mechanisms. Our systematic and longitudinal analysis of *Mnx1* function during murine pancreas organogenesis and into the adult uncovered novel stage-specific roles for *Mnx1* in endocrine lineage allocation and β -cell fate maintenance. Inactivation in the endocrine-progenitor stage shows that *Mnx1* promotes β -cell while suppressing δ -cell differentiation programs, and is crucial for postnatal β -cell fate maintenance. Inactivating *Mnx1* in embryonic β -cells (*Mnx1* ^{Δ beta}) caused β -to- δ -like cell transdifferentiation, which was delayed until postnatal stages. In the latter context, β -cells escaping *Mnx1* inactivation unexpectedly upregulated *Mnx1* expression and underwent an age-independent persistent proliferation. Escaper β -cells restored, but then eventually surpassed, the normal pancreatic β -cell mass, leading to islet hyperplasia in aged mice. *In vitro* analysis of islets isolated from *Mnx1* ^{Δ beta} mice showed higher insulin secretory activity and greater insulin mRNA content than in wild-type islets. *Mnx1* ^{Δ beta} mice also showed a much faster return to euglycemia after β -cell ablation, suggesting that the new β -cells derived from the escaper population are functional. Our findings identify *Mnx1* as an important factor in β -cell differentiation and proliferation, with the potential for targeting to increase the number of endogenous β -cells for diabetes therapy.

KEY WORDS: β -cell versus δ -cell fate selection, Endocrine lineage diversification, β -cell fate maintenance, β -cell proliferation, Islet

INTRODUCTION

Similar to most other organ systems, lineage determination and cell specification in the pancreas involve a highly ordered series of events, consisting of appropriately programmed extrinsic signaling pathways that regulate hierarchies of cell-intrinsic transcriptional networks. Various dynamically regulated genes encoding transcription factors have been linked to specific steps in endocrine cell subtype specification, differentiation and maturation (Pan and Wright, 2011). A comprehensive understanding of these processes could provide important advances in the current effort to develop β -cell replacement or regeneration therapies for patients with diabetes.

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The homeodomain-protein-encoding gene motor neuron and pancreas homeobox 1 (*Mnx1*, formerly *Hb9* or *Hlxb9*), encodes a transcription factor required for the development of multiple tissues, including pancreas. In the mouse, *Mnx1* is expressed in the motor neurons, notochord, the entire dorsal endoderm and the ventral pre-pancreatic endoderm at the embryonic day 8 (E8) (Tanabe et al., 1998; Li et al., 1999). Endodermal *Mnx1* expression is transient and forms a dorsal-ventral gradient at E9.5 (Sherwood et al., 2009), with expression persisting in both pancreatic buds at E10.5, and being downregulated between E10.5 (Li et al., 1999) and E12.5 (Harrison et al., 1999). In the adult mouse pancreas, *Mnx1* is specifically expressed in mature β -cells (Harrison et al., 1999; Li et al., 1999).

Global inactivation of *Mnx1* leads to dorsal pancreatic bud agenesis, while the ventral bud develops normally (Harrison et al., 1999; Li et al., 1999). By contrast, using *Pdx1* cis-regulatory sequences to induce high-level *Mnx1* mis-expression over the entire early pancreatic epithelium results in highly deficient pancreas organogenesis, and the pancreatic mesenchyme seems to adopt a stomach/intestinal mesenchymal state (Li and Edlund, 2001). Together, these studies emphasize that the early endodermal *Mnx1* expression, in both timing and level, must be tightly controlled for proper dorsal pancreas specification.

In addition to its role in dorsal pancreas specification, global *Mnx1* null mutants have a nearly threefold increase in δ -cells, and the remaining β -cells in the ventral pancreas are immature, with reduction or absence of β -cell maturation markers (Harrison et al., 1999; Li et al., 1999). Thus, these initial studies suggested that *Mnx1* regulates β -cell differentiation and maturation. Furthermore, *Mnx1* homozygous mutation was recently shown to cause permanent neonatal diabetes mellitus in humans (Bonfond et al., 2013; Flanagan et al., 2014), suggesting a potentially conserved role of *Mnx1* in β -cell function between mouse and human.

The limited number of studies on *Mnx1* are mostly from over a decade ago, and, while indicating its essential nature in pancreas organogenesis, they did not focus on the endocrine progenitor or β -cell-specific requirements for this factor, or relate its activity to the more recent advances in our understanding of pancreatic endocrine-cell ontogeny and fate maintenance. Here, we report the inactivation of *Mnx1* in distinct contexts using Cre driven from the endocrine-progenitor stage using transgenic gene-regulatory sequences from *neurogenin-3* (*Ngn3*^{Cre}), and in the β -cell itself using *insulin* (*RIP2*^{Cre}). We performed an extensive, systematic and longitudinal analysis to characterize the resulting effects on endocrine cells from organogenesis on into adult life. We identify *Mnx1* as an endocrine-precursor-stage instructor of β -cell lineage allocation, and it is crucial for maintaining the β -cell against conversion to a δ -like (somatostatin-producing) phenotype. The incomplete inactivation of *Mnx1* within the insulin-producing cell pool led to the presence of escaper β -cells within islets populated with increased δ -like cell numbers. The escaper cells upregulated *Mnx1* expression and displayed a large,

persistent increase in proliferation lasting into aged mice. Our findings identify *Mnx1* as another β -cell-programming factor that initiates and maintains β -cell-specific gene expression programs and represses alternative endocrine-lineage programs. These eminent functions in β -cell differentiation and proliferation render *Mnx1* a potentially important therapeutic target, particularly in reprogramming other cell types into β -cells, or in stimulating β -cell proliferation.

RESULTS

Novel *Mnx1* expression in Pax6⁺ endocrine precursors

Previous studies showed that early pancreatic *Mnx1* expression is transient and temporally regulated (Harrison et al., 1999; Li et al.,

1999), yet its expression pattern during organogenesis at that time was incompletely characterized, because it was not placed in reference to the many, more recently described, regulators of endocrine-lineage differentiation. We therefore re-examined *Mnx1* expression, focusing on stages of early pancreas development between E10.5 and 14.5. *Mnx1* protein was detected in essentially all cells of the dorsal and ventral pancreatic buds at E10.5, and excluded from the duodenum (red line, Fig. 1A). In E11.5 tissue, dorsal-bud *Mnx1*-positivity was notably heterogeneous compared with *Pdx1*, whereas ventral-bud expression was downregulated (Fig. 1B). In contrast to previous reports, *Mnx1* was still detectable at E12.5, but was now restricted to tip domains of the pancreatic epithelium, as

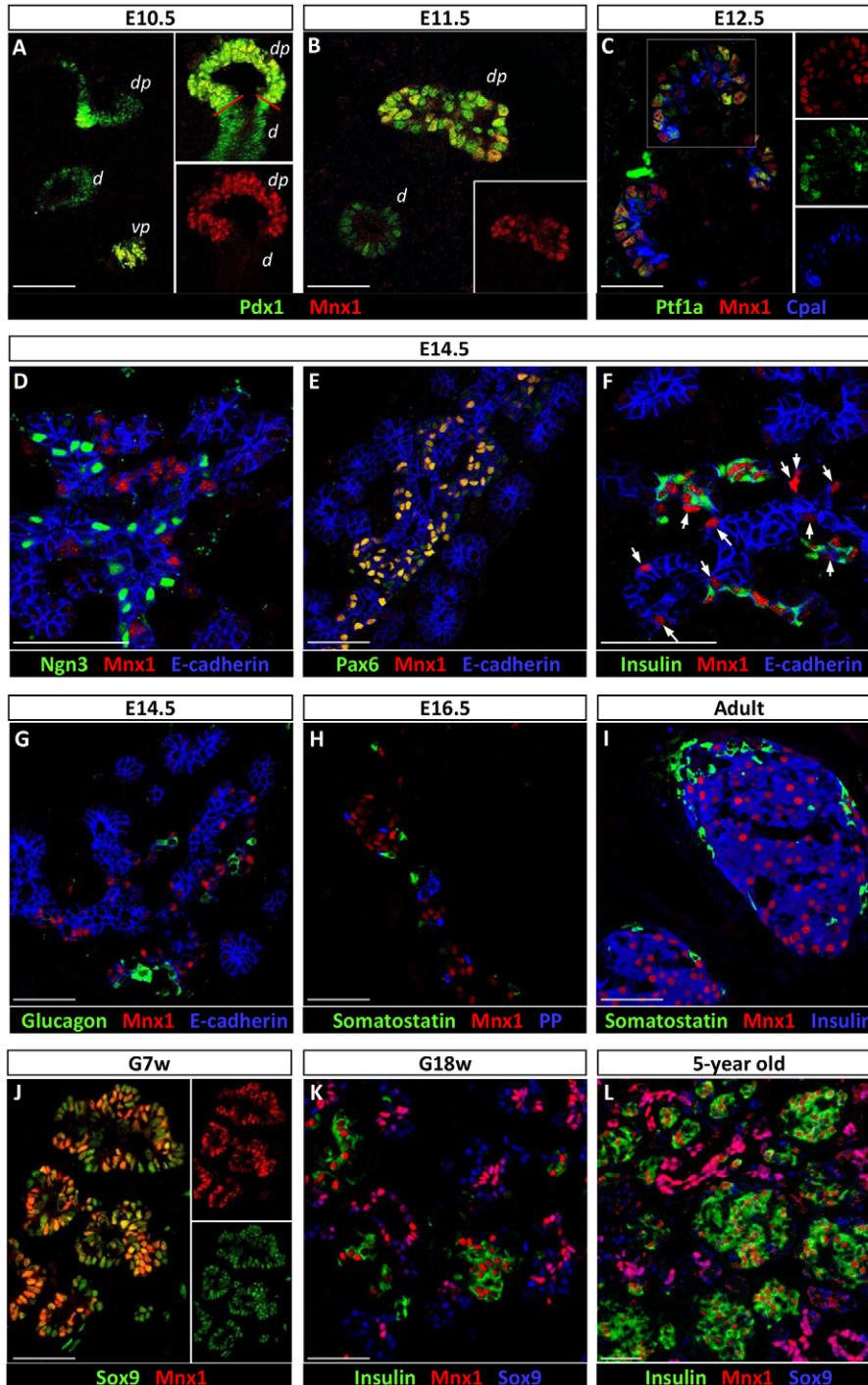


Fig. 1. Immunodetection of *Mnx1* in developing and adult mouse and human pancreas.

