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Pancreatic Islet Progenitor Cells in Neurogenin 3-Yellow Fluorescent Protein Knock-Add-On Mice

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The basic helix-loop-helix transcription factor Neurogenin 3 (NGN3) controls endocrine cell fate specification in uncommitted pancreatic progenitor cells. *Ngn3*-deficient mice do not develop any islet cells and are diabetic. All the major islet cell types, including insulin-producing β-cells, derive from *Ngn3*-positive endocrine progenitor cells. Therefore, the characterization of this population of immature cells is of particular interest for the development of novel strategies for cell replacement therapies in type 1 diabetes. To explore further the biology of islet progenitor cells we have generated a mouse in which *Ngn3*-expressing cells are labeled with the enhanced yellow fluorescent protein (EYFP) using a knock-add-on strategy. In this approach, the *EYFP* cDNA is introduced into the 3′-untranslated region of the proendocrine transcription factor, Neurogenin 3, without deleting any endogenous coding or regulatory sequences. In $Ngn3^{EYFP/+}$ and $Ngn3^{EYFP/EYFP}$ mice, the EYFP protein is targeted to Ngn3-expressing progenitors in the developing pancreas, and islets develop normally. Islet progenitors can be purified from whole embryonic pancreas by fluorescence-activated cell sorting from $Ngn3^{EYFP/+}$ mice and their development can be monitored in real time in pancreas explant cultures. These experiments showed that endocrine progenitors can form *de novo* and expand, *in vitro*, in the absence of signals from the surrounding mesenchyme, suggesting that endocrine commitment is a default pathway. The $Ngn3^{EYFP}$ mice represent a valuable tool to study islet cell development and neogenesis in normal and diabetic animals as well as for the determination of the conditions to generate β-cells *in vitro*. (*Molecular Endocrinology* 18: 2765–2776, 2004)

GENE INACTIVATION IN the mouse has generated significant progress in our understanding of the hierarchy of transcription factors controlling the successive steps of islet cell differentiation (1, 2). This knowledge can now be translated to specifically label and characterize in depth islet progenitor cells at the molecular and cellular level. Such lineage labeling can be realized by genetically engineering mice to express various autofluorescent reporter proteins under the control of regulatory elements of transcription factors regulating key steps in islet cell development. Several studies have demonstrated the feasibility of monitoring simultaneously multiple spectrally distinct fluorescent proteins noninvasively within a single animal (3–5). Such a compound transgenic mouse or rainbow mouse will be instrumental in the purification and determination of the transcriptome and proteome of specific subtypes of islet progenitor cells. Moreover, multispectral visualization in live samples of pancreas cultures will also be possible, and the effect of extrinsic factors on the expansion and differentiation of various populations of tagged islet progenitors will be able to be tested. Finally, such tools will be of central importance for studies aimed at generating β-cells *in vitro* starting from various sources of plastic stem/progenitor cells to monitor normal islet gene expression and function. We believe that in the perspective of the generation of such a rainbow mouse, one essential feature is the faithful recapitulation of the spatiotemporal expression of the tagged genes by the fluorescent reporter proteins. Due to the potential lack of important regulatory elements, as well as positional effects, this cannot always be achieved by traditional promoter approaches or BAC transgenesis. An additional problem is that the random insertion of the foreign DNA within the genome might interfere with the function of other genes. Both of these problems are potentially circumvented using a more sophisticated and long term knock-in approach in which the endogenous gene is replaced by a fluorescent reporter pro-

Abbreviations: CMV-Cre, Cre recombinase under the control of cytomegalovirus promoter; dpc, days post coitus; E15.5, embryonic d 15.5; EGFP, enhanced green fluorescent protein; ES cell, embryonic stem cell; EYFP, enhanced yellow fluorescent protein; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; IRES, internal ribosome entry site; Ngn3, neurogenin 3; UTR, untranslated region.

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tein by homologous recombination in embryonic stem (ES) cells. In most cases, inactivation of one allele of islet-specific transcription factors does not impair endocrine differentiation. However, one cannot exclude that in a compound rainbow mouse, the multiple allelic combination of the different XFP variants targeted to the locus of genes controlling pancreatic endocrine cell development might generate an islet phenotype. Because of these considerations and with the perspective of the future generation of such a technicolor mouse, we decided to produce a mouse in which islet progenitor cells have been genetically labeled with the enhanced yellow fluorescent protein (EYFP) using a knock-add-on strategy. In this approach, the *EYFP* cDNA is introduced into the 3′-untranslated region (UTR) of the proendocrine transcription factor Neurogenin 3 (Ngn3) without deleting any endogenous coding or regulatory sequences.

The Neurogenin 3 transcription factor has emerged as a master gene controlling endocrine cell fate decision in pluripotent pancreatic progenitors during embryogenesis. *Ngn3*-deficient mice lack islets of Langerhans as well as islet precursor cells, and they develop diabetes and die postnatally (6). Lineage tracing experiments demonstrated that all the islet cell types, including the insulin-secreting β-cells, derive from Ngn3-expressing cells (7). These data, together with other studies (8–10), thus clearly demonstrate that *Ngn3* is the earliest marker of pancreatic epithelial cells committed toward an islet cell fate. *Ngn3* has also been shown recently to control enteroendocrine commitment in the developing and adult intestinal epithelium (11), whereas gastric endocrine cell differentiation is only partially dependent on *Ngn3* function (11, 12). For an in-depth characterization of the biology of immature islet progenitor cells, the issues of identification, visualization, and isolation of islet progenitor cells must be addressed. We thus decided to generate genetically labeled mice that would permit purification of pancreatic but also intestinal and gastric endocrine progenitor cells. Single transgenic mice, expressing the enhanced green fluorescent protein (EGFP) driven by *Neurogenin3* regulatory sequences using promoter (7) and knock-in (12) approaches have been reported previously. We describe here the production and characterization of a mouse generated by a knock-add-on strategy in which Ngn3-expressing cells are tagged with EYFP. We demonstrate the usefulness of this approach for the future generation of multiple fluorescent mice as well as for the study, in real time, of the development of islet progenitor cells in pancreatic explant cultures.

