Pancreatic neurogenin 3-expressing cells are unipotent islet precursors

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Pancreatic islet endocrine cells arise during development from precursors expressing neurogenin 3 (Ngn3). As a population, Ngn3+ cells produce all islet cell types, but the potential of individual Ngn3+ cells, an issue central to organogenesis in general and to in vitro differentiation towards cell-based therapies, has not been addressed. We performed in vivo clonal analyses in mice to study the proliferation and differentiation of very large numbers of single Ngn3+ cells using MADM, a genetic system in which a Cre-dependent chromosomal translocation labels, at extremely low mosaic efficiency, a small number of Ngn3+ cells. We scored large numbers of progeny arising from single Ngn3+ cells. In newborns, labeled islets frequently contained just a single tagged endocrine cell, indicating for the first time that each Ngn3+ cell is the precursor of a single endocrine cell. In adults, small clusters of two to three Ngn3+ progeny were detected, but all expressed the same hormone, indicating a low rate of replication from birth to adult stages. We propose a model whereby Ngn3+ cells are monotypic (i.e. unipotent) precursors, and use this paradigm to refocus ideas on how cell number and type must be regulated in building complete islets of Langerhans.

KEY WORDS: Cell lineage, Clonal analysis, Islet, Mosaic, Progenitor, Transgenic, Mouse

INTRODUCTION

How organs are assembled with the required numbers of correctly organized and specialized cell types, a process that involves flux control from a progenitor state, is a question of general relevance in biology. The pancreas is both an endocrine and exocrine gland. Its three main cellular components, namely acini, ducts and endocrine islets, derive from a common endodermal progenitor cell that expresses two transcription factors: Pdx1 and Ptf1a (Gannon et al., 2000; Herrera et al., 2002; Kawaguchi et al., 2002). Downstream in the differentiation cascade, endocrine precursors are characterized by the transient expression of the bHLH transcription factor neurogenin 3 (Ngn3; Neurog3) (Gu et al., 2002; Herrera et al., 2002). These Ngn3+ cells are subsets of the entire population of Pdx1+ endodermal epithelial cells, and they first appear at embryonic day (E) 8.5, peak at E15.5 and are undetectable at birth. Loss-of-function studies have revealed that *Ngn3* expression is necessary for islet development (Gradwohl et al., 2000). Ngn3+ cells differentiate into the four adult islet endocrine cell types: β-, α-, δ- and PP cells. These cells produce insulin, glucagon, somatostatin and pancreatic polypeptide (PP; Ppy), respectively. A fifth cell type (ε-cell), which produces the hormone ghrelin, is transiently observed in developing pancreata (Prado et al., 2004).

It has been known for many years that islets are polyclonal: each islet originates from a pool of progenitors, as demonstrated with mouse balanced chimeras and transgenic mice (Deltour et al., 1991; Kockel et al., 2006; Swenson et al., 2009), and more recently in human islets (Scharfmann et al., 2008). This is probably the case for exocrine acini as well (Delacour et al., 2004), although this is still controversial (Swenson et al., 2009).

It remains unknown whether every single Ngn3+ cell is a multipotent stem or progenitor cell (i.e. able to give rise to different islet cell types) or is, on the contrary, a unipotent committed cell

(each cell differentiating into only one cell type) (Fig. 1A). The differentiation potency of Ngn3+ cells can be explored in vivo by tracing the fate of single cells (Fig. 1B).

Previous reports have suggested the existence of conditions that impose differentiation restrictions on Ngn3+ cells. For instance, transgenic modulation of Ngn3 expression has revealed the existence of temporal windows of differentiation for Ngn3+ cells (Johansson et al., 2007). Early in development, Ngn3+ cells mainly commit towards α-cell fates (Johansson et al., 2007; Schwitzgebel et al., 2000), whereas at later stages, because of the different, non-autonomous signaling received in the different tissue context, Ngn3 expression results in the formation of all the endocrine cell types (Johansson et al., 2007). In another study, the expression of Nkx6.1 before Ngn3 was shown to be required for acquisition of the β-cell fate (Nelson et al., 2007).

Here, we performed an in vivo clonal analysis of Ngn3-expressing cells. We generated mosaic mice in which very few Ngn3+ cells were discretely labeled at isolated clonal density. This was achieved via an *Ngn3-Cre*-based somatic chromosomal translocation in MADM [mosaic analyses with double markers (Zong et al., 2005)] mice. MADM is the mouse version of the well-known *Drosophila* MARCM technique (Lee and Luo, 1999). We characterized the clonal progeny of labeled cells during their life, in newborns and adults, by scoring very large numbers of individual cell-marking events in multiple mice (Fig. 1B). There are two requisites to performing such an in vivo clonal analysis: first, cell tagging must be a rare and unbiased event; second, the identity of tagged cells must be unequivocally defined. The MADM reporter system developed by Zong and colleagues (Zong et al., 2005) addresses both concerns. The power of this method is that it intentionally uses the high-count evaluation of a very rare mosaic labeling to describe the overall behavior of the progenitor population under test with respect to its proliferative potential and fate allocation. Briefly, one *Rosa26* allele bears the N-terminal part of the enhanced green fluorescent protein (EGFP) coding sequence followed by the C-terminus of the red fluorescent protein (RFP), with a loxP site in between (*Rosa26^{GR}*). The second allele has the converse arrangement of RFP/GFP fragments (*Rosa26^{RG}*) (see Fig.