(A,B) Immunolabeling comparison of *Pdx1* with *Mnx1* in the embryonic mouse pancreas at E10.5 (A) and E11.5 (B). (C) *Mnx1* detected in *Ptf1a*⁺ *Cpa1*⁺ tip MPC at E12.5. (D) *Mnx1* is absent in *Ngn3*⁺ endocrine progenitors, but present in *Pax6*⁺ endocrine precursors (E). (F) *Mnx1* can be found in *insulin*⁺ developing β -cells as early as E14.5. Arrows indicate *Mnx1* expression in *insulin*-endocrine precursors. (G,H) *Mnx1* protein was not detected in α -cells (G), δ -cells or in PP cells (H). (I) *Mnx1* is restricted to β -cells in adult mouse pancreas. (J) In human fetal pancreas, *Mnx1* is detected in the *Sox9*⁺ MPC population at gestational week 7 (G7w). (K,L) In G18w (K) and 5-year-old tissue (L), *Mnx1* was immunodetected in β -cells and *Sox9*⁺ trunk/duct cells. Scale bars: 50 μ m.

shown by co-labeling of *Mnx1* against *Ptf1a* or *Cpa1* (Fig. 1C). The numbers of *Mnx1*⁺*Ptf1a*⁺*Cpa1*⁺ cells, however, decreased over time to become relatively scattered among the tip epithelial domains. The distribution of these *Mnx1*⁺*Ptf1a*⁺*Cpa1*⁺ cells was similar to the distribution of tip multipotent progenitor cells (MPC) between E12.5 and E14.5, which are *Ptf1a*⁺*Sox9*⁺*HNF1β*⁺ (Pan et al., 2013). These data provide new insight by indicating that *Mnx1* is not uniformly downregulated between E10.5 and 12.5, but continues to mark tip MPC upon tip-trunk compartmentalization, thus highlighting the dynamic expression of *Mnx1* during early pancreas organogenesis.

At E14.5, *Mnx1* was detected in cells that co-expressed the endocrine precursor marker *Pax6* (Fig. 1E). This marking of the endocrine precursor population during the secondary transition was previously not described. In addition, *Mnx1* was found in insulin⁺ β-cells as early as E14.5 (Fig. 1F; arrows indicate *Mnx1*⁺insulin⁻ endocrine precursors). In no case did we detect *Mnx1* expression in *Neurog3*⁺ cells (Fig. 1D), glucagon⁺ cells (Fig. 1G), somatostatin⁺ or pancreatic polypeptide⁺ (PP, also known as Ppy – Mouse Genome Informatics) cells (Fig. 1H), suggesting that *Mnx1* is not expressed in the *Neurog3*⁺ endocrine progenitors, α-, δ- or PP-cells. In the adult pancreas, the vast majority of the β-cells was *Mnx1*-immunopositive. Only one or two insulin⁺ cells per islet were apparently *Mnx1*⁻, as is typical for many immunodetection analyses of transcription factors (TFs). If they are truly *Mnx1*⁻, the significance of this minute population is unknown.

We also studied human pancreatic tissue to gain insight into the level of functional conservation. In human pancreas, *Mnx1* was detected in *Sox9*⁺ cells as early as gestational week 7 (G7w, equivalent to mouse E10.5, Fig. 1J; Pan and Wright, 2011), suggesting that *Mnx1* also marks early MPC in human fetal pancreas. In later-stage tissue, *Mnx1* was found in insulin⁺ β-cells and *Sox9*⁺ trunk epithelial cells at G18w (equivalent to mouse E16.5; Fig. 1K), and was maintained in β-cells and *Sox9*⁺ ducts in juvenile pancreas samples (5 years old; Fig. 1L). These are the first observations to provide insight into similarity and distinctions in *Mnx1* expression patterns between human and mouse pancreas development.

Mnx1 suppresses δ-cell fate and promotes β-cell fate during endocrine differentiation

The multiphase and cell type-specific *Mnx1* patterns described above suggested several possible functions during organogenesis. To begin to determine cell type-specific *Mnx1* functions, we used a floxed allele (*Mnx1*^{fl/fl}) in which the homeodomain-encoding exon 3 was flanked by *LoxP* sites. We confirmed that *Mnx1*^{fl/fl} could produce functional nulls by crossing to the germ-line *E11A*^{Cre} deleter line to derive *E11A*^{Cre};*Mnx1*^{fl/fl} mice (Fig. S1A). These mice phenocopied the global null phenotypes (Harrison et al., 1999; Li et al., 1999), with dorsal pancreas agenesis and normal ventral pancreas formation (Fig. S1C) compared with *Mnx1*^{fl/fl} (Fig. S1B).

Our new placement of *Mnx1* in *Pax6*⁺ endocrine precursors led us to determine how *Mnx1* integrates into the transcriptional hierarchies controlling lineage allocation during endocrine diversification. To determine *Mnx1* function in the *Pax6*⁺ endocrine precursor state, we crossed *Mnx1*^{fl/fl} to a *Ngn3*-*Cre* BAC transgenic line to generate *Ngn3*-*Cre*;*Mnx1*^{fl/fl} mice (hereafter denoted *Mnx1*^{Δendo}; Fig. 2A). Because *Neurog3* is expressed earlier than *Pax6*, this model inactivated *Mnx1* function in all endocrine cells, including *Pax6*⁺ precursors, as early as E9.5 (Schonhoff et al., 2004). Pups and pancreata from *Ngn3*-*Cre*, *Mnx1*^{fl/fl} and *Mnx1*^{Δendo} genotypes displayed similar blood-glucose levels, gross morphology and endocrine cell proportions; thus, in

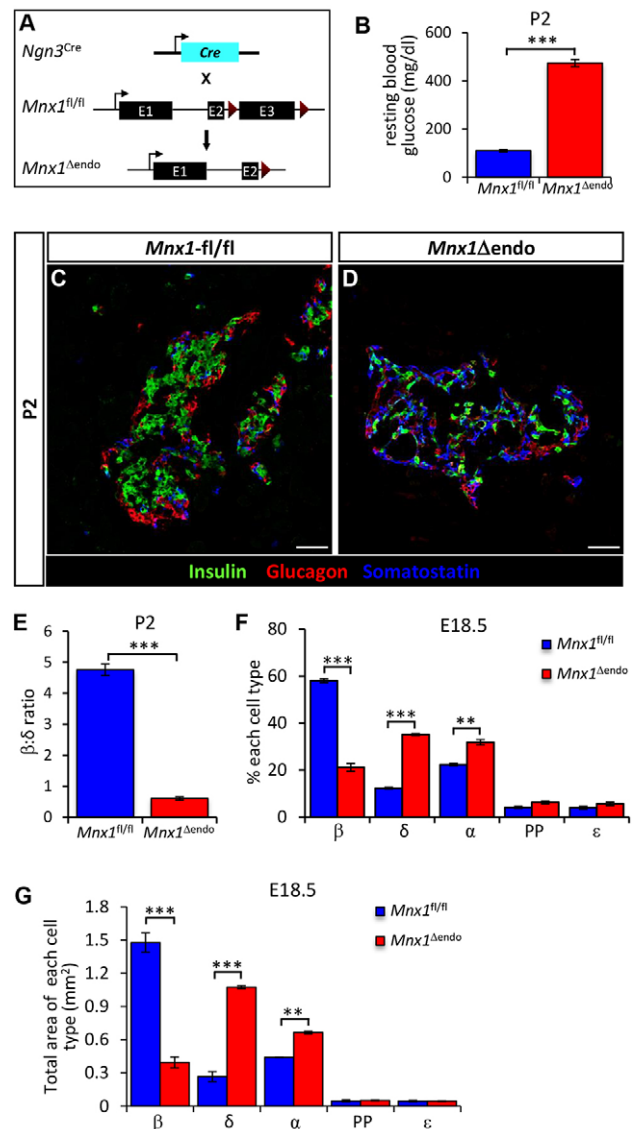


Fig. 2. Dramatic increase in δ-cell numbers and significant decrease in β-cell numbers in *Mnx1*^{Δendo}. (A) Schematic of conditional *Mnx1* deletion in endocrine progenitors with *Ngn3*-*Cre*. (B) Random blood-glucose measurement showed that *Mnx1*^{Δendo} mutant pups were hyperglycemic at P2 ($n=10$). (C, D) Immunolabeling with insulin, glucagon and somatostatin showed a dramatic increase in δ-cell number, concomitant with a decrease β-cell number at P2. (E) Significant decrease in β:δ cell ratio in *Mnx1*^{Δendo} mutants at P2 ($n=4$). (F, G) Quantification of endocrine cell types fraction (F) and area (G) at E18.5, indicating significant increase in δ-cells at the expense of β-cells in *Mnx1*^{Δendo} pancreas ($n=4$). Scale bars: 50 μm. Data shown as mean±s.e.m. * $P<0.05$, ** $P<0.005$, *** $P<0.001$.

all data shown herein, *Mnx1*^{Δendo} will be compared with their respective age-matched *Mnx1*^{fl/fl} littermates. *Mnx1*^{Δendo} mutant pups developed extreme hyperglycemia already at postnatal day 2 (P2) (Fig. 2B), which probably explains their inability to survive past P10, and suggests functional deficits in or decreased numbers of β cells.