RESULTS

Generation of Ngn3-EYFP Mice

We have generated a mouse line expressing the enhanced yellow fluorescent protein (EYFP) targeted to the *Ngn3* locus using a knock-add-on approach. Briefly, a floxed internal ribosome entry site (IRES)-EYFP-Puromycine cassette has been introduced in the 3′-UTR of the mouse *Ngn3* gene by homologous recombination in ES cells (Fig. 1). This cassette insertion does not delete any sequence from the *Ngn3* locus. In previous efforts, a 3′-flanking region from the mouse *Ngn3* gene failed to target a *LacZ* reporter in Ngn3-expressing cells (Orvain, C., A. Dierich, and G. Gradwohl, unpublished observation). This observation suggested that the 3′-region lacks elements that regulate tissue-specific expression of the *Ngn3* gene, indicating that the introduction of the EYFP gene should not interfere with the normal regulation of *Ngn3* transcription. Two correctly targeted ES clones, as determined by Southern blot hybridization (Fig. 1C), have been injected into blastocysts. The resulting chimeric mice successfully transmitted the mutation to their progeny. The excision of the puromycin resistance gene (*pac*, puromycin *N*-acetyltransferase) has been performed by breeding the $Ngn3^{EYFP\text{-}Pac/+}$ mouse with a transgenic mouse expressing the Cre recombinase under the control of the cytomegalovirus promoter (CMV-Cre) (13) (Fig. 1D). We observed no obvious difference either in the expression pattern or in the fluorescence intensity of the EYFP protein in the mice carrying or not carrying the *pac* gene. Using a strictly identical strategy, we also generated EGFP-tagged mice (data not shown).

EYFP Is Targeted to Ngn3-Expressing Cells in the Pancreas

To determine whether the EYFP expression reflects the distribution of the Ngn3-expressing cells, we first examined directly the intrinsic EYFP fluorescence in developing embryonic pancreas. As shown in Fig. 2A, EYFP fluorescence was detected both in the dorsal and ventral embryonic pancreas in a pattern reminiscent of Ngn3-expressing cells, *i.e*. isolated or small clusters of cells located along the epithelial duct-like structures. To localize EYFP cells more precisely, we performed immunofluorescence experiments for EYFP on cryosections of pancreas at different developmental stages in $Ngn3^{EYFP/+}$ embryos (Fig. 2B). The EYFP expression mirrors the dynamic expression of *Ngn3*, first detected at the bud stage: the number of EYFP+ cells peaks at embryonic d 15.5 (E15.5) dpc (days post coitus) and decreases thereafter. At E18.5, only rare EYFP-expressing cells are detected in the developing pancreas as was previously observed for Ngn3 protein (data not shown). To further characterize EYFP+ cells, we performed double immunofluorescence for EYFP and either Ngn3 or endocrine hormones. With an antiserum specific for Ngn3, we first compared the overlap between EYFP- and Ngn3-expressing cells. As expected and shown in Fig. 3A, the EYFP protein is targeted to Ngn3+ progenitor cells starting from the early bud stage at E10.5 dpc, where most of the Ngn3 expressing cells coexpress EYFP. At

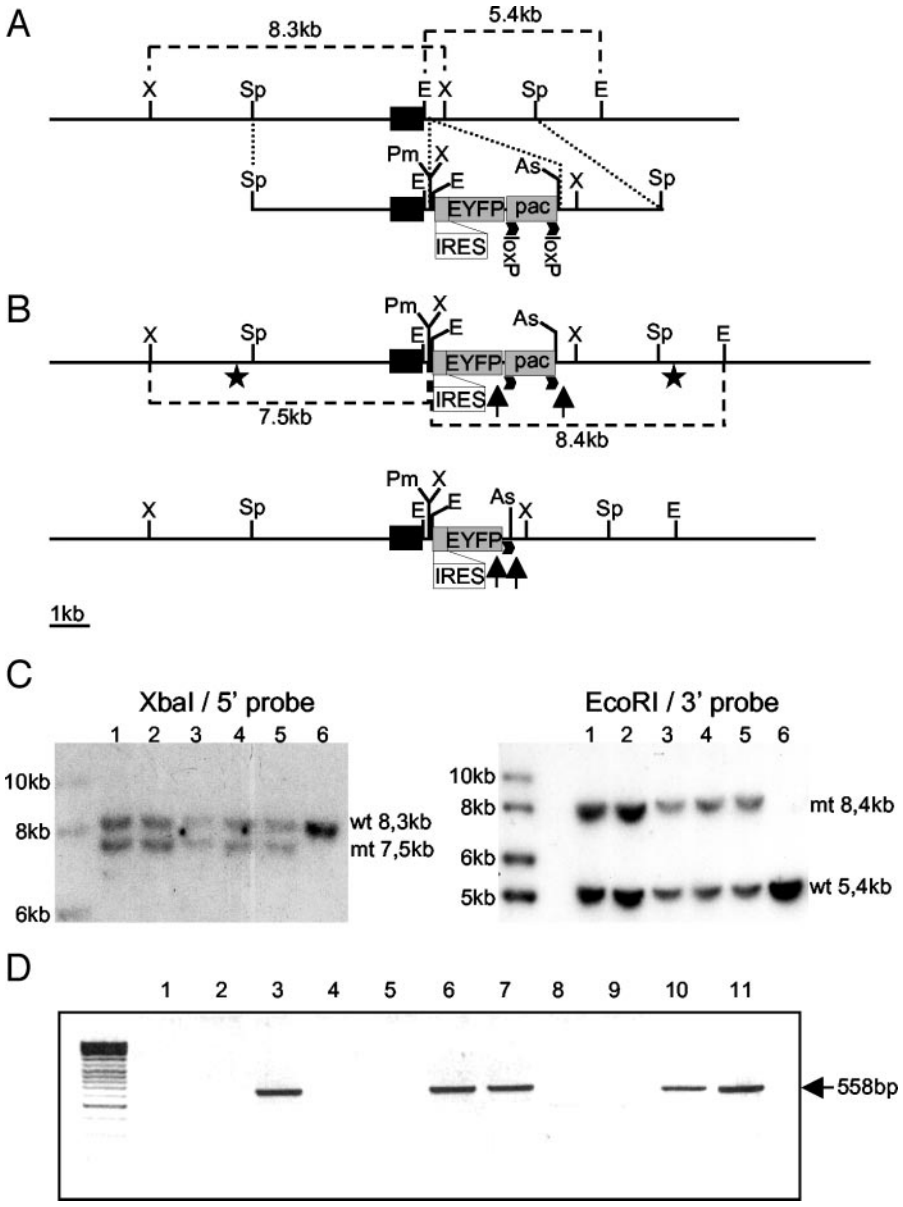

Fig. 1. Strategy for the Introduction of the EYFP Gene into the 3′-UTR of Ngn3 Locus by Homologous Recombination

Several independent correctly targeted ES cells clones could be established. A, Maps of the wild-type *Ngn3* locus and the targeting construct. B, EYFP-targeted *Ngn3* allele before and after the excision of the floxed puromycin resistance gene (*pac*) by the Cre recombinase. *Stars* in panel B indicate the position of the 5′- and 3′-external probes used for Southern blot analysis. *Black box* indicates the *Ngn3* coding sequence, the IRES-EYFP sequence, and puromycin resistance gene (pac); the loxP sites are indicated as well. C, Genotype analysis by Southern blots of six independent recombinated ES cell clones by hybridization with a 5′- and 3′-external genomic probe. D, Representative genotyping by PCR of a litter from intercrosses between $Ngn3^{EYFP/+}$ heterozygous and CMV-Cre mice with primers EYFP-pA-5′ and Ngn3–3′ as described in *Material and Methods*. When the pac gene has been successfully excised by the CRE recombinase, a 558-bp PCR band is amplified. Positions of primers are indicated by *arrows*. Wt, Wild type; mt, mutant; X, *Xba*I; Sp, *Spe*I; E, *Eco*RI; Pm, *Pme*I; As, *Asc*I.