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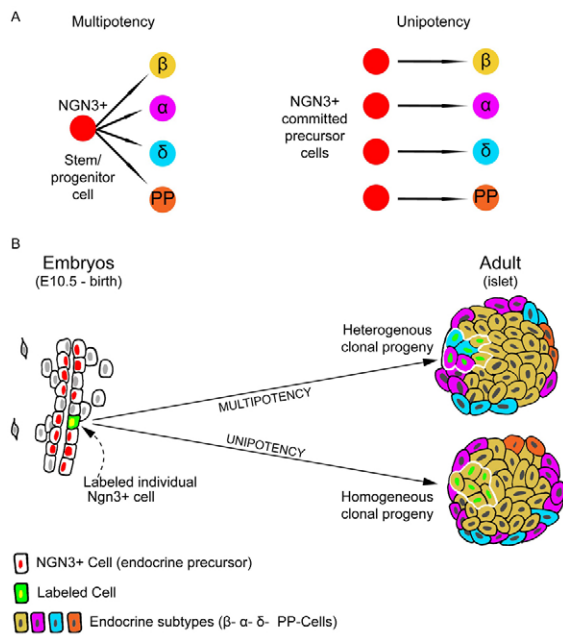
Fig. 1. In vivo clonal analysis to determine the differentiation potency of Ngn3⁺ cells. (**A**) Ngn3⁺ cells can be multipotent or unipotent. (**B**) Clonal analysis of Ngn3⁺ cells. A small fraction of Ngn3⁺ cells are genetically labeled during pancreatic development, such that the clonal progeny of individual labeled cells can be followed from embryogenesis to adulthood. If the labeled endocrine precursor is multipotent, its labeled clonal progeny in the adult islet should express different hormone genes. Conversely, if the labeled endocrine precursor is unipotent, then its descendants will only produce one hormone.

S1 in the supplementary material). Cre-mediated recombination in *Rosa26^{GR/RG}* mice restores two functional fluorescence markers: EGFP and RFP. This somatic chromosomal translocation is purposely rare, only occurring in a small number of cells expressing Cre from the *Ngn3-Cre* transgene (the rarity is because of the arm exchange between single loxP sites in the separate GR and RG chromosomes). When the translocation occurs in a quiescent cell (in G0) it becomes double-colored (i.e. doubly labeled, hereafter termed DL). A DL cell is thus a cell tagged before DNA replication. If such a DL cell subsequently re-enters the cycle, its progeny will be DL as well (see Fig. S1A in the supplementary material). However, in an actively cycling cell, the translocation is likely to occur after S phase, and thus between chromatids of homologous chromosomes. Therefore, the two daughter cells will be different: either one green and one red, or, alternatively, one DL and the other unlabeled (for further details, see Fig. S1B in the supplementary material).

Our results reveal that every Ngn3⁺ cell becomes one endocrine cell at birth. We also show that the predominance of β-cells over α- and δ-cells in adult islets results from there being a greater number of Ngn3⁺ cells committed to the β-cell lineage during development and from higher β-cell proliferation rates with age.

MATERIALS AND METHODS
Mice
GR/RG, *Ngn3-Cre* and *Rosa26-EYFP* mice have been described elsewhere (Herrera et al., 2002; Srinivas et al., 2001; Zong et al., 2005). *Ngn3-Cre* transgenics were obtained by pronuclear microinjection of a transgene containing a 5.7 kb fragment of the human *NGN3* promoter [provided by M. German (Lee et al., 2001)]. *Ngn3; Rosa26^{GR/RG}* mice (abbreviated to *Ngn3-*

GR/RG in the text) were obtained at the expected Mendelian frequency by breeding *Ngn3; RG/RG* females to *GR/GR* males. We analyzed embryos at E11.5, E15.5 and E17.5, as well as neonates and adults at 2 and 10 months of age. All strains were in a highly enriched C57/BL6 background and were provided standard rodent diet ad libitum, according to the guidelines of the Direction Générale de la Santé of the canton of Geneva.

Tissue sampling, immunofluorescence (IF) and immunohistochemistry (IHC)
We analyzed ~3600 islets from 218 pancreatic sections of dorsal and ventral lobes, which were obtained from 16 mice. Dissected pancreata were always embedded and oriented in the same way, so that the maximum pancreatic surface was cut on every section, always encompassing both dorsal and ventral lobes. By random multiple cutting, some sections hit the islets at their edge, whereas others hit the middle of islets. From this large sample, we estimated the frequency of cell and islet labeling, as well as the composition and the dynamic change with age in the size and shape of the clusters of labeled cells (see the Statistical Appendix in the supplementary material). Some samples from newborns were analyzed on consecutive serial sections that covered at least 50 µm in total, so as to analyze whole islets.