To uncover potential causes of the hyperglycemia in P2–P5 *Mnx1*^{Δendo} mutants, we analyzed the islet hormone-expressing cell types using immunofluorescence. The *Mnx1*^{Δendo} mutant pancreas at P2 displayed a significant decrease in insulin⁺ β-cell numbers, and a concomitant, striking increase in somatostatin⁺ cells compared with control *Mnx1*^{fl/fl} (Fig. 2C, D; quantified in Fig. 2E). Analysis of

endocrine-cell numbers at prenatal stages (E18.5) revealed a threefold decrease in the insulin⁺ β -cell population (~58% in control, ~21% in mutant), accompanied by a threefold increase in somatostatin⁺ cells (~12% in control, ~35% in mutant; Fig. 2F,G). These late-gestation tissues also showed a slight, but significant, increase in glucagon⁺ α -cells (~22% in control, ~31% in mutant), whereas PP and ϵ -cell numbers had not changed substantially (Fig. 2F).

Because previous studies suggested that the remaining β -cells in the ventral pancreas of *Mnx1* global-null mice were immature – being Glut2⁻ and Nkx6.1⁻, and Pdx1^{LO} (Harrison et al., 1999; Li et al., 1999) – we further evaluated the remaining 20% *Mnx1*-deleted β -cells (Fig. S2H,I) in *Mnx1*^{Δendo} tissue with respect to several informative β -cell-specific TFs. In contrast to previous findings in the global null, the nuclear Pdx1 and Nkx6.1 signal in *Mnx1*^{Δendo} mutant β -cells at P2 was equivalent to control β -cells (Fig. S2A-D). Because MafA nuclear localization is usually well-established by P4, and is indicative of mature β -cell status (Guo et al., 2013), we tested for its presence and subcellular localization in mutant β -cells. MafA was cytoplasmic in the remaining insulin⁺somatostatin⁻ cells, unlike the nuclear localization in control β -cells (Fig. S2E,F); thus, we concluded that the remaining insulin⁺ β -like cells in the *Mnx1*^{Δendo} mutants were immature and probably poorly functional. The early-lethality phenotype precluded testing for the degree of maturity at a physiological level, as even normal β -cells are not fully mature at this age. Taken together, we conclude that *Mnx1* promotes β -cell fate and suppresses δ -cell fate, with this allocation occurring in the Pax6⁺ endocrine-precursor stage.

***Mnx1* loss in Pax6⁺ endocrine precursors leads to aberrant lineage allocation and postnatal β -to- δ cell transdifferentiation**

The dramatic decrease in the β -to- δ cell proportion seen in *Mnx1*^{Δendo} pancreatic tissue could result from either aberrant lineage allocation in the Pax6⁺ endocrine-precursor pool, or from β -to- δ cell transdifferentiation. To distinguish between these two possibilities, we examined β - and δ -cell numbers during relatively late stages of the secondary transition, when there is a large wave of β - and δ -cell commitment. We observed a dramatic decrease in β -cell number as early as E16.5 in *Mnx1*^{Δendo} mutant pancreas (Fig. 3B). Conversely, at the same stage, both populations of Hhex^{HI}somatostatin⁻ cells (likely δ -cell precursors) and Hhex^{HI}somatostatin⁺ (δ - or δ -like) cells were markedly increased over control (Fig. 3A-D. Note: duct cells were Hhex^{LO}). We rarely found somatostatin⁺insulin⁺ double-positive cells at E16.5. We extended this analysis to P2 to determine if the Hhex^{HI}somatostatin⁻ δ -cell precursor number continued to increase over the perinatal period. Surprisingly, the number of Hhex^{HI}somatostatin⁻ δ -cell precursors was similar to the control at this stage, but the number of Hhex^{HI}somatostatin⁺ δ cells was greatly increased, suggesting that a substantial proportion of the earlier Hhex^{HI}somatostatin⁻ δ -cell precursors (observed at E16.5) had differentiated into Hhex^{HI}somatostatin⁺ cells (Fig. 3E,F). In addition, we also observed a population of cells that were insulin⁺Hhex⁺somatostatin⁺, suggestive of β -to- δ cell transdifferentiation. (Validating this potential transdifferentiation by lineage tracing would require dual-level lineage tracing, because endocrine cells would all be EYFP-labeled in the *Mnx1*^{Δendo};*ROSA26R*^{EYFP} pancreata.) These analyses suggest that the increased δ / δ -like cell number observed in *Mnx1*^{Δendo} mutants occurred by both lineage reallocation from the Pax6⁺

endocrine precursors and by transdifferentiation of *Mnx1*⁻ β -like cells into δ -like cells (Fig. 3G); these mechanisms are not mutually exclusive.

We also noted an overall decrease in total hormone⁺ endocrine-cell area (Fig. 3H), although the total acinar area was not changed significantly in *Mnx1*^{Δendo} mutants at E18.5 (Fig. S2G). Consistent with this observation, this stage also showed an overaccumulation of Pax6⁺ hormone-negative endocrine precursors (Fig. 3I). Because Ngn3⁺ cell numbers were unaffected (data not shown), this evidence suggests a significant block in the differentiation program of *Mnx1*-deficient endocrine-precursor cells (Fig. 3J).

***Mnx1* is required for β -cell fate maintenance**

The expression of *Mnx1* in β -cells long after their specification suggests a continued important role in β -cell differentiation and function. To address this issue, we used *RIP2*-Cre transgenic mice to derive *RIP2*-Cre;*Mnx1*^{fl/fl} mice (denoted as *Mnx1*^{Δbeta} hereafter; Fig. 4A). The *Mnx1*^{Δbeta} newborn pups had slightly, but noticeably, higher glycemia, persisting through 1 month of age, but returning to normal as mice aged (Fig. S3A-D). To explore the potential defects in β -cell differentiation or function in *Mnx1*^{Δbeta} mutants, we tested for hormone-producing cell types in *Mnx1*^{Δbeta} and control mice. Our analysis began with 4-month-old mice, in which the *Mnx1*^{Δbeta} mutant islets had substantially increased δ -cell numbers (Fig. 4B-D). Glucagon⁺ α -cells were also slightly increased, but far less than the δ -cells. Although the proportion of β -cells was slightly reduced compared with *Mnx1*^{fl/fl} control tissue, the number of β -cells quantified by β -cell area on extensive sectional analysis was similar to controls (Fig. 4B-D; Fig. S4D); the issue of β -cell repopulation from escaper β -cells is discussed below. PP and ϵ -cell numbers were also similar between mutant and controls (data not shown).

The gain in δ - and α -cell populations in *Mnx1*^{Δbeta} mutants could have arisen by *de novo* neogenesis, or by transdifferentiation of *Mnx1*-inactivated β -cells. We generated *Mnx1*^{Δbeta};*ROSA26R*^{EYFP} mice in order to use lineage tracing to dissect the sequence of events leading up to the *Mnx1*^{Δbeta} phenotype described above. At 2 months of age, EYFP labeling in *RIP2*-Cre;*ROSA26R*^{EYFP} control islets was restricted to β -cells, with none in somatostatin⁺ cells, whereas in *Mnx1*^{Δbeta} islets, ~56% of EYFP⁺ cells were somatostatin⁺ (δ -like) cells (Fig. 4E,F; Fig. S4E), thus derived from β -cells that lost *Mnx1*. A small proportion of glucagon⁺ α -cells were also EYFP⁺ (~12% of total EYFP⁺ cells), indicative of their β -cell origin. We conclude that *Mnx1* is required for β -cell fate maintenance, and that in its absence, β -cells lose their identity and transdifferentiate, predominantly into δ -cells and, to a lesser extent, into α -cells.

In earlier-stage analysis (E18.5), *Mnx1* protein was diminished in all Cre-reporting β -cells of *Mnx1*^{Δbeta} mice (EYFP⁺*Mnx1*⁻ cells; Fig. S5C,D). Although the Cre activity from *RIP2*-Cre begins in β -cells formed as early as E13.5 (Gannon et al., 2000), we did not observe any β -to- δ or β -to- α transdifferentiation before birth (Fig. S5A,B). The earliest detection of such transdifferentiation was at P5, when a minor number of EYFP⁺somatostatin⁺ cells were detected (representative islet shown in Fig. 4H), as well as insulin⁺Hhex⁺ or insulin⁺Hhex⁺somatostatin⁺ transitional cell states (Fig. S5E,F). The proportional representation of insulin⁺ cells was similar to control at P5. Consistent with our previous findings in the context of forced TF expression within the embryonic endocrine lineages (Yang et al., 2011), our observations suggest that *Mnx1*-deficient β -cells receive some type of postnatal stimulus to allow their conversion toward other endocrine-hormone-expressing states.