E13.5, 88% and at E15.5, 83% of the Ngn3-expressing cells are labeled with EYFP, whereas not all of the EYFP expressing cells colabel for Ngn3 at all stages analyzed. At E13.5 the EYFP reporter is exclusively expressed in immature cells that do not express endocrine hormones (Fig. 3B). However, at E10.5 and at E15.5 dpc, approximately 12% of the EYFP-positive cells costain for insulin or glucagon (Ins/Glu). In summary, these studies show that in the EYFP knock-add-on *Ngn3* mice we generated, the EYFP transgene is, as expected, targeted to Ngn3 progenitors in the developing pancreas. A fraction of more mature cells is also labeled by the reporter protein, which is likely due to differences in the stability and/or turnover between Ngn3 and YFP proteins.

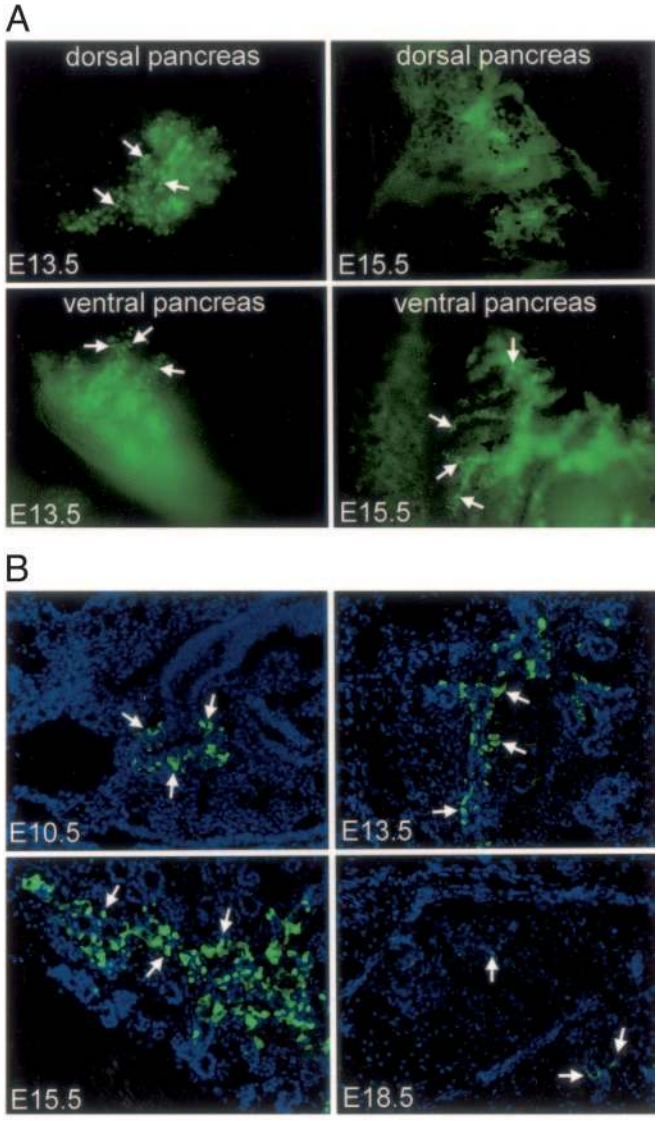

Fig. 2. EYFP Expression in the Pancreas from Ngn3$^{EYFP/+}$ Mice at Different Developmental Stages

A, Pancreases were harvested from *Ngn3*$^{EYFP/+}$ mice and intrinsic EYFP fluorescence observed in dissected dorsal and ventral pancreas at E13.5 and E15.5. *Arrows* indicate clear single EYFP-expressing cells. B, Immunofluorescence with an anti-EGFP antibody also recognizing EYFP showing the specific expression of EYFP in the pancreatic epithelium at different developmental stages. EYFP+ cells can be detected in a pattern reminiscent of Ngn3-expressing cells as soon as E10.5 in the dorsal pancreatic bud, showing its highest expression at E15.5, after which it gradually decreases. At E18.5 only a very few EYFP+ cells can still be detected. *Arrows* indicate clear EYFP+ cells. Magnification, ×20.

Labeling of Enteroendocrine and Neural Progenitor Cells in the Gastrointestinal Tract and Developing Central Nervous System (CNS)

Ngn3 is also expressed in isolated progenitors of the endocrine lineage in the embryonic and adult gastrointestinal tract. Intrinsic EYFP fluorescence of *Ngn3*-expressing cells can be observed in the embryonic intestine of *Ngn3*$^{EYFP/+}$ mice at E15.5 dpc (data not shown). The weak intensity of EYFP fluorescence and the paucity of *Ngn3*-expressing cells in the adult digestive tract hampered the identification of individual EYFP+ cells in the adult intestinal epithelium and the glandular stomach. Immunodetection with an antibody against EYFP/EGFP, however, clearly revealed rare and isolated EYFP+ cells in the adult duodenal crypts and the gastric epithelium of newborn mice in a pattern reminiscent of *Ngn3* expression in enteroendocrine progenitors (Fig. 4, A–C). As expected, we also successfully targeted the EYFP reporter protein to developing neural progenitors in the central nervous system (Fig. 4D) where *Ngn3*-expressing cells have also been described.