Collected pancreata were rinsed in cold PBS and fixed overnight at 4°C in 4% paraformaldehyde. For IF, specimens were cryoprotected for 12 hours in 30% sucrose in PBS, and embedded in OCT prior to cryostat (Leica CM1900) sectioning (10 µm). For IHC, collected pancreata were dehydrated and embedded in paraffin prior to microtome (Leica RM2135) sectioning (5 µm). Primary antibodies used for immunostaining were: rabbit anti-EGFP (Invitrogen, 1/100, used to detect both EYFP and EGFP); goat anti-chromogranin A (Santa Cruz, 1/100); guinea-pig anti-insulin (Dako, 1/400); mouse anti-glucagon (Sigma, 1/1000); rabbit anti-glucagon (Zymed, 1/100); goat anti-glucagon (Santa Cruz, 1/100); rabbit anti-somatostatin (Dako, 1/200); goat anti-Myc tag (Novus, 1/100); rabbit anti-pHH3 (Upstate, 1/500); mouse anti-Ki67 (BD Biosciences, 1/200); mouse anti-Ngn3 (BCBC 2013, 1/4000); rabbit anti-Ngn3 (BCBC 2011, 1/500); goat anti-amylase (Santa Cruz, 1/100); rabbit anti-PP (Bachem T-4088, 1/200); guinea-pig anti-PP (Linco 4041-01, 1/100); goat anti-PP (Novus 100-1793, 1/1000).

For IHC and IF, sections were either dewaxed or rehydrated, permeabilized for 20 minutes in 0.1% Triton X-100, then washed in 3% BSA, 0.1% Tween 20 in PBS. A supplementary antigen-retrieval treatment was required for anti-Ngn3, anti-Ki67 and anti-pHH3, which consisted of microwaving the slides in citrate buffer (10 mM Na-citrate pH 6) for 5 minutes at 600 W, followed by 15 minutes at 300 W, before incubation with blocking solution. Primary antibodies were incubated for 1 hour at 37°C in a wet chamber. After washing in PBS-T (0.1% Tween 20 in PBS), sections were incubated with specific secondary antibodies conjugated with Alexa Fluor 488 or 647 (Invitrogen) or with Cy3 (Jackson ImmunoResearch). For anti-Myc tag antibody, TSA amplification (PerkinElmer) was performed according to the manufacturer's recommendations. For chromogranin A, secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch, 1/500) were used with 3,3-diaminobenzidine tetrahydrochloride (DAB) as substrate. The endocrine (chromogranin A⁺) area was measured on four to five different sections each, separated by 250 µm for 2- and 10-month-old animals (*n*=5) and separated by 150 µm for P0 animals (*n*=3). Mitotic indexes were assessed from the percentage of cells positive for Ngn3 and pHH3, as calculated on four different sections separated by 50 µm (*n*=3). Tissues were mounted in PVA (Lennette, 1978) or Neo-Mount (Merck) and examined with a Nikon epifluorescence microscope (Eclipse TE200) and a Leica confocal microscope (TCS SPE DM 5500). Tissue surface and cell numbers were measured using ImageJ software (NIH).

Genomic DNA PCR
Genomic DNA was extracted from mouse tails using DirectPCR-Tail (Viagen Biotech) according to the manufacturer's instructions. PCR primers and programs were as described (Zong et al., 2005) for *Rosa^{RG/GR}* mice.

Statistical analysis
Results are reported as the mean ± s.e.m. Statistical analyses were performed using Prism 4.0 software (GraphPad).

RESULTS

Analysis of the clonal descent of individual Ngn3-expressing cells

We previously generated *Ngn3-Cre* transgenic mice in which Ngn3^{+} cells can be genetically 'tagged' by Cre recombinase from the beginning of pancreas development; this allowed us to determine that *Ngn3* expression is a feature of islet precursor cells (Herrera et al., 2002) (see Fig. S2 in the supplementary material). Interestingly, in these mice, Ngn3^{+} cells never simultaneously co-expressed the Cre-induced reporter molecule (YFP in *Ngn3-Cre; R26-YFP* mice) and Ngn3 protein (see Fig. S2B in the supplementary material). This suggest that Ngn3 expression is transient and short-lived, as there are only 2 hours between the induction of *Cre* transcription and Cre-mediated recombination at loxP sites [as determined in transgenic zebrafish with an inducible promoter (Thummel et al., 2005)]. Moreover, all reporter-positive cells were also hormone-positive in *Ngn3-Cre; R26-YFP* mice (see Fig. S2B in the supplementary material), indicating that the gap between Ngn3 extinction and the initiation of hormone gene expression is also brief.

Here, to explore the differentiation potential of a single Ngn3-expressing cell, we performed a high-count analysis of a highly mosaic (clonal) labeling method. We generated *Ngn3-Cre; Rosa26^{GR/RG}* (henceforth *Ngn3-GR/RG*) transgenic mice (see Materials and methods) (Fig. 2A). In 2-month-old adults, we found that 85% of labeled cells were doubly labeled (DL), indicating that recombination in the embryo mainly occurred in quiescent Ngn3^{+} cells (Fig. 2B; see Fig. S1 in the supplementary material). This correlates with previously published observations showing that most Ngn3^{+} cells are not proliferative at the time of Ngn3 expression (Gu et al., 2002) (see Fig. S3A,B in the supplementary material). Indeed, whereas Pdx1^{+} cells proliferate actively during pancreas ontogeny [10% of Pdx1^{+} cells were positive for phosphorylated histone H3 (pHH3[+])] (Bonal et al., 2009), we found that only 0.5% of Ngn3^{+} cells were pHH3[+] (see Fig. S3A in the supplementary material). No labeled cells were found outside of the islets.