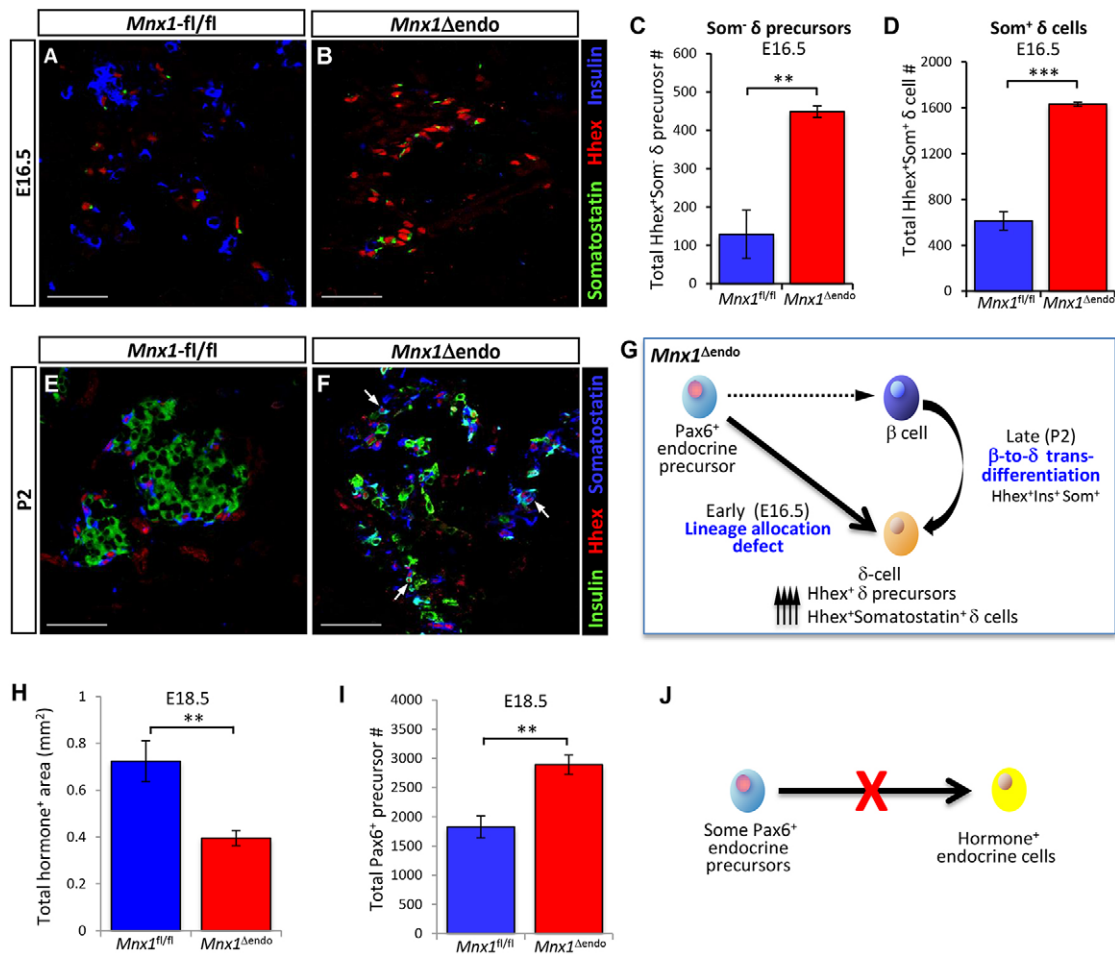


Fig. 3. Embryonic endocrine lineage-allocation defect, followed by postnatal β -to- δ transdifferentiation as two major routes to increased δ -cell numbers. (A,B) Representative sections from E16.5 pancreata with immunodetection of Hhex, insulin and somatostatin illustrate the increase in Hhex⁺ somatostatin⁻ δ -cell precursors in *Mnx1^{Δendo}* mutants. (C,D) Quantitative analysis of E16.5 *Mnx1^{fl/fl}* and *Mnx1^{Δendo}* pancreata indicate a dramatic increase in total number Hhex⁺somatostatin⁻ δ -cell precursors (C) and total Hhex⁺somatostatin⁺ δ -cell numbers (D) in *Mnx1^{Δendo}* mutant. (E,F) Immunofluorescence analysis of Hhex, insulin and somatostatin on P2 pancreas demonstrates the presence of Hhex⁺insulin⁺somatostatin⁺ cells (white arrows) in *Mnx1^{Δendo}* pancreas tissue, an indirect indicator of β -to- δ transdifferentiation. (G) Model showing early stage endocrine lineage-allocation defects and late-stage β -to- δ transdifferentiation when *Mnx1* is inactivated in endocrine progenitors. (H–J) Quantification indicates that total endocrine area was reduced in *Mnx1^{Δendo}* mutant (H), but total Pax6⁺ endocrine precursors were increased in *Mnx1^{Δendo}* mutant at E18.5 (I), indicating failure of a subset of Pax6⁺ precursors to differentiate towards hormone-producing endocrine cells in the absence of *Mnx1* (J). Scale bars: 50 μ m. Data are shown as mean \pm s.e.m. ** P <0.005, *** P <0.001.

The massively increased δ/δ -like cell number in *Mnx1^{Δbeta}* mutants could result in overproduction of somatostatin, which is a well-known inhibitor of insulin secretion (Alberti et al., 1973). There are limited mature δ -cell markers, and the β -cell-derived δ -like cells all expressed Hhex (Zhang et al., 2014) and somatostatin (Fig. S5F). Quantitative analysis showed a 3.5-fold greater representation of δ -cells over controls, but *in vitro* assays on isolated islets showed that somatostatin content and secretion from mutant islets were >10-fold higher than controls, suggesting overproduction and hypersecretion of somatostatin (Fig. 4I,J). The somatostatin secretion in mutant islets was not regulated by glucose, unlike in controls, but was substantially potentiated by the cAMP analog 3-isobutyl-1-methylxanthine (IBMX) (Fig. 4I). Thus, the δ/δ -like cells in mutant islets are functionally distinct from wild-type δ -cells.

β -cells escaping *Mnx1* inactivation in *Mnx1^{Δbeta}* mutants repopulate the islet

We found evidence that incomplete *Mnx1* inactivation across the β -cell population was a result of lack of Cre production in 15–20% of

the β -cells in RIP2-Cre islets, which might be caused by the *RIP2*-Cre transgene being packaged in such a way that it cannot be activated, leading to the variegated expression (Fig. S6). This represents an important finding, because the lack of Cre production was not caused by an inability in some cells to express both insulin genes, as *Ins1* and *Ins2* mRNA were highly upregulated in these Cre⁻ β -cells (Fig. 6C; Fig. S8C; see below). Such incomplete recombination was followed by subsequent islet repopulation by β -cells that did not undergo *Mnx1* inactivation – hereafter ‘escaper β -cells’. At P15, insulin⁺somatostatin⁺ cells were much more pervasive, although the [insulin⁺somatostatin⁺ plus insulin⁺] pool was still approximately equivalent to the control insulin⁺ pool (data not shown). At 4 months of age, this double-positivity was vastly resolved to hormone monopositivity; that is, insulin⁺ or somatostatin⁺ cells (Fig. 4B,C and Fig. 5G–N). The number of insulin⁺ β -cells was very similar to that in controls in 4-month-old *Mnx1^{Δbeta}* pancreas (Fig. S4D), and the vast majority were Cre⁻ and EYFP⁻, suggesting derivation from escaper β -cells (Fig. 4E,F and Fig. 5A,B; Fig. S6A–C). *RIP2*-Cre functions in ~85% of β -cells, as judged by our Cre immunolabeling (Fig. S6A) and by previously