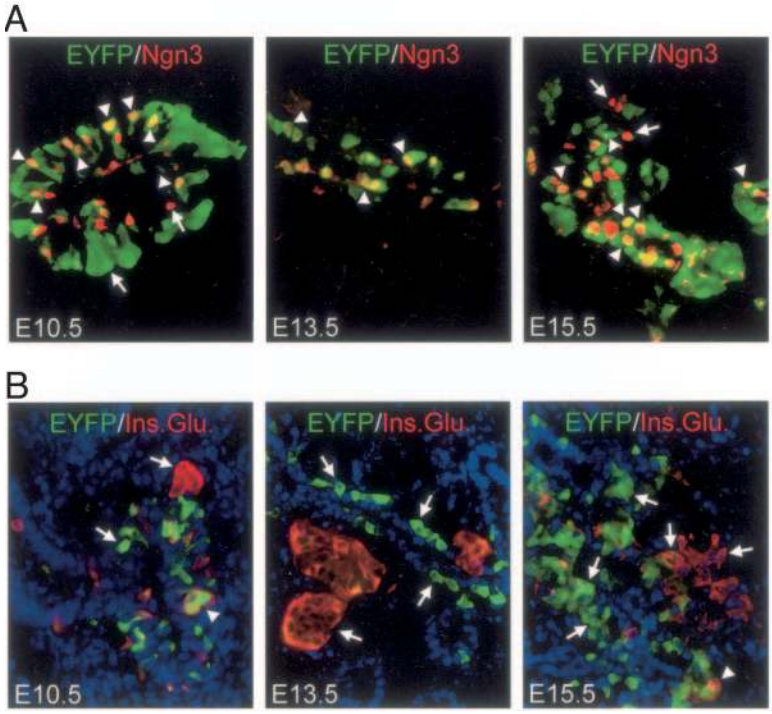

Fig. 3. Labeling of Ngn3-Expressing Cells with EYFP in the Developing Pancreas

A, Double immunofluorescence for EYFP (*green*) and Ngn3 (*red*) on cryosections of pancreas from *Ngn3*$^{EYFP/+}$ mice at different embryonic stages. EYFP is coexpressed in most of the Ngn3+ cells at E13.5 and E15.5. B, Double immunofluorescence for EYFP (*green*) and insulin/glucagon (*red*). EYFP is mostly expressed in undifferentiated islet progenitors cells that do not costain for hormones. EYFP is coexpressed with insulin and glucagon (Ins/Glu) in some cells at E10.5 and E15.5 (*arrowheads*), whereas at E13.5 its expression is exclusive. The fact that not all cells coexpress EYFP and Ngn3 might be due to a longer stability of the EYFP protein and/or the low sensitivity of the Ngn3 antibody. *Arrows* in panels A and B indicate examples of EYFP, Ngn3, or Ins/Glu single positive cells, whereas *arrowheads* indicate cells either coexpressing EYFP and Ngn3 or EYFP and Ins/Glu. Magnification, ×40.

Ngn3$^{EYFP/EYFP}$ Mice Do Not Develop Diabetes

Ngn3 mutant mice develop diabetes and very rapidly die at early postnatal stages. To determine whether, as expected, the insertion of the EYFP in the 3′-UTR of *Ngn3* locus does not interfere with *Ngn3* proendocrine function we generated *Ngn3*$^{EYFP/EYFP}$ mice. In contrast to *Ngn3*-deficient mice the *Ngn3* $^{EYFP/EYFP}$ animals develop normally and reach adulthood. Islets of Langerhans can clearly be observed (Fig. 5A), and we did not find any difference in the urine and blood glucose content between wild-type and transgenic mice. Moreover, ip glucose tolerance tests performed on 8- to 10-wk-old mice showed no significant difference in the response between wild-type, *Ngn3*$^{EYFP/+}$, and *Ngn3* $^{EYFP/EYFP}$ mice (Fig. 5B). Thus, islet cells are correctly specified demonstrating that the introduction of the EYFP tag at the *Ngn3* locus does not perturb the normal function of this gene during islet cell development.

Fluorescence-Activated Cell Sorting (FACS) Analysis of EYFP-Labeled Endocrine Progenitor Cells

The opportunity to isolate and study the population of Ngn3-expressing islet and enteroendocrine progenitor cells is one of the main purposes of the *Ngn3*$^{EYFP/+}$ mouse. To test whether endocrine progenitors can be purified, embryonic pancreases were dissociated to single cells by enzymatic digestion and subjected to FACS. The EYFP-labeled endocrine progenitor cells could readily be detected and purified by FACS (Fig. 6). Approximately 1300 EYFP+ cells can be purified from one E15.5 pancreas. Although intrinsic EYFP fluorescence was weaker in the intestine than in the pancreas, an average of 1700 EYFP+ enteroendocrine progenitors were successfully purified from each E15.5 small intestine (data not shown). This result demonstrates that a single-copy insertion of EYFP expressed under the control of *Ngn3*-regulatory sequences provides enough intrinsic fluorescence to detect and sort single EYFP-expressing cells.

Development of EYFP-Labeled Islet Progenitors Cells *in Vitro* in Real Time

The labeling of islet progenitor cells with noninvasive fluorescent markers provides a unique tool to follow, *in vitro* and in real time, the induction and expansion of endocrine progenitor cells. We developed explant cultures of pancreatic epithelium dissected from

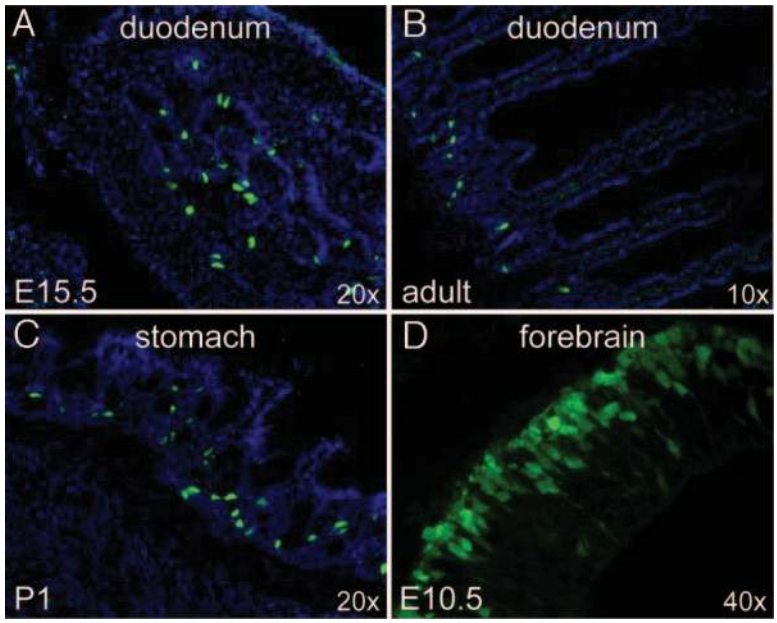

Fig. 4. EYFP Expression in Organs outside the Pancreas in Ngn3$^{EYFP/+}$ Mice

Fluorescence immunohistochemistry showing that EYFP expression follows the normal expression pattern of Ngn3 in organs outside the pancreas. EYFP+ cells can easily be detected in enteroendocrine progenitors in the duodenum during embryogenesis (A) and in the adult (B); in the latter it is restricted, like Ngn3, to the crypts as well as in the postnatal stomach (C). D, EYFP expression in neural progenitors of the developing forebrain neuroepithelium.