Ngn3-expressing cells are unipotent

DL endocrine cells represent the clonal progeny of tagged individual Ngn3^{+} cells in newborn and adult mice. Whereas DL cells existed mainly as isolated single cells at birth (see below), they often formed small and compact groups, or 'clusters', within the islets of adult mice. All DL cells found within a given islet (as determined on pancreatic sections) were arbitrarily considered to form one single cluster, and thus to represent the clonal descendents of one single Ngn3-expressing cell. In *Ngn3-GR/RG* mice, DL cells constituted 1% of the total islet cell population. This proportion was constant throughout life, from embryos to adults (at 2 and 10 months of age) (Fig. 2C), which indicates that the induced chromosomal translocation was not deleterious for DL cells.

Approximately 30% of all islets analyzed throughout the pancreas, and with no particular locational bias, contained DL cells (in keeping with the rarity and randomness of the labeling event). This figure remained unchanged between 2 and 10 months of age (Fig. 2D).

We explored the differentiation potency of Ngn3^{+} cells by assessing the expression of insulin, glucagon or somatostatin by adult clonal DL cells, using confocal immunofluorescence to accurately determine the co-expression status. We could not study the PP cell population owing to a lack of reliable antibodies (see Fig. S4 in the supplementary material); alternatively, or in addition, these results might reflect actual co-expression of PP in some α-cells. If all DL cells of a single cluster contain the same
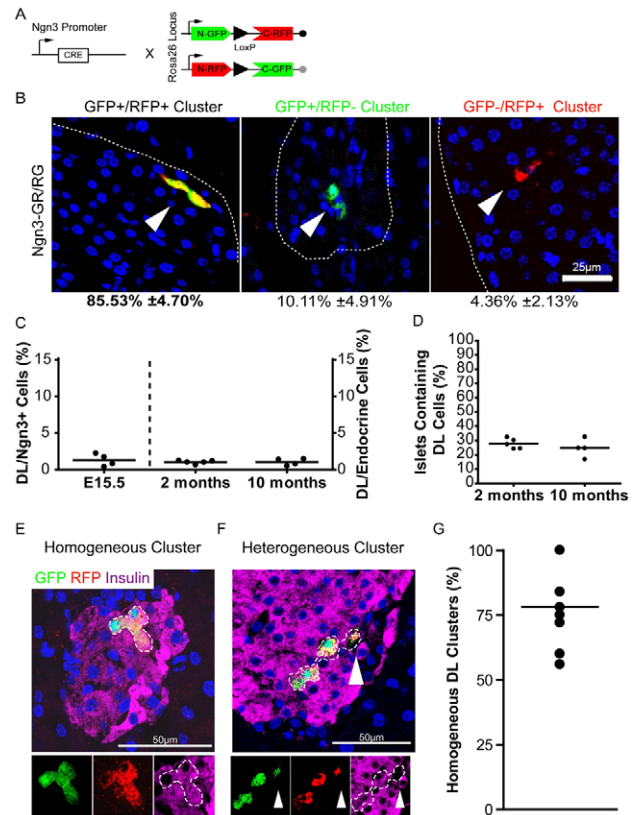
Fig. 2. Ngn3+ tagged cells principally form homogeneous clusters of doubly labeled (DL) endocrine cells. (**A**) The *Ngn3-Cre* and *Rosa26^{GR/RG}* transgenes used to generate *Ngn3-Cre; Rosa26^{GR/RG}* (*Ngn3-GR/RG*) mice by backcrossing. (**B**) Confocal micrographs of 2-month-old *Ngn3-GR/RG* islets stained for EGFP (green) and RFP (red), showing the proportions of DL (EGFP+ RFP+) cells and of cells positive for EGFP or RFP (*n*=5 mice; 426 clusters of labeled cells scored). Islets are outlined. (**C**) The proportion of DL cells relative to Ngn3+ cells (shown on the left scale; E15.5 embryos, *n*=4, 600 cells scored per embryo) or to total endocrine cells (right scale; adults, *n*=4-5 mice, 60,000 and 150,000 cells scored at 2 and 10 months, respectively). About 1% of cells are labeled; this proportion remains constant at any stage analyzed (ANOVA, *P*>0.05). Note that *Ngn3* expression has ceased when cells become DL (see Fig. S2B in the supplementary material). (**D**) Proportion of islets containing DL cells in 2- and 10-month-old mice (800-1200 islets scored per group). (**E,F**) Confocal micrographs of islets from *Ngn3-GR/RG* mice containing clusters of cells stained for EGFP (green), RFP (red) and insulin (purple). Clusters of clonal DL cells are outlined. The cluster in E is formed by three β-cells and is therefore homogeneous, whereas that in F is heterogeneous because it is formed by three β-cells and one non-β unidentified endocrine cell (arrowhead). (**G**) The proportion of homogenous clusters of DL cells in 2-month-old adult islets (each dot represents one individual; *n*=6 mice, 465 islets scored).