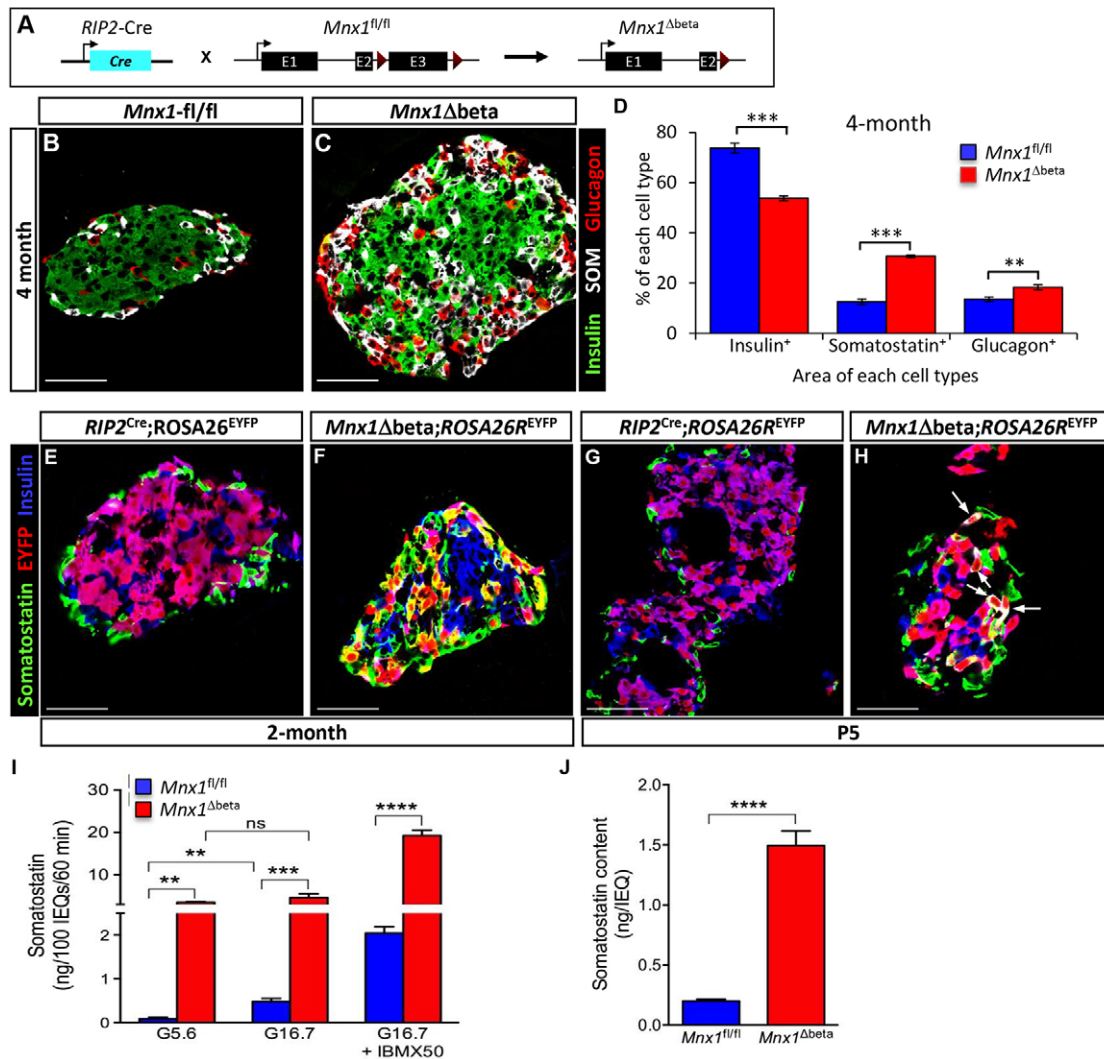
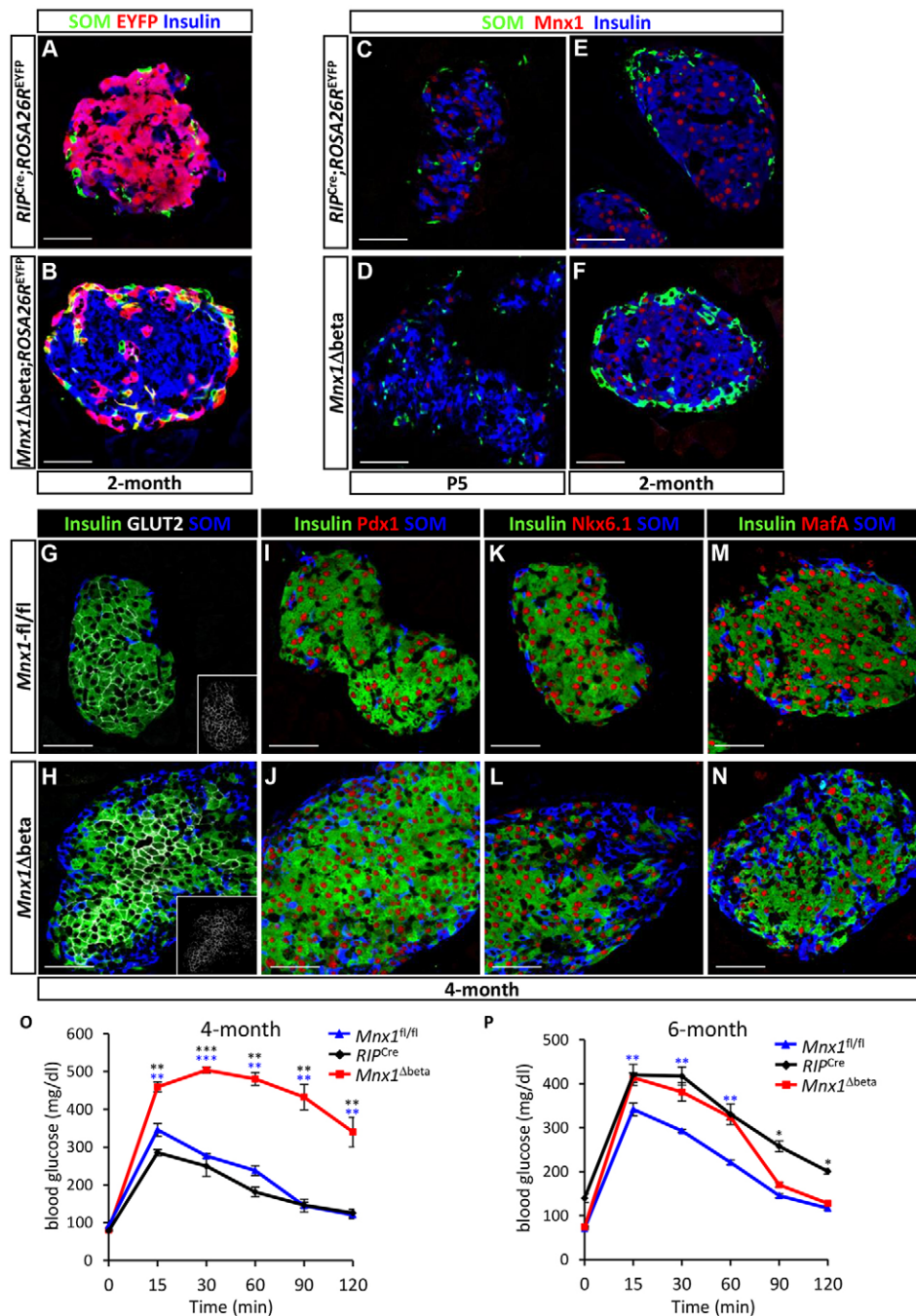


Fig. 4. β -to- δ cell transdifferentiation in $Mnx1^{\Delta\beta}$ mutants. (A) Schematic showing β -cell-specific $Mnx1$ deletion using $RIP2$ -Cre transgenics. (B,C) Immunolabeling with insulin, somatostatin and glucagon showed an increase in δ - and α -cells, as well as larger islet size in the $Mnx1^{\Delta\beta}$ islets. (D) Quantitative analysis of β -, δ - and α -cell fraction indicate an increased in δ - and α -cell populations concomitant with decreased in percentage of β -cells ($n=3$). (E,F) Lineage-tracing analysis using $ROSA26R^{EYFP}$ reporter show co-expression of EYFP and somatostatin, indicating that a subset of δ -cells in the $Mnx1^{\Delta\beta}$ mutant are derived from β -cells. (G,H) β -to- δ transdifferentiation, as indicated by $EYFP^+$ somatostatin⁺ insulin⁺ cells (arrows), can be detected as early as P5. (I,J) Measurement of islet somatostatin secretion (I) and total somatostatin content (J) *in vitro* indicate that mutant δ -cells hypersecrete somatostatin and actively synthesize more somatostatin compared with control islets ($n=3$). Somatostatin secretion was normalized to islet number. Scale bars: 50 μ m. Data are shown as mean \pm s.e.m. ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$.

reported $ROSA26$ -based β -galactosidase production after $ROSA26R$ activation (Gannon et al., 2000), which agrees with our direct estimation of the efficiency of $RIP2$ -Cre-inactivation of $Mnx1$ in $Mnx1^{\Delta\beta}$ mice, using $Mnx1$ immunodetection. At P5, around 85% of β -cells were $Mnx1^-$ and ~15% were $Mnx1^+$ (Fig. 5C,D; Fig. S5C,D); these data also indicate efficient deletion of $Mnx1$. At 2 and 4 months of age, almost all β -cells in $Mnx1^{\Delta\beta}$ mice were Cre^- and $Mnx1^+$ (Fig. 5E,F; Fig. S6C), and the β -cell proportional representation in islets was reproducibly similar to controls, suggesting that the initial 15% escaper β -cells had repopulated the islets (more directly addressed in the following section below). We propose that in the $Mnx1^{\Delta\beta}$ pancreas, β -cells gradually transdifferentiate into δ - (and α -) cells after birth, and escaper β -cells expand in number to repopulate the islets. The elevated ad-lib-feeding glycemia in mice between birth and 1 month of age occurs over the active β -to- δ transdifferentiation phase, wherein there are insufficient escaper β -cells to maintain euglycemia. As escaper

β -cells repopulated the islets, ad-lib-feeding blood glucose improved with age (Fig. S3A-D).

The putative escaper cells produced normal, mature β -cell-specific markers, such as $Glut2$ (Fig. 5G,H), $Pdx1$ (Fig. 5I,J), $Nkx6.1$ (Fig. 5K,L) and $MafA$ (Fig. 5M,N) compared with 4-month-old controls. The 4-month-old $Mnx1^{\Delta\beta}$ mice did show abnormal glucose clearance by intraperitoneal glucose tolerance testing (IPGTT; Fig. 5O), but this improved by 6 months of age (Fig. 5P). The glucose intolerance was not a result of peripheral insulin resistance, because there was a normal insulin-tolerance response (Fig. S7A). We reasoned that the increase in δ -cell number and somatostatin secretion in $Mnx1^{\Delta\beta}$ islets caused impaired insulin secretion and the glucose-intolerance phenotype. We tested this idea by insulin secretion assays on *in vitro*-cultured islets from 6-month-old mice. We chose this stage to assure the completion of all β -to- δ transdifferentiation, and to allow sufficient time for the functional maturation of the repopulating



escaper β-cells. Our data demonstrated that even in the presence of high somatostatin levels (Fig. 4I), insulin secretion was not blocked. Instead, more insulin was being produced in the mutant islets, and then secreted in response to glucose or glucose + IBMX, as shown by insulin secretion assay (Fig. 6A) and total plasma insulin levels *in vivo* (Fig. 6B). Consistent with these results, *Ins1* and *Ins2* mRNAs were highly upregulated in mutant islets (Fig. 6C; Fig. S8C), possibly as a result of increased *Mnx1* mRNA and protein levels (Fig. 6D; Fig. S8A, B), because *Mnx1* is a potent regulator of insulin transcription and secretion (Shi et al., 2013). Altogether, escaper β-cells in the *Mnx1*^{Δββ} islets were mature, they produced and secreted more insulin, and became insensitive to the inhibitory effect of somatostatin on insulin secretion.