Ngn3$^{EYFP/+}$ E12.5 (d 0) embryos and removed from the surrounding mesenchyme. The development of islet progenitor cells can thus be followed in the same explant over the time of the culture period by simply monitoring the EYFP fluorescence. As shown in Fig. 7, at d 1 only a few islet progenitor cells are observed; their number next increases, peaks at d3–d4, and decreases at later stages when the number of α- and β-cells increases (data not shown). Like *in vivo*, this expression pattern reflects the wave of *Ngn3*+ cells observed during the secondary transition phase in the developing pancreas. To gain a more dynamic insight into endocrine progenitor cell development, time-lapse image acquisition of EYFP-labeled living cells was performed on E12.5 dpc embryonic pancreas epithelium between d2 and d4 of culture (see video published as supplemental data on The Endocrine's Society's Journals Online web site at http://mend.endojournals.org). In this sequence the expansion of the EYFP+ endocrine progenitor cells can clearly be observed during this period, with cells turning on and then off the EYFP protein, illustrating the transient expression of the *Ngn3* gene in the developing endocrine lineage. Importantly, the division of EYFP-labeled cells into two EYFP+ cells can clearly be observed in some cases, which indicates that NGN3-EYFP+ endocrine progenitors are formed not only *de novo* but also by division from existing Ngn3-EYFP+ cells giving rise to two equally Ngn3-EYFP+ daughter cells.

Effects of the Mesenchyme on the Development of EYFP-Labeled Endocrine Progenitor Cells

Recent studies have shown that, *in vitro*, the mesenchyme may not be required for the differentiation of the endocrine pancreas but rather necessary for the development of the exocrine counterpart (14, 15). Moreover, it was suggested that signals from the mesenchyme stimulate epithelial cell proliferation but repress the development of the pancreatic epithelium into endocrine cells. To determine directly the effects of the mesenchyme on the development of endocrine progenitor cells, pancreatic rudiments dissected from *Ngn3$^{EYFP/+}$* E12.5 embryos were grown either with or without mesenchyme (Fig. 8A). The numbers of both immature (EYFP+) and differentiated endocrine cells (insulin or glucagon+) per explant were counted on d3 and d5 of culture (Fig. 8B). We found, as previously observed by others, that islet cell differentiation is repressed in the presence of mesenchyme. At d5, for example, we counted about 3 times more insulin- and glucagon-producing cells when the mesenchyme was removed. In contrast, at d3 no major differences were observed in the development of the EYFP+ islet progenitor cells in the pancreatic epithelia grown with or without the mesenchyme. Their number was initially high and decreased thereafter concomitantly with the increase of differentiated cells. At d5, however, we observed a slightly higher number of progenitor cells in the absence of mesenchyme. These results suggest

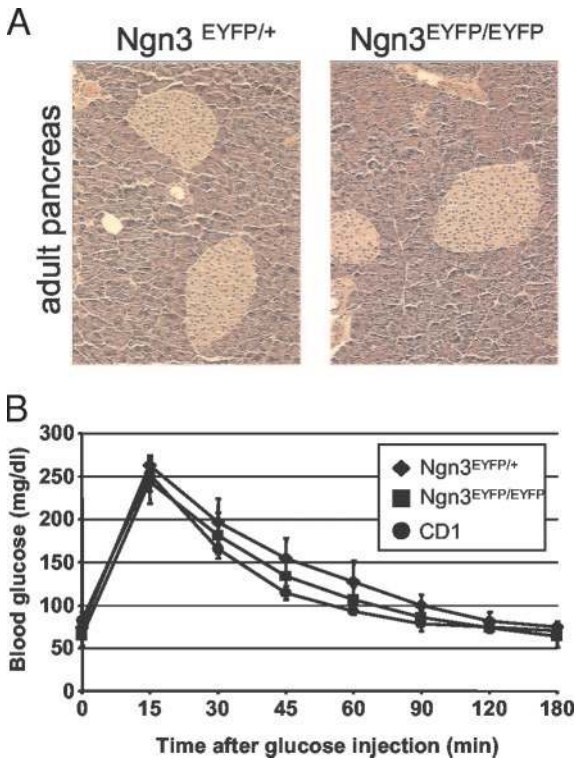

Fig. 5. Normal Islet Development and Function in Ngn3$^{EYFP/EYFP}$ Mice

Hematoxylin-eosin staining of heterozygote (*Ngn3*$^{EYFP/+}$) and homozygote (*Ngn3*$^{EYFP/EYFP}$) adult pancreas showing normally developed Islets of Langerhans. B, ip glucose tolerance test showing a normal glucose response of heterozygote (*Ngn3*$^{EYFP/+}$) and homozygote (*Ngn3*$^{EYFP/EYFP}$) adult mice. Sex- and age-matched wild-type CD1 mice were used as control.

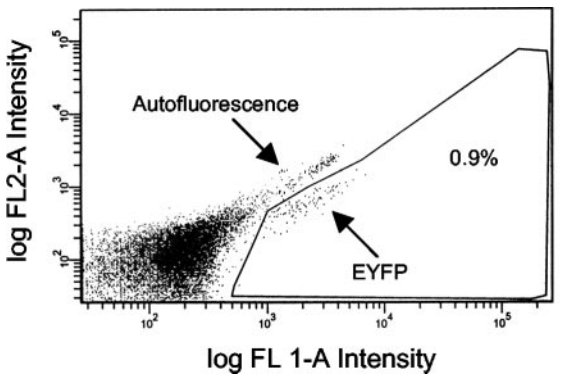

Fig. 6. Flow Cytometric Analysis of a Single-Cell Suspension Prepared from Whole Pancreas of E15.5 ngn3$^{eYFP/+}$ Mutant Mouse Embryos

Fluorescence intensities are expressed in arbitrary units on a four-decade logarithmic scale. Of the pancreatic cells from the *Ngn3*$^{eYFP/+}$ mouse at E15.5, 0.9% express EYFP (visible due to the shift of the emission spectrum to the *green* compared with the autofluorescence of the negative cells). x-Axis, log FL 1-A intensity (530/30); y-axis, log FL 2-A intensity (585/42).

that the generation of islet progenitor cells does not require signals from the mesenchyme and that endocrine fate commitment would be a default pathway. They also suggest that the mesenchyme does not massively repress the development of EYFP-progenitors *in vitro* in our experimental system.

DISCUSSION

An important step in our understanding of islet cell development has been the identification of the islet cell progenitor marker, the transcription factor *Ngn3*. Indeed, Ngn3-expressing cells can give rise to all the four islet cell types *in vivo*, and the inactivation of this gene in mice has proven its essential function in controlling islet fate in uncommitted pancreatic endodermal cells. The extensive study of this population of progenitor cells is thus of particular interest in the light of the important international effort to develop a β-cell replacement therapy for type 1 diabetes. The identification of this marker allows the labeling of *Ngn3*-expressing progenitor cells, which is essential for their purification and subsequent characterization both *in vivo* and *in vitro*. Here we have generated a mouse line in which islet progenitor cells expressing Ngn3 transcription factor have been genetically labeled with EYFP using a knock-add-on approach. The genetically modified animals appear healthy, and functional pancreatic islets develop in mice heterozygous or homozygous for the EYFP transgene. This demonstrates that the expression of EYFP protein in the developing islet progenitor cells is not toxic and that insertion of the reporter gene in the 3′-UTR region of *Ngn3* does not impair the proendocrine function of *Ngn3*. Thus the knock-add-on approach we have adopted here could potentially be applied for other genes to genetically label a specific cell population in the developing pancreas. This approach combines the advantages of ensuring the tissue-specific expression of the reporter gene without simultaneously inactivating the function of the targeted gene at the same time. Robust fluorescence was observed in the developing pancreas of *Ngn3*$^{EYFP/+}$ mice after dissection or when grown *in vitro*, and the background fluorescence was low and clearly distinguishable from the true signal. This demonstrates that one copy of the EYFP gene expressed under the control of *Ngn3*-regulatory sequences is enough to monitor islet progenitor cell development using standard fluorescence microscopy. After standard fixation procedure of the tissue, intrinsic fluorescence of the EYFP could not be detected, but the EYFP protein can be visualized by immunofluorescence.