hormone, the cluster is homogeneous; this indicates that the precursor was unipotent. If not, it is heterogeneous, meaning that it derives from a multipotent progenitor (Fig. 2E,F; Fig. 1B). The majority of DL clusters were homogeneous at 2 months (75%, on average), independently of whether they comprised α-, β- or δ-cells (Fig. 2G). Some clusters (~25%) were heterogeneous, but we determined mathematically that these arose from the initial fusion of two or three homogeneous clusters (for example, of homogeneous insulin-expressing and homogenous glucagon-

expressing clusters). The relative probabilities of having one, two or three independent recombination events per single islet can be calculated, knowing that somatic chromosome translocation in $Ngn3^+$ cells is rare (Fig. 3A). Improbable events occur with a Poisson distribution (see the Statistical Appendix in the supplementary material): the probability of one, two or three recombination events per islet in *Ngn3-GR/RG* mice was 85%, 14% and 1%, respectively (Fig. 3A, 'expected' values). These expected frequencies are in accordance with those observed for pancreatic sections at birth, namely that 78% of labeled islets contained only one DL cell, 17% contained two and 5% had three DL cells (Fig. 3A) (χ^2 test, P=0.2).

It was striking that in newborns, labeled islets frequently contained just a single tagged endocrine cell. We confirmed this observation by analyzing serial consecutive sections covering whole newborn islets, over a depth of at least 50 μm. Of the 14 labeled islets fully explored, ten contained only one DL cell, from top to bottom.

The probability of observing homogeneous DL clusters for each islet cell type was then calculated knowing (1) the estimated chance (15%) of having more than one DL cell per islet at birth and (2) the relative abundance of each islet cell type [70-80% are β-cells, 15-20% α-cells, 5% δ-cells and 1% PP cells in newborns and adults (Herrera et al., 1991; Orci, 1982)]. We found that the observed and expected frequencies were similar for every islet cell type (Fisher's exact test, P>0.05) (Fig. 3B). This mathematical 'dissection' suggests that heterogeneous clusters of DL cells are in fact chimeric: they most likely result from the merging of two or more clonal clusters derived from adjacent DL cells that are present initially within the same islet at birth.

If heterogeneous clusters are the progeny of at least two recombined $Ngn3^+$ cells instead of one, then they would be expected to be more widely spread and to be larger (i.e. composed of more cells) than homogenous clusters. Indeed, this was the case: the average distance between the two most separated cells in a cluster was doubled in heterogeneous clusters as compared with homogeneous clusters, as was the number of cells per cluster (Fig. 3C,D).

Whereas the vast majority of labeled $Ngn3^+$ cells in *Ngn3-GR/RG* mice yielded DL endocrine cells, 15% of tagged endocrine cells were not DL, but either $EGFP^+$ or RFP^+ (single-labeled, termed SL) (Fig. 2B). This proportion suggests that Cre-mediated recombination occurred after DNA replication in ~20% of $Ngn3^+$ cells (see the Statistical Appendix in the supplementary material).

The presence of SL cells did not alter our main conclusion: like DL clusters, SL clusters were homogeneous (76%) and small (two-celled at 2 months; 30 SL clusters scored), irrespective of whether they were formed by β-, α- or δ-cells, thus suggesting that their tagged parental $Ngn3^+$ cells are unipotent. However, because in most cases these green-only and red-only clusters were not found simultaneously within the same islet, it cannot be determined whether these labeled proliferating $Ngn3^+$ cells are unipotent (as with DL clusters) or bipotential (each daughter having a different fate and 'migrating' into a different islet); alternatively, or in addition, it is also possible that in some instances one of the two labeled daughter cells died owing to aberrant chromosomal translocation.