Continued proliferation of escaper β-cells and islet hyperplasia in aged *Mnx1*^{Δββ} mice

During the course of analysis, we realized that average islet size seemed slightly larger in *Mnx1*^{Δββ} mutants at 4 months (Fig. 5H, J, L, N) and 6 months of age (data not shown), suggesting possible increased β-cell replication, transdifferentiation of β-cells from other endocrine lineages, or β-cell neogenesis. The latter two possibilities would need to be determined by a currently unavailable two-tier lineage tracing strategy, presumably including a non-Cre method. Nevertheless, our detection of ~twofold increase in β-cell proliferation at 1 and 4 months of age (Fig. 6E, F) was consistent with the time when active β-to-δ transdifferentiation occurred, and the Cre⁻ escaper β-cells had started to repopulate the islet (Fig. S6). Interestingly, the absence

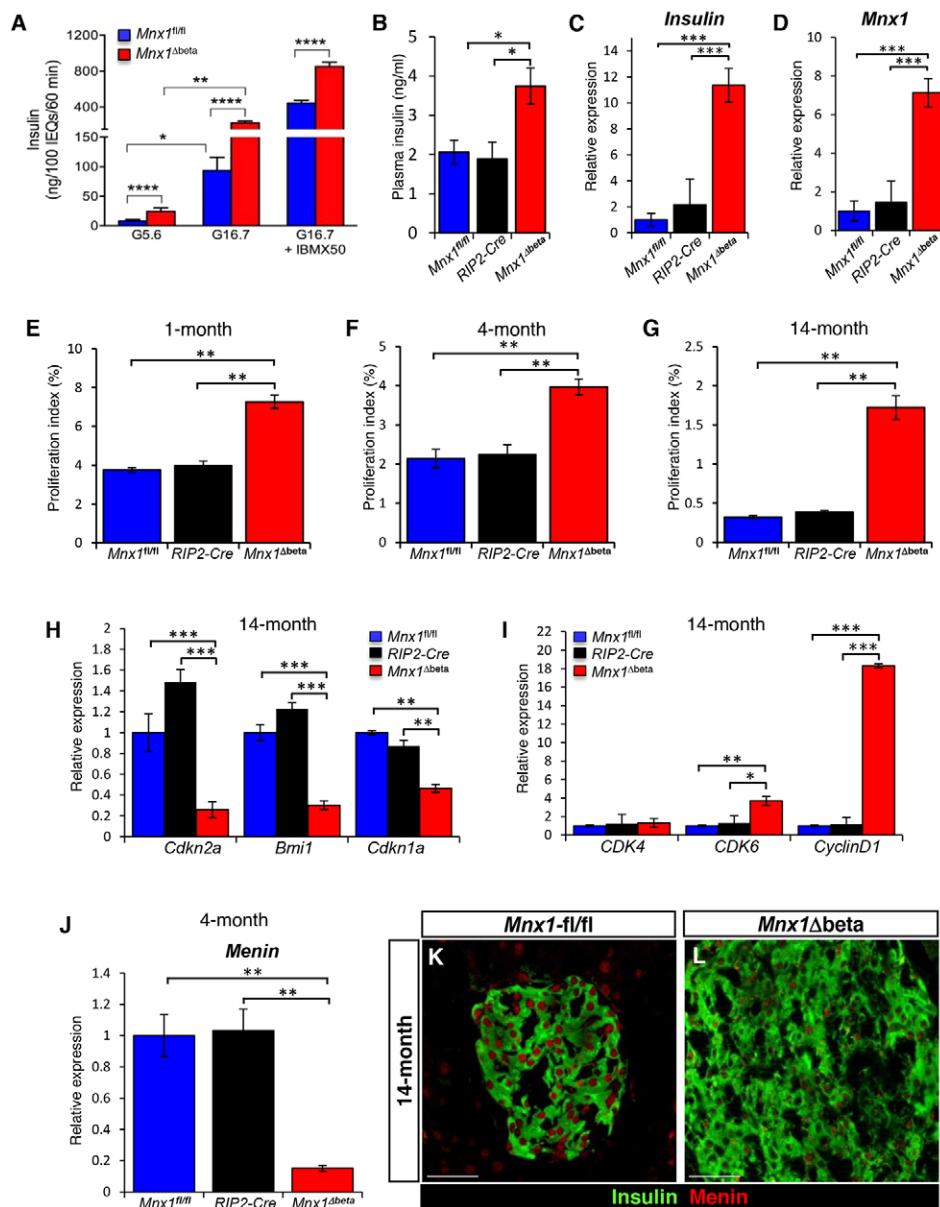


Fig. 6. Escaper β -cells secrete and produce more insulin, and continue to proliferate in $Mnx1^{\Delta\beta}$ aged mice via downregulation of menin. (A,B) Measurement of insulin secretion from isolated islets (A) and plasma insulin levels (B) indicate that $Mnx1^{\Delta\beta}$ β -cells secrete more insulin ($n=3$). Insulin secretion was normalized to islet number. (C,D) qRT-PCR analysis indicates significantly increased mRNA expression for *Ins2* (C) and *Mnx1* (D) in $Mnx1^{\Delta\beta}$ β -cells ($n=4$). (E-G) $Mnx1^{\Delta\beta}$ β -cells showed increased proliferation as determined by Ki67 at 1 month ($n=4$; E), 4 months ($n=4$; F) and 14 months ($n=4$; G). (H,I) qRT-PCR analysis show decreased expression of cell-cycle inhibitors *Cdkn2a*, *Bmi1* and *Cdkn1a* (H), and increased expression of cell-cycle-positive regulators *Cdk6* and *Ccnd1* (I). (J) qRT-PCR analysis on 4-month-old islets RNA shows that *menin* mRNA expression was also downregulated in the $Mnx1^{\Delta\beta}$ islets. (K,L) Immunolabeling of menin showed that menin protein was significantly reduced in $Mnx1^{\Delta\beta}$ β -cells at 14 months. Menin protein level was similar in $Mnx1^{fl/fl}$ and *RIP2-Cre* β -cells, indicating no effect on Menin from the transgenic Cre driver. Scale bars: 50 μ m. Data are shown as mean \pm s.e.m. * $P<0.05$, ** $P<0.005$, *** $P<0.001$, **** $P<0.0001$.

of Cre (the *RIP2-Cre* remained inactive) and the increased proliferation of escaper β -cells in $Mnx1^{\Delta\beta}$ mutants were persistent, with ~ 5 -fold increase in Ki67⁺ β -cells in 14-month-old mutant islets (Fig. 6G; Fig. S6D-F). These data were supported by qRT-PCR analysis showing decreased expression of several cell-cycle inhibitors (*Cdkn2a*, *Bmi1*, *Cdkn1a*), whereas positive cell-cycle regulators (*Cdk6* and *Ccnd1*) were markedly increased (Fig. 6H,I). In addition, menin, a β -cell proliferation inhibitor and tumor suppressor, was downregulated at both mRNA (Fig. 6J) and protein levels (Fig. 6K,L) in escaper β -cells in $Mnx1^{\Delta\beta}$ mice as early as 4 months of age, and its decreased expression was maintained in 14-month-old mice. This finding is consistent with a recent report that menin interacts with and inhibits *Mnx1* in regulating MIN6 cell line proliferation (Shi et al., 2013). The increased β -cell proliferation in $Mnx1^{\Delta\beta}$ mice ultimately led to islet hyperplasia (Fig. 7E,F), and doubling of endocrine and β -cell numbers at 14 months (Fig. 7A,B). The observed differential in proliferation index is sufficient to cover the increased β -cell number occurring over the prolonged period

from 4 to 14 months in $Mnx1^{\Delta\beta}$ islets. The differential in β -cell proliferation could have been even greater at the untested intermediate time points. Both δ - and α -cell numbers were also increased because of the β -cell transdifferentiation (Fig. 7C,D). Considering that only $\sim 15\%$ of β -cells retained *Mnx1*-positivity at P5 in $Mnx1^{\Delta\beta}$ mutants, there was a remarkable ~ 13 -fold increase in β -cell number (*Mnx1⁺insulin⁺*) between P5 and 14 months of age compared with controls. Even with the doubled β -cell number, the fraction of β -cells within islets remained similar to control because of the increase in other cell types (Fig. S8C). Thus, persistent replication of escaper β -cells, likely involving downregulation of menin and bypassing the progressive inhibition of β -cell proliferation in normally aging mice, eventually led to islet hyperplasia (Fig. 7H).

Escaper β -cells alleviate hyperglycemia upon STZ treatment at 6 months of age

We reasoned that, if the cell progeny from escaper β -cells were functionally mature and maintained such proliferative capability in