In *Ngn3*$^{EYFP/+}$ mice, more then 80% of the pancreatic cells expressing the Ngn3 protein during the secondary transition phase, when most of the endocrine cells are generated, are successfully labeled with EYFP reporter protein. The remaining Ngn3+/EYFP-negative cells might still express EYFP protein at levels

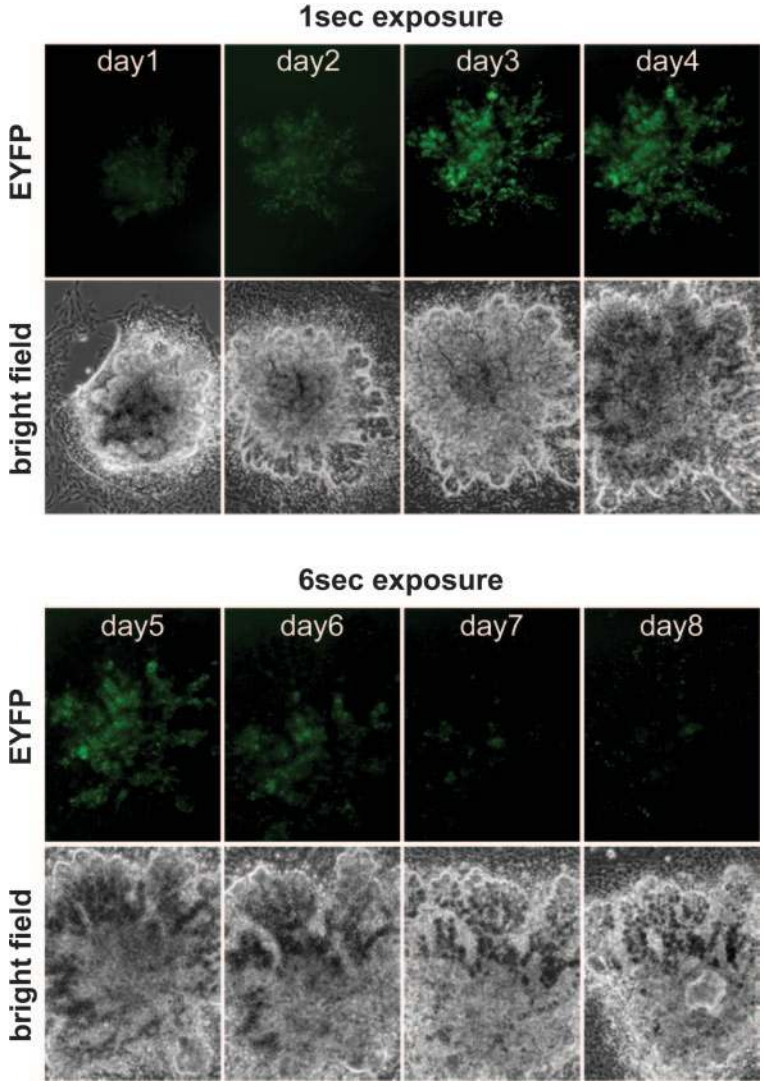

Fig. 7. Pancreas Explant Cultures of Ngn3$^{EYFP/+}$ Embryos

E12.5 dorsal pancreas was dissected and, after removal of the mesenchyme, the epithelium was subsequently cultured for 8 d on collagen-coated Petri dishes. From d1 to d4 the number of EYFP-labeled cells (EYFP) progressively increases (exposure time, 1 sec) after which (d5 to d8) it rapidly decreases (exposure time, 6 sec). EYFP expression pattern mimics the *in vivo* wave of *Ngn3*-expressing islet progenitor cells during pancreas development. Bright-field images show the overall development of the explant. Magnification, ×20.

that are not detected by the anti-EGFP polyclonal antibody which also recognizes EYFP. Alternatively, these Ngn3+ cells are recently born and do not yet express the reporter due to the slower maturation of the EYFP protein (21). This might be the main reason why at E10.5, where Ngn3 expression has just recently started, a slightly higher percentage of Ngn3+ cells (20%) do not costain with EYFP compared with later stages. The proportion of Ngn3-/EYFP+ cells might reflect the lack of sensitivity of the Ngn3 antibody, which would not detect all the Ngn3-expressing cells. An additional possibility, which does not exclude the previous hypothesis, is that some more mature progenitor cells are labeled due to a greater stability of the EYFP protein maintained for a short period in cells that have transiently expressed Ngn3. Supporting this hypothesis a fraction of differentiated islet cells (EYFP+/GLU+; EYFP/INS+) coexpress the reporter protein. Similar observations have already been made in transgenic mice in which the expression of the β-galactosidase gene is driven by a human (19) or a mouse (11) Ngn3 promoter fragment, also finding Ngn3 and β-galactosidase single positive cells as well as occasionally β-cells coexpressing insulin and β-galactosidase. The latter problem could be bypassed by using destabilized fluorescent reporter proteins when designing future strategies to label specific stages of developing islets cells. Using time lapse video (see supplemental data), we could monitor in real time the development of EYFP+ cells in cultures of pancreas explants, mim-