In summary, we observed that in most cases (1) newborn islet cells are $Ngn3^+$ cells that differentiate without division such that one $Ngn3^+$ cell becomes one islet cell at birth, and (2) in islets of adult mice, DL cells form clusters of homogeneous composition. These two results suggest that cells expressing *Ngn3* are unipotent.
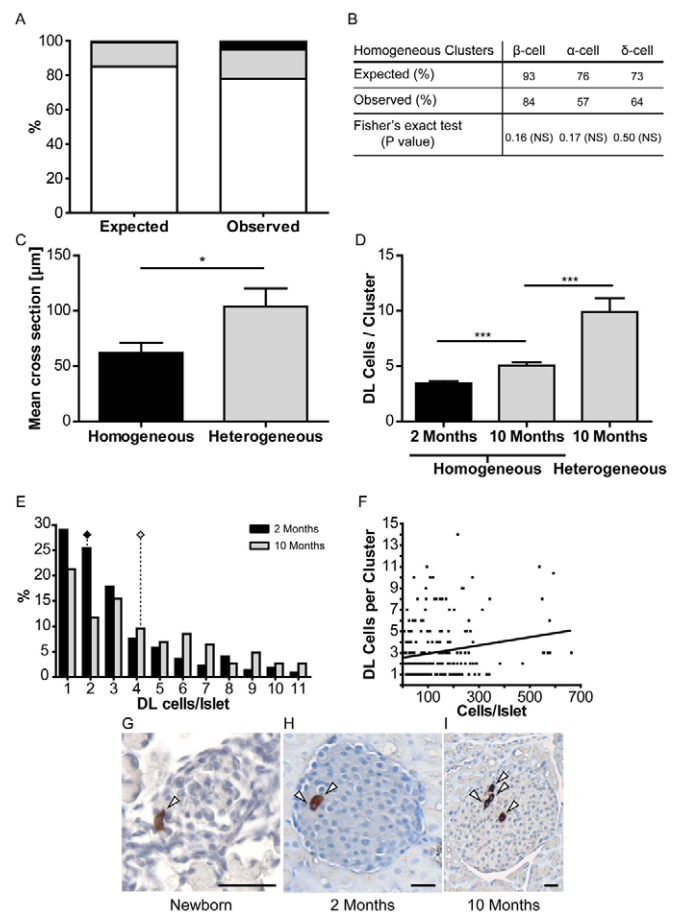
Fig. 3. DL islet cells are found as isolated single cells at birth. (**A**) Expected recombination events predicted by the Poisson distribution (left) and recombination events per islet observed at birth (right). White, gray and black areas represent the proportion of islets containing one, two or three DL cells per islet (n=4, 150 DL cells), respectively. (**B**) Proportion of homogenous clusters of DL cells, calculated per islet cell type with the knowledge that 22% of islets contain more than one cluster of DL cells. Expected and observed proportions of homogeneous clusters were compared using Fisher's exact test. No statistical difference was found. This indicates that the observed frequencies of homogeneous clusters correspond to those expected if labeled $Ngn3^+$ cells were unipotent. n=4 mice per group, 100-200 islets per group; NS, not significant. (**C**) Mean cross-section diameter of homogeneous and heterogeneous clusters. Heterogeneous clusters are more dispersed (15 clusters per group, from four mice, *P<0.05). (**D**) Size (number of cells) of homogeneous and heterogeneous clusters at 2 and 10 months. Note the increased size of homogeneous clusters between 2 and 10 months of age. At 10 months, heterogeneous clusters are twice the size of homogeneous clusters (n=4-5 mice, greater than 50 clusters per group, ***P<0.001). (**E**) Number of DL cells per cluster in adult islets. The prevailing size of DL cell clusters changes with age in 2- and 10-month-old transgenic mice. Diamonds indicate the median cluster size; n=4-5 mice per age group; 220 and 340 clusters of DL cells were scored in 800 and 1200 islets, respectively. (**F**) DL cluster size and islet size are not correlated. The average number of cells per cluster is the same in small and large islets (coefficient of determination: R^2=0.036; 223 labeled islets scored of all sizes, from five mice). (**G-I**) Paraffin sections of P0, 2- and 10-month-old islets from *Ngn3-GR/RG* mice stained for EGFP (DAB) and counterstained with Hemalun. At birth (G), one isolated DL cell is shown (arrowhead), whereas one cluster of two adjacent DL cells is seen at 2 months (H), and another of four DL cells at 10 months (I). Scale bars: 25 μm in G; 50 μm in H,I.

Adult islet cells replicate slowly and have a long life span

The size and shape of DL clusters provide information about their origin. Because DL cells were generally found as single isolated cells in newborns (Fig. 3A,G), most clusters of DL cells in adult islets were the clonal progeny of a single DL islet cell, which re-entered the cell cycle postnatally. In *Ngn3-GR/RG* adults, DL cells formed small and compact clusters. This reveals that during normal islet homeostasis, endocrine cell proliferation and migration are limited. With age, the average number of cells per DL cluster increased. In 2-month-old adults, 30% of DL cells were still isolated, while the rest were in clusters (the average cluster size was two cells) (Fig. 3E,H). Interestingly, there was no correlation between cluster size and islet size: the average number of cells per cluster was the same in small and large islets (Fig. 3F).

At 10 months, 20% of DL cells remained as single isolated cells, but the clusters contained on average three to four DL cells (Fig. 3E,H,I). Since the level of islet cell death is very low in healthy, normal adults (Porter et al., 1998), this confirms that islet cells have a long life span and that there is limited islet cell proliferation under normal conditions, as reported previously (Teta et al., 2005; Teta et al., 2007; Tsubouchi et al., 1986).

The proportion of DL cells among the total per endocrine pancreas (1%) and the proportion of islets that contained DL clusters (30%) remained unchanged throughout life (up to 10 months, the period of analysis). Similarly, the total number of islets per mouse pancreas was constant between 2 and 10 months of age: we estimated that there were ~700 islets per mouse pancreas (Fig. 4A). Thus, the reported increase in DL cell cluster size with ageing (Fig. 3D,E) implies that the average islet size must increase accordingly. Indeed, we found that the mean islet volume increased 1.5-fold between 2 and 10 months (Fig. 4B). This correlates with the observed lower frequency of small islets in aged individuals (Fig. 4C).

Taken together, these observations indicate that under normal conditions: (1) endocrine cell proliferation during embryonic and postnatal life is very low; (2) islet cells have a long life span and little migratory potential; (3) the total number of islets remains constant during adult life (between 2 and 10 months); and (4) islet size increases with age.