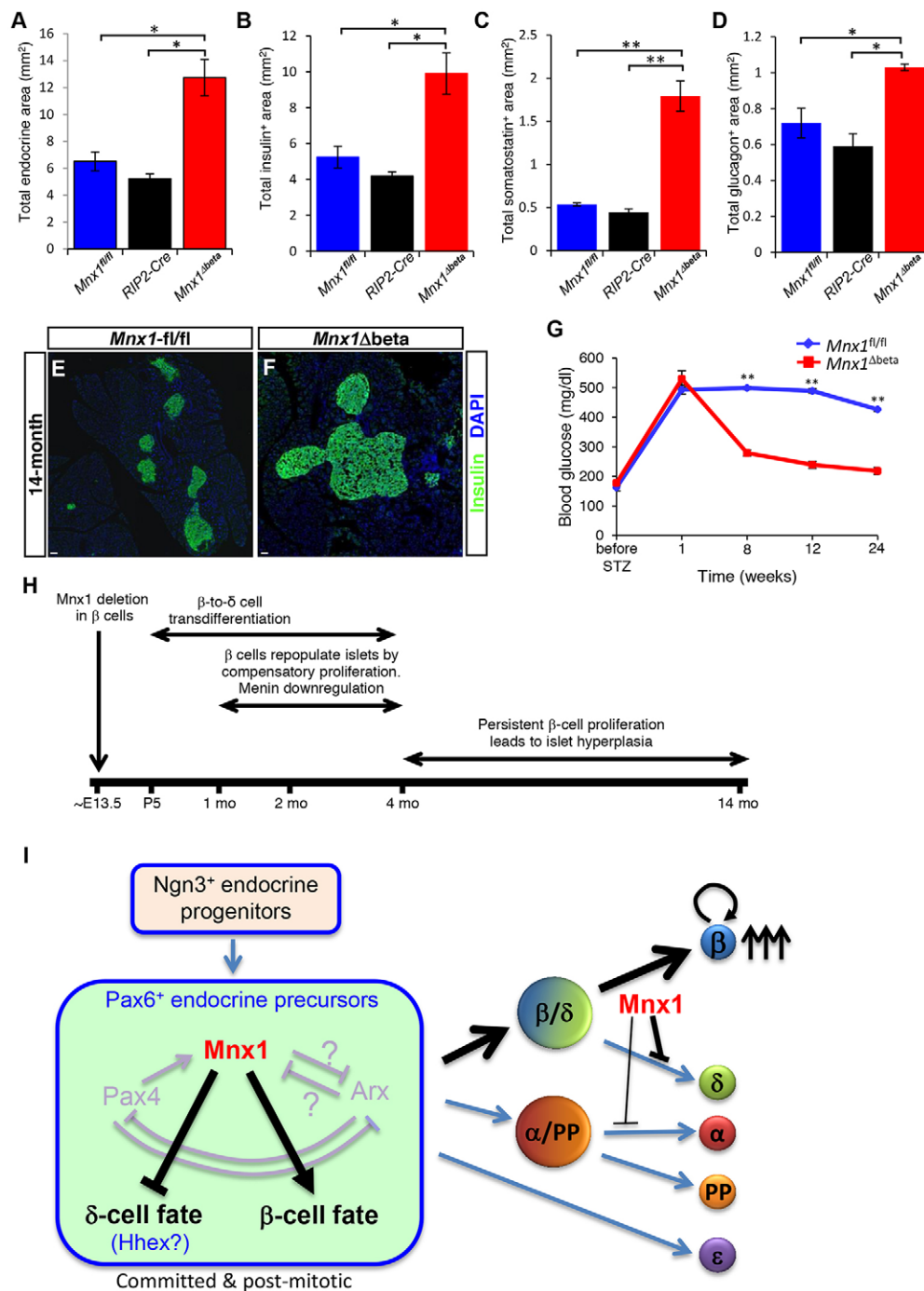


Fig. 7. Sustained proliferation of escaper β-cells leads to islet hyperplasia in *Mnx1^{Δbeta}* aged mice, and alleviates hyperglycemia following STZ treatment. (A-D) Quantification of total area of hormone⁺ (A), insulin⁺ (B), somatostatin⁺ (C) and glucagon⁺ (D) area demonstrate a twofold increase in total endocrine, insulin⁺ and glucagon⁺ area, in addition to the approximately fourfold increase in somatostatin⁺ area. (E,F) Immunolabeling with insulin shows the presence of hyperplastic islet in *Mnx1^{Δbeta}* pancreata. (G) Measurement of resting blood glucose over 6-month period upon streptozotocin (STZ) treatment showed the return of blood glucose to normal levels 2 months post-STZ treatment compared with *Mnx1^{fl/fl}* mice. (H) Summary of processes occurring in *Mnx1^{Δbeta}* mutant. (I) Model showing novel Mnx1 function in promoting β-cell fate and suppressing δ-cell fate in the Pax6⁺ endocrine precursors. Mnx1 is continuously required to maintain β-cell fate in the developing β-cells. When Mnx1⁻ β-cells transdifferentiate into δ-cells, a subset of Mnx1⁺ β-cells start to repopulate islets by proliferation, leading to islet hyperplasia in aged mice. Scale bars: 100 μm. Data are shown as mean±s.e.m. **P*<0.05, ***P*<0.005.

older mice, they might also be capable of repopulating the islet and restoring euglycemia in the experimental setting of ablating most pre-existing escaper β-cells in aged mice. We killed β-cells with two high doses of streptozotocin (STZ) in 6-month-old control and mutant mice and monitored blood glucose over six months. As in our previous study (Pan et al., 2013), mice with 70-80% STZ-mediated β-cell ablation often exhibited hyperglycemia (of >350 mg/dl) within one week post-injection. STZ-injected control mice remained hyperglycemic for up to 24 weeks post-STZ treatment. By contrast, *Mnx1^{Δbeta}* mice returned to euglycemia within 8 weeks post-STZ (Fig. 7G). These data indicate that functional escaper β-cells in aged mice were more capable than controls in repopulating the islets and alleviating hyperglycemia.

DISCUSSION

Here, we present evidence on the differential function of Mnx1 across several development stages in different pancreatic islet cell types. Apart from its initial function in dorsal pancreas specification, Mnx1 appears to be a lineage-allocation factor, regulating cell-fate choice in Pax6⁺ endocrine precursors to become β-cells instead of δ-cells. As development progresses, Mnx1 has an essential role in β-cell fate maintenance. We also discovered a previously unanticipated, non-autonomous role of Mnx1 in β-cell proliferation. These unexpected results not only allow us to begin to elucidate the molecular mechanisms regulating β-cell proliferation via the Mnx1-menin route, but also highlight the potential implication of δ-cells and intra-islet communication in β-cell proliferation.

Mnx1 as a β -cell lineage-allocation factor

Loss of *Mnx1* function in endocrine progenitors resulted in mutant pups with hyperglycemia at early postnatal stages. This phenotype is similar to the recently reported human homozygous *Mnx1* mutation that resulted in permanent neonatal diabetes, suggesting a highly conserved *Mnx1* function between mouse and human. The presence of normal exocrine mass in this human with *Mnx1* mutation strongly suggested that the dorsal pancreas still formed, and that the *Mnx1* requirement was at either the endocrine-precursor stage or in the Sox9⁺ bipotent progenitor pool, showing an equivalent role in β -cell specification in human and mouse (Bonnefond et al., 2013). It is noteworthy that the persistent *Mnx1* expression we found in human fetal and adult duct cells implies a possible function in maintaining the ductal lineage. This hypothesis awaits future testing, probably *in vitro*.

Our data demonstrated a marked increase in δ -cell numbers concomitant with reduced β -cell numbers in *Mnx1* ^{Δ endo} mutants that was caused by defective early-lineage allocation in the endocrine precursors and postnatal β -to- δ cell transdifferentiation. These data extend the findings of Li et al. (1999) in the global *Mnx1* null mutants, and show that *Mnx1* is required in the endocrine precursor, but not MPC population for β -cell lineage allocation and differentiation. Our results are in contrast, however, to findings in zebrafish, in which *Mnx1* is required to suppress α -cell fate while promoting β -cell fate, with the most important difference being that δ -cell numbers were distinctly unaffected in morphants (Dalgin et al., 2011). This discrepancy is probably due to a slight difference in gene regulatory modules controlling endocrine-cell differentiation between mouse and zebrafish. Nevertheless, in both species, *Mnx1* is a novel lineage-allocation factor for β -cells.

The dramatic increase in δ -cell numbers in the *Mnx1* ^{Δ endo} mutants suggests that *Mnx1* is required to suppress the δ -cell program in endocrine precursors (Fig. 7I). Recent studies from Zhang et al. (2014) demonstrated that loss of *Hhex* in endocrine progenitors and adult δ -cells led to a complete loss of δ -cells, indicating that *Hhex* is a principal determinant of δ -cell fate during endocrine lineage allocation and postnatal δ -cell fate maintenance. Prior studies in *Drosophila* and mouse reported that *Mnx1*, via its tinman repressor domain, acts predominantly as a repressor in neuron subtype determination (Broihier and Skeath, 2002; William et al., 2003; Lacin et al., 2014). These studies, together with our observation of a substantial increase in *Hhex*⁺ δ -cell precursor numbers in the *Mnx1* ^{Δ endo} mutants, lead us to hypothesize that *Mnx1* might also act as a repressor in the pancreas, suppressing δ -cell programming through direct or indirect inhibition of *Hhex* expression in endocrine precursors and β -cells. In addition, the presence of potential *Hhex* binding sites in *Mnx1* regulatory regions (Klaus Kaestner, personal communication) further suggests that cross-repressive interactions establish and maintain their mutually exclusive expression in the β - versus δ -cell. A similar mutual inhibition of TFs in endocrine cell fate determination was previously reported for *Pax4* and *Arx* in β - versus α -cell differentiation (Collombat et al., 2003).

A remarkable increase in δ -cell numbers at the expense of β -cells, which occurs in the *Mnx1* ^{Δ endo} mutant, was also observed in *Pax4*^{-/-}*Arx*^{-/-} and *Arx*^{-/-}*Nkx2.2*^{-/-} double-null mutants (Collombat et al., 2005; Kordowich et al., 2011; Mastracci et al., 2011), suggesting possible genetic interaction between *Mnx1*, *Pax4*, *Arx* and *Nkx2.2*. In the *Pax4*^{-/-} pancreas, *Mnx1* expression is undetectable in β -cells (Wang et al., 2004), indicating that *Mnx1* might act downstream of *Pax4* in promoting β -cell fate. Furthermore, we observed a slight increase in α -cell numbers in

the *Mnx1* ^{Δ endo} mutants, suggesting that *Mnx1* also functions to suppress *Arx* and prevent α -cell program activation in endocrine progenitors or precursors (Fig. 7I). Future valuable insight could be gleaned from elucidating the epistatic relationship of *Mnx1* with these TF genes, and how it fits into the transcriptional hierarchy controlling endocrine lineage diversification.

Mnx1 in β -cell fate maintenance

Inactivation of *Mnx1* in the developing β -cells leads to postnatal β -to- δ -cell, and, to a lesser extent, β -to- α -cell, transdifferentiation, indicating that *Mnx1* is required continuously to maintain β -cell fate after specification. The activation of *Hhex* and *Arx* (not shown) in β -cells upon *Mnx1* deletion suggests that *Mnx1* uses a repressive mechanism similar to those described above in endocrine precursors to suppress δ - and α -cell programs in β -cells. Future identification of the types of co-factors that form the *Mnx1* repressor complex will provide insights on the epigenetic regulation and molecular mechanisms of how *Mnx1* maintains β -cell fate. Likewise, inactivating *Mnx1* in mature β -cells with inducible systems will help addressing *Mnx1* function in this population in the future.