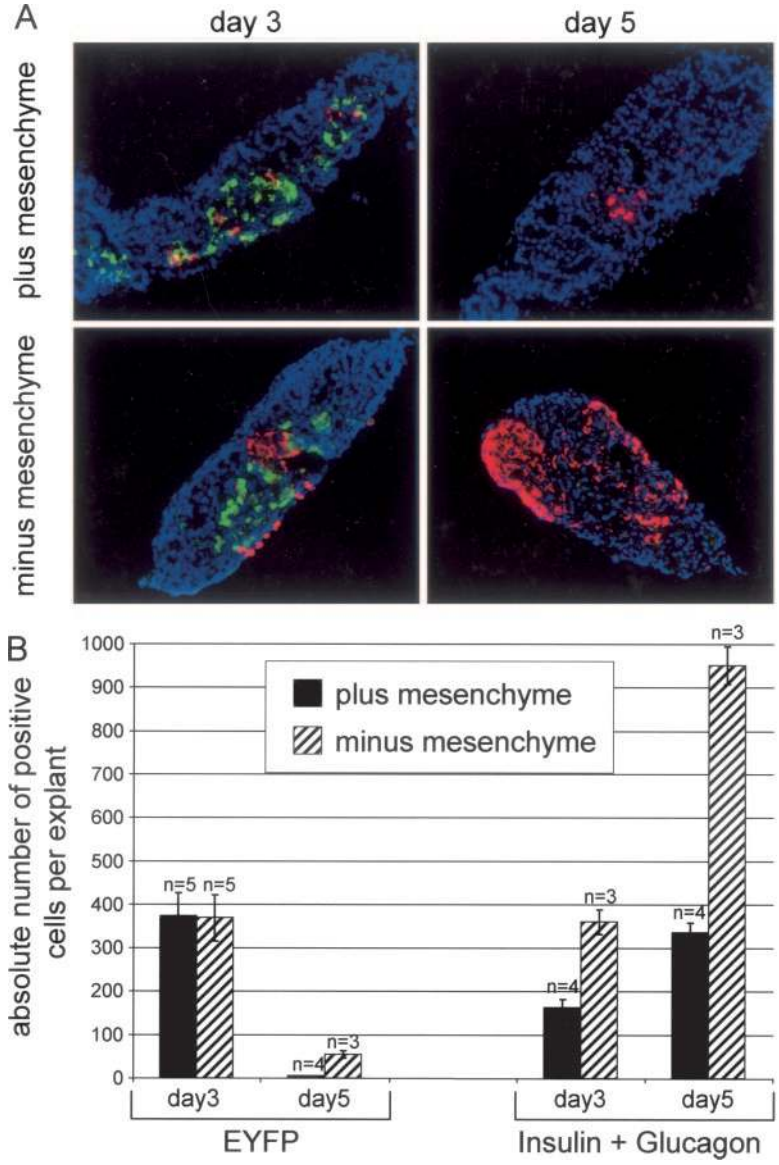

Fig. 8. Effects of the Pancreatic Mesenchyme on the Development and Differentiation of EYFP-Labeled Islet Progenitor Cells *in Vitro*

$Ngn3^{EYFP/+}$ pancreatic E12.5 dpc explants were cultivated *in vitro* with or without their surrounding mesenchyme for 3 or 5d, and the expression of EYFP (*green*) and insulin and glucacon (Ins/Glu, *red*) was analyzed by immunofluorescence on cryosections. B, Quantitative analysis of pancreatic development *in vitro*. The absolute number of EYFP and insulin/glucagon-expressing cells was analyzed in explants of $Ngn3^{EYFP/+}$ embryos surrounded by its mesenchyme (*black bars*) or depleted of it (*hatched bars*), after 3 and 5 d in culture. Each data point represents the mean ± SEM of three to five (n) rudiments. The repressive effect of the mesenchyme on the generation of α- and β-cells can clearly be seen, whereas the development of EYFP+ islet progenitor cells is almost unaffected at d 3.

icking the wave of generation of islet progenitor cells during E13.5 and E15.5. We could also observe that EYFP+ cells can give rise to two EYFP+ daughter cells, suggesting that Ngn3 endocrine progenitors can also divide into two equivalent cells. This demonstrates the usefulness of this experimental system to follow the effect of extrinsic factors on the induction and expansion of islet progenitor cells, simply by monitoring the fluorescence. We took advantage of our explant cultures to follow the EYFP-labeled endocrine progenitor cells in the presence or absence of the surrounding mesenchyme, a source of signaling molecules (15). In both conditions, islet progenitor cells developed in cultures to a similar level. In contrast, the differentiation of insulin- and glucagon-expressing cells was clearly repressed in the presence of the mesenchyme as previously reported (14). These results suggest that endocrine cell fate commitment is occurring independently of signals from the mesenchyme or that growth factors present in the fetal calf

serum (FCS) are sufficient to trigger endocrine fate decisions in cultures of embryonic pancreases. Moreover, these data suggest that in our experimental system, the repressing effects of the mesenchymal signals on islet cell development act by inhibiting the differentiation of islet progenitor cells into hormone-producing cells rather than on the generation of these progenitors from uncommitted epithelial cells.

We believe that the *Ngn3*$^{EYFP/+}$ mice will be a useful tool to isolate and characterize islet progenitor cells at the molecular and cellular levels as well as Ngn3+ progenitors from different origins such as the intestine, stomach, and neural epithelium. Indeed, we will be able to define the gene expression profiles of purified progenitors and their differentiation potential *in vitro*. Moreover, compound technicolor mice can be generated by crossing the *Ngn3*$^{EYFP/+}$ mice with other genetically engineered mouse lines in which different fluorescent proteins have been tagged to key genes controlling the development of islet cells and/or mice in which β-cells are labeled with GFP (16). Such rainbow mice will be instrumental for the purification of subpopulations of pancreatic epithelial cells and the characterization of the sequential maturation steps of islet cells by functional genomics, in particular to identify specific cell surface markers. Moreover, such rainbow mice can be used as a unique source of multipotent stem cells in experiments aimed at generating β-cells *in vitro*. Indeed, the appropriate culture conditions that would recapitulate normal islet cell development could be easily monitored in real time because the activation of the proper gene cascade would also turn on the different fluorescent reporters.

MATERIALS AND METHODS

Targeted Introduction of EYFP into the Ngn3 Locus

Using ET (RecE/RecT) recombination in bacteria (17) an *Asc*I and a *Pme*I recognition site was introduced into the 3′ UTR of a 7.8-kb *Spe*I–*Spe*I genomic fragment from the Ngn3 locus using the primers 5′-TTC TGG CTT TCA CTA CTT GGA TCC CTA GCC CTC TCA CAG GGC TTA ACT AGG CCG TTT AAA CCG AAT AAA TAC CTG TGA CGG AAG and 3′-AGA GCA GCC CCC AAT GTC TGC TGT GCG CAG CAG CAA GGG TAC CGA TGA GAA GGC GCG CCT AAC GAC CCT GCC CTG AAC CGA CGA. An "IRES-EYFP-PGKpA-loxP-SV40 early-promoter-pac-HSVtkpA-LoxP" cassette containing the EYFP reporter and the Puromycin resistance gene *pac* was subcloned into the *Asc*I and *Pme*I site. The IRES-EYFP-PGKpA-loxP-SV40 early-promoter-pac-HSVtkpA-LoxP cassette was generated by replacing the IRES-EGFP sequence from the pIGuX1 plasmid (generously provided by F. Stewart) with the IRES-EYFP sequence from pIRES-EYFP Clontech vector (CLONTECH, Palo Alto, CA). Homologous recombination in ES cells and generation of chimeric mice were performed at the ICS (Mouse Clinical Institute), Illkirch, France. The final targeting vector was linearized with an external unique *Not*I restriction site and electroporated into R1 ES cells (18), and puromycin selection was performed (1.5 μg/ml during 10 d). Homologous recombination events were detected in resistant colonies by Southern blotting using a 1.2-kb 3′-external probe generated from a *Kpn*I–*Not*I Ngn3 genomic subclone. Independently targeted ES cell clones (n = 44) were obtained from 298 clones analyzed and further examined with a 0.48-kb 5′-external probe generated from a Ngn3 genomic clone by PCR using the primers 5′-CCC CTC TTC TCC CTT TGT TC and 3′-ACA CAT GGA TTT GGC ACT GA. Two correct targeted independent ES cell clones (K20/79, K20/119) were used to generate chimeras by standard procedures. Germline transmission was obtained by crossing the chimeras with C57BL/6J females, and the colony was amplified by crossing heterozygous animals with CD1 mice. Genotyping was performed by PCR of genomic DNA obtained from postnatal tails by using the primers Ngn3 5′and 3′ as described by Gradwohl *et al.* (6) to detect the wild-type allele and EYFP 5′-CCT GAA GTT CAT CTG CAC CAC and EYFP 3′-TTG TAG TTG TAC TCC AGC TTG TGC to detect the mutant allele (300 bp). Heterozygous females were crossed with CMV-Cre males (13) to excise the puromycin resistance *pac* gene. When successful excision of the *pac* gene occurred, a 558-bp PCR product is amplified using the primer EYFP-pA 5′-CAT TTT GAA TGG AAG GAT TGG and Ngn3 3′ from genomic tail DNA of the offspring.