DL β-cells form larger clusters

Given that the proportion of the different islet cell types in newborns and adults is constant and that the proliferation of endocrine cells before birth is limited (Nekrep et al., 2008) (see Fig. S3A-C in the supplementary material), we concluded that islet formation involves fixed proportions of Ngn3+ cells being allocated to each islet cell lineage. In particular, this means that β-cell-specified Ngn3+ cells are more abundant than the others. Indeed, the majority of DL cell clusters were composed of β-cells (72% at 2 months and 83% at 10 months, in 229 homogeneous DL clusters analyzed for the presence of insulin). This precisely corresponds to the proportion of β-cells found in islets (Orci, 1982), further suggesting that during pancreas ontogeny the majority of Ngn3+ cells differentiate into β-cells.

On average, and at any age, clusters composed of DL β-cells were always larger than those composed of other cell types (Fig. 4D). Since the level of islet cell death is very low (Porter et al., 1998), this suggests that β-cells proliferate more rapidly than non-β-cells, which agrees with previous reports (Tsubouchi et al., 1986). We also noticed that for each of the various endocrine islet cell types, the average labeled cluster size was very reproducible from animal to animal and throughout the pancreas volume (with no location or islet
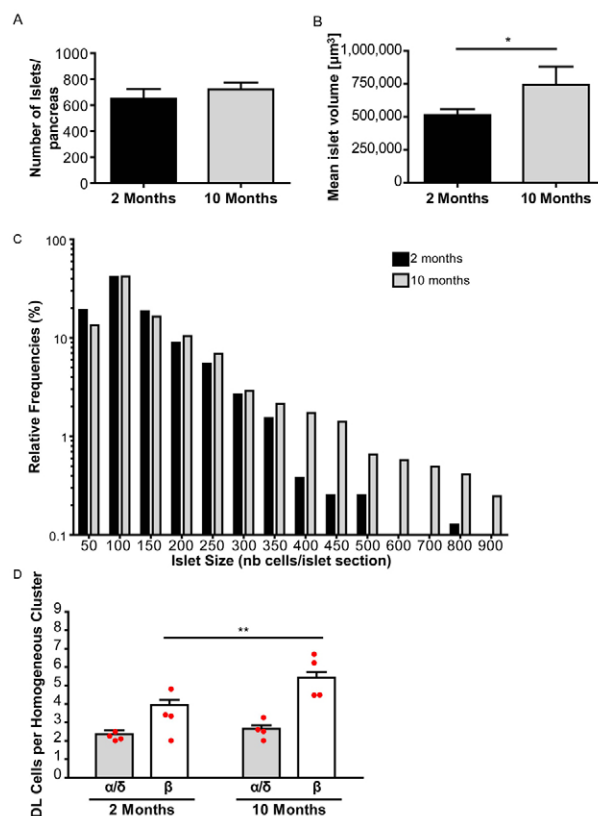
Fig. 4. Islets are larger in aged mice. (**A**) The number of islets per pancreas is constant throughout life (*n*=3 mice per group, with 250-550 islets sampled per group; see Materials and methods). (**B**) Average islet volume increases with age (*n*=4-5 mice per group, with 800-1200 islets scored per group; *P<0.05). (**C**) Frequency distribution of islet size from 2- and 10-month-old mice (*n*=5 mice per group; 60,000-150,000 islet cells, with 800-1200 islets scored per group). (**D**) Average number of cells per homogeneous cluster of DL cells relative to cell composition and age. Homogenous clusters composed of β-cells (white bars) are larger than clusters of non-β-cells (gray bars) at 2 and 10 months [44-132 clusters scored (bar chart, **P<0.01); *n*=4-5 mice per group (red dots, *P*=0.057)]. The size of the β-cell clusters increases with age.

size dependencies), suggesting that there are no subpopulations of proliferating versus non-proliferating islet cells. In a recent study using the same MADM mice to label adult β-cells, Brennand and co-workers also showed evidence indicating that in adult animals, all β-cells exhibit the same proliferation rate (Brennand et al., 2007).

In summary, our observations imply that the predominance of β-cells over α-, δ- and PP cells in adult islets results from (1) a greater number of Ngn3+ cells committed to the β-cell lineage during embryonic stages (clusters of DL β-cells are more abundant than clusters of DL non-β-cells) and (2) a measurably higher replication rate of β-cells as compared with non-β-cells between 2 and 10 months of age (clusters of DL β-cells are larger than those of DL non-β-cells).

DISCUSSION

The results reported here regarding the developing and adult pancreas illustrate, at a more general level, the power of the mouse MADM technique to (1) define the differentiation potential of

progenitor cells in vivo at the single-cell level as opposed to in entire cell populations, and (2) dissect the dynamics of cell mass establishment during development and cell mass maintenance throughout life, and in disease.