We noticed that the *Mnx1* ^{Δ beta} mice still had impaired IPGTT at 4 months old, although, by this age, β -cell mass was already restored to normal. In addition, the defect in glucose clearance in this mutant was not a result of peripheral insulin resistance (Fig. S7A). Thus, the newly formed escaper β -cells might not be fully functional at this stage, a suggestion consistent with the finding of lower *MafA* levels in an appreciable number of these escaper β -cells. An alternative explanation is that the newly formed escaper β -cells within the islet (at 4 months) are relatively sensitive to the somatostatin-based inhibition of insulin secretion but, with aging, develop increased resistance to the inhibitory effect after long-term adaptation to high somatostatin levels. Nonetheless, these escaper β -cells apparently became fully functional by 6 months of age, as indicated by *in vitro* insulin secretory assay, and based on their ability to restore euglycemia after STZ-induced β -cell ablation in aged mice.

Unexpected β -cell compensatory growth in *Mnx1* ^{Δ beta}: β -cell proliferation-promoting signals

One surprising finding was the compensatory islet repopulation and eventual β -cell hyperplasia, which we propose arose from the Cre⁻ β -cells escaping *Mnx1* inactivation. We do not think that these large numbers of insulin-producing cells arose by transdifferentiation from an alternate minor endocrine lineage, namely the δ -cell, such as reported by Chera et al. (2014). There are differences between our study and theirs. Postnatal δ -to- β transdifferentiation observed by Chera et al. occurred under extreme (95–95%) β -cell diphtheria toxin-based ablation. The presence of substantial numbers of functional β -cells in the *Mnx1* ^{Δ beta} mice (15% escaper β -cells plus 85% of still-transdifferentiating insulin⁺somatostatin⁺ β -cells) should be insufficient to generate the signals that stimulate δ -to- β transdifferentiation. All other β -cell-directed Cre tools (*Pdx1*-CreER, *MIP*-CreER, *RIP*-CreER) have at best 80–90% recombination efficiency and therefore also create a situation in which the β -cell pool could include cells derived from, for example, wild-type δ -cells. Detecting such a potential contribution would require a currently unavailable second level of non-Cre-based δ -cell-specific lineage tracing. Conversely, strong evidence in favor of escaper β -cells being the primary repopulating pool is the doubling of β -cell proliferation observed when β -to- δ cell transdifferentiation was occurring at 1 month, and at 4 months, when the process was slowing down. This β -cell proliferation was

even more pronounced (four- to fivefold) at 14 months in association with the β -cell hyperplasia in aged $Mnx1^{\Delta\beta}$ mice.

Similar β -cell compensatory growth occurs in mice double-heterozygous for insulin receptor and insulin receptor substrate 1 ($IR^{+/-}IRS1^{+/-}$), and in liver-specific insulin-receptor knockout (LIRKO) mice (Michael et al., 2000; Kido et al., 2000). Whereas $IR^{+/-}IRS1^{+/-}$ and LIRKO mice develop hyperglycemia and peripheral insulin resistance at 6 months, the $Mnx1^{\Delta\beta}$ mutants remained euglycemic and only developed mild insulin resistance and glucose intolerance at 20 months of age (Fig. S7B,C). Thus, $Mnx1^{\Delta\beta}$ mutants serve as a new model to study the mechanisms of compensatory and persistent β -cell proliferation without hyperglycemia and insulin resistance.

It will take much more work to identify the exact signal(s) that stimulated β -cell expansion in the $Mnx1^{\Delta\beta}$ mutants, which comprises compensatory growth to restore the β -cell mass towards normal over the first 16 weeks of life, and later-stage islet hyperplasia associated with persistent β -cell proliferation (Fig. 7H). It is possible that the large numbers of δ -cells derived from β -cell transdifferentiation, and which seem substantially different from normal δ -cells, based on their hypersecretory state, produce novel signals to stimulate β -cell proliferation by acting locally (intra-islet) or systemically. Future co-transplantation of mutant δ -cells together with wild-type islets under the kidney capsule could be one way of addressing this possibility.

Previous studies also suggest several other possible influences. The hyperglycemic state in P5- to 1-month-old $Mnx1^{\Delta\beta}$ mutant mice might stimulate an initial compensatory phase of β -cell proliferation, because elevated glucose levels trigger modest β -cell replication in young mice (Salpeter et al., 2010, 2011). Nevertheless, as glycemia in $Mnx1^{\Delta\beta}$ mice had reproducibly returned to normal by 2 months of age, we presume that the longer-term sustenance of escaper β -cell proliferation arises from different stimuli.

The lower level of menin (an inhibitor of β -cell proliferation; Karnik et al., 2007) could directly or indirectly contribute to the persistent β -cell proliferation of escaper β -cells in older $Mnx1^{\Delta\beta}$ mice. Aged $Men1^{+/-}$ mice have hyperplastic islets and develop insulinomas (Shi et al., 2013; Desai et al., 2014). Menin interacts physically with Mnx1 to inhibit β -cell proliferation, and menin downregulation in MIN6 or human insulinoma cells causes Mnx1 accumulation, with the stabilized phospho-Mnx1 having anti-apoptotic characteristics and positively regulating genes that modulate insulin levels (Shi et al., 2013; Desai et al., 2014). Thus, menin downregulation probably increases $Mnx1$ mRNA and protein levels, and the increased Mnx1 then feeds forward to increase insulin expression, as well as inducing cell-cycle positive-acting factors and reducing cell-cycle-inhibitor expression. It will be valuable in the future to elucidate better which signal(s) downregulate(s) menin expression and linkages with how Mnx1 regulates β -cell proliferation. Prior findings showed that insulin signaling via the Foxo1/Pdx1/insulin pathway is a predominant influence in compensatory β -cell growth in LIRKO mice (Okada et al., 2007). Therefore, the elevated insulin production and secretion in $Mnx1^{\Delta\beta}$ mutant escaper β -cells could act in an autocrine or paracrine manner to increase β -cell proliferation. Together, the combined menin downregulation, elevated Mnx1 and increased insulin production might all contribute to causing the persistent proliferation of escaper β -cells. Understanding how these factors interact to drive the cell-cycle forward and bypass the age-inhibitory effect on β -cell proliferation could help to discover ways to restore endogenous β -cells or β -cell function in diabetic patients.

MATERIALS AND METHODS

Mice

The $Mnx1$ floxed allele was described in Harrison et al. (1999). $Ngn3$ -Cre (Schonhoff et al., 2004), $RIP2$ -Cre (Gannon et al., 2000), $Ella^{Cre}$ (Lakso et al., 1996) and $ROSA26R^{EYFP}$ (Srinivas et al., 2001) mice were described previously. Animals and embryos were PCR-genotyped. All experiments were under protocols approved by Vanderbilt University IACUC.

Tissue preparation and immunostaining

Embryonic or adult mouse pancreas and human fetal pancreas were fixed (2–4 h, 4% paraformaldehyde, 4°C), and, for cryosections, were washed twice in cold PBS, sucrose-equilibrated (30%, 4°C, overnight) and OCT-embedded (Tissue-Tek, Sakura). Immunofluorescence staining was performed on 10- μ m cryosections (Kawaguchi et al., 2002). Antibodies used are shown in Table S1.

Data collection, morphometric and statistical analysis

Images from Zeiss confocal (LSM 510 META upright) or Apotome were analyzed with LSM Image Browser and Zeiss Axiovision 4.8 software, respectively. As there was no difference in cell size between endocrine cell types in control and mutant, measurements of endocrine cell area were performed based on whole-section imaging with scanning (ScanScope FL; Aperio Technologies) followed by systematic Quantification analysis performed by the NIH ImageJ software. For endocrine cell number and area at E16.5 and E18.5, every sixth section (at least 60 μ m separated; \sim 20 sections per tissue) was counted. For adult pancreas, every twentieth section (at least 200 μ m apart; \sim 15–20 sections per tissue) was counted. β -cell proliferation was assessed by either Ki67 labeling (6000–8000 β -cells/tissue analyzed). For all quantitative analyses, $n \geq 3$ mice were used per group. Comparisons between two groups were carried out by Student's *t*-test. Data are mean \pm s.e.m. Statistical significance was assigned when $P < 0.05$.

RNA extraction and qRT-PCR

RNA isolation (Trizol, Invitrogen), DNase treatment (Ambion), cDNA synthesis and qPCR (SYBR Green, Bio-Rad) were performed using *GAPDH* as housekeeping gene and the primer-probe sets shown in Table S2. Three to four samples per genotype per stage were collected, and qPCR was performed at least twice on each sample to determine ΔC_T . Results were subjected to Student's *t*-test to determine significance ($P < 0.05$).

Glucose tolerance testing, islet insulin and somatostatin secretion

Intraperitoneal glucose-tolerance testing (2.0 g glucose injected per kg body weight) was performed after a 14- to 16-h fast as described (Fujitani et al., 2006; Brissova et al., 2014). Islets were isolated by collagenase P digestion of the pancreas and handpicked under microscopic guidance to nearly 100% purity. Analysis of islet function using static incubation system was performed by the Vanderbilt Islet Procurement Core (P30 DK020593) as described previously (Dai et al., 2012). Insulin and somatostatin concentration in the culture medium, and insulin and somatostatin content in islet extracts, were determined by radioimmunoassay (insulin, RI-13K, Millipore; somatostatin, RK-060-14, Phoenix Pharmaceuticals).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.C.P. developed the concept, designed and performed experiments and data analysis, and wrote the manuscript. M.B. performed experiments and data analysis and edited the manuscript. A.C.P. performed manuscript editing. S.P. provided the published $Mnx1$ floxed allele. C.V.E.W. developed the concept, performed manuscript writing and editing.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.126011/-/DC1>

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