Live Imaging

Pancreases were dissected in ice-cold 1× PBS from Ngn3$^{EYFP/+}$ embryos in E13.5, E15.5, E18.5, and adult mice. The intrinsic EYFP expression from dissected pancreas or cultivated pancreatic explants was analyzed using a Leica MZFLIII stereo-dissecting microscope or a Leica DMIRE2 microscope (Leica Corp., Deerfield, IL), respectively, with a YFP filter, and pictures were taken with a CoolSnap HQ camera (Roper Scientific, Tucson, AZ) and the MetaView software (Universal Imaging Corp., Downingtown, PA).

Immunohistochemistry

Immunofluorescence experiments were performed on cryosections as described previously (20). The following primary antibodies were used: rabbit anti-GFP at 1:500 (Molecular Probes, Eugene, OR), mouse antiinsulin at 1:1000 (Sigma Chemical Co., St. Louis, MO), mouse antiglucagon at 1:2000 (Sigma). The guinea pig anti-Ngn3 was used at 1:1000 and was generated by the Sander laboratory against the same glutathione-*S*-transferase-Ngn3 fusion protein as described by Schwitzgebel *et al.* (10). Secondary antibodies used were: Alexa 488 antirabbit at 1:500 (Molecular Probes), Cy3 antiguinea pig and Alexa 568 antimouse at 1:500 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; and Molecular Probes, respectively). Images were taken on an Olympus AX60 fluorescence microscope equipped with a F-view Olympus camera and SIS Olympus software (Olympus Corp., Lake Success, NY).

Culture of Embryonic Pancreas

Dorsal pancreases were dissected from Ngn3$^{EYFP/+}$ embryos on E12.5. The pancreatic epithelium was separated from its surrounding mesenchyme by incubating the pancreases in DMEM/F12 (1:1) (Life Technologies, Gaithersburg, MD) supplemented with 0.8 mg/ml of Collagenase IAS (Sigma) at 37 C for 25 min. The collagenase is then substituted by FCS: DMEM/F12 (1:1). The epithelium was then mechanically removed from the surrounding mesenchyme using needles on a gel in a Petri dish [0.25% Agar, 25% HBSS (Life Technologies), 75% F12/DMEM]. Pancreatic epithelia were cultivated on rat tail collagen I coated Petri dishes (6 μg collagen/1 cm^2) in F12/DMEM medium supplemented with 10% FCS (Life Technologies), Pen/Strep (Life Technologies), 2 mM L-glutamine (Life Technologies), and 0.04 mg/ml gentamycin (Life Technologies). Cultures were maintained at 37 C in a humidified atmosphere of 95% air-5% Co_2, and the medium

was changed every 2 d. For the immunohistochemistry analysis, pancreatic explants were fixed in *p*-formaldehyde 4% on ice for 15 min, followed by three washes with ice-cold PBS for 5 min, and equilibration in 20% sucrose for 10 min. Finally, explants were embedded in OCT compound (Tissue-Tek, Sakura, Japan) and frozen on dry ice, and consecutive sections of 6 μm were collected on Superfrost Plus (Menzel Glaser) slides.

Quantitative Analysis

To determine the number of each cell type per pancreatic rudiment, serial 6-μm sections were cut and collected. To avoid analyzing and counting the same cell twice, one of two consecutive sections (*i.e*. sections separated by 12 μm) were collected on a parallel prepared glass slide. One of the two glass slides was then analyzed by immunocytochemistry for a given antigen. The results of each experimental point were obtained by quantifying the absolute number of each pancreatic cell type in at least three pancreatic rudiments.

Glucose Tolerance Test

Glucose tolerance was tested in overnight-fasted mice by ip injection with 2 g/kg body weight of 15% D-glucose. Circulating glucose was measured in tail blood at 0, 15, 30, 45, 60, 90, 120, and 180 min using a GlucoMen Sensor (A. Menarini Diagnostics, Firenze, Italy).

Preparation of Single-Cell Suspensions from Dissected Pancreases and Flow Cytometric Cell Sorting

Dissected pancreases from E15.5 ngn3$^{EYFP/+}$ embryos were incubated in a solution of 0.05% (vol/vol) trypsin-EDTA (Life Technologies) in PBS at 37 C for 5min. The digestion was stopped by adding F10 medium containing 10% (vol/vol) FCS. The resulting suspension of single cells was washed twice with PBS and filtered through a 70-μm nylon mesh. Flow cytometric analysis and sorting of EYFP-labeled progenitors were carried out using a FACS Vantage SE DiVa high-speed cell sorter with DiVa software (Becton Dickinson Biosciences, San Jose, CA). EYFP-expressing cells were detected after excitation with the 488-nm line of an Argon Ion Laser (200 mW) using the signal area of the FL 1 (530/30 nm band pass) and FL 2 (585/42 nm band pass) channels, separated by a 560 short pass dichroic filter (corresponding to the standard fluorescein isothiocyanate and phycoerythrin channels of the sorter).

Sorting of the EYFP-positive cells was performed using a 100-μm flow cell at 40 psi and 52 kHz. The samples and sorted fractions were kept at 4 C. All cells within the gate, as shown in Fig. 6, were sorted as positives.

Video Time-Lapse Analysis

Ngn3$^{EYFP/+}$ pancreatic explants were prepared as described above. Video time-lapse analysis were started on d 2 of culture using a Leica DMIRB2 microscope with a YFP filter, and pictures were taken every 30 min with a CoolSnap HQ camera and the MetaMorph software (see supplemental data).

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