Ngn3+ cells are unipotent precursors

All of the Ngn3+ cells taken together as a population are multipotent, yet at the single-cell level we have observed with MADM that each Ngn3+ cell becomes just one endocrine cell at birth, indicating that Ngn3+ cells are unipotent and mainly non-proliferating (Fig. 5A). Our results do not reveal directly whether islet cell fate allocation occurs before, during, or after Ngn3 expression, but together with previous results indicating a requirement for Nkx6.1 expression before that of Ngn3 for acquisition of the β-cell fate (Nelson et al., 2007), they strongly suggest that Ngn3 expression might be a feature of cells that are already pre-committed or pre-biased towards one of the various islet cell lineages. We need to determine the timing of cell fate specification and of the allocation of cell number relative to the Ngn3+ stage; to determine, for instance, whether there is a correlation between cell fate and location along the epithelial cord domain, and what factors (autonomous and non-autonomous) affect their behavior and therefore regulate the relative proportions of the mature cell types.

The Ngn3+ cell has commonly been referred to as a 'stem' or 'progenitor' cell, yet given its unipotent character and low-level proliferation, it seems more appropriate to institute a renaming of this state as 'precursor'.

Ngn3 is also expressed in intestinal cells that give rise to at least 15 different types of entero-endocrine cells (Jenny et al., 2002). Further studies will help to determine whether intestinal Ngn3+ cells are unipotent like pancreatic Ngn3+ cells.

Ngn3+ cells exhibit a low level of proliferation

The 1:1 progression from the Ngn3+ to the differentiated islet cell state at birth (i.e. Ngn3+ to endocrine) correlates with the reported low proliferation rates of Ngn3+ cells and of embryonic endocrine cells (Georgia et al., 2006; Gu et al., 2002; Nekrep et al., 2008) (see Fig. S3A,B in the supplementary material). This result, together with the fact that at birth, islet cells, whether labeled or not, express only one hormone, reveals that endocrine cell expansion during pancreatic development before birth is the consequence of progenitor differentiation rather than the result of proliferation of early endocrine cells, as previously predicted (Herrera et al., 1991). Consequently, the control of Ngn3+ cell number is a process that determines the number of endocrine cells at birth. Our observations suggest that the total number of endocrine cells per pancreas at birth should be similar to the sum of the Ngn3+ cells formed during all stages of pancreas development.

As each Ngn3-expressing cell gives rise to a single differentiated endocrine cell at birth, the pool of precursors of each islet cell type must be different as well. For instance, in order to reach the expected 70-80% of β-cells, the majority of Ngn3+ cells must be 'β-specified', a smaller proportion of them must then be 'α-specified', and so on. In *Ngn3-GR/RG* mice, we found that the majority (~80%) of DL clusters comprised β-cells. This result fits with a model in which the definitive proportions of islet cells are determined by the size of their respective pools of progenitors.

Determination of cell fate choice

In the developing pancreas, α-cells appear first, followed by PP, δ- and β-cells (Herrera et al., 1991). This process occurs following a pattern defined by the establishment of temporal competence
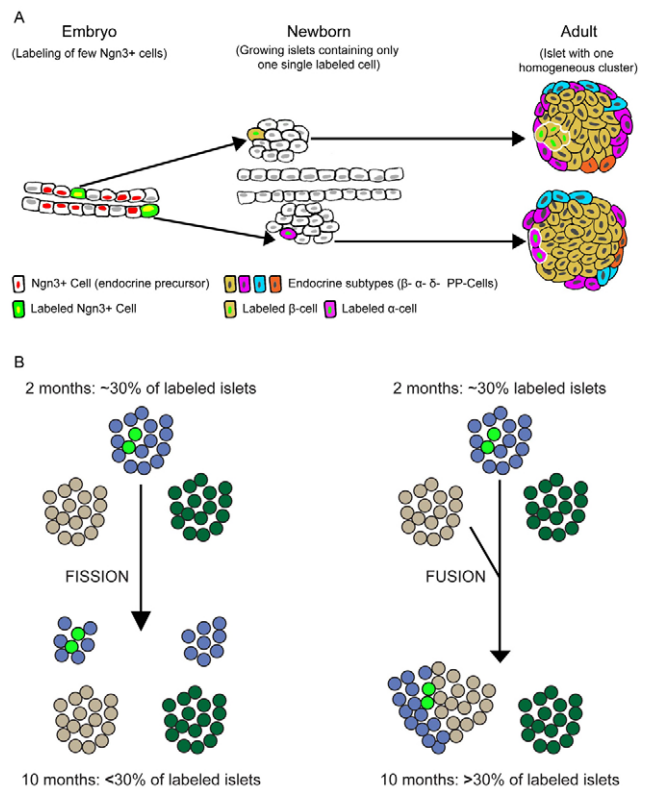
Fig. 5. An overview of islet development and homeostasis.
(**A**) One Ngn3+ cell becomes one endocrine cell at birth. Soon after Ngn3 expression, labeled endocrine precursor cells differentiate into hormone-expressing cells, without division. Individual, labeled, hormone-expressing cells present at birth within early islets proliferate during postnatal life. β-cells proliferate more than non-β-cells, resulting in the formation of larger homogeneous clusters of labeled β-cells. (**B**) Normal islet homeostasis does not involve islet fission or fusion. In the absence of islet cell neogenesis (i.e. the proportion of labeled cells is constant), islet fission would result in a decreased proportion of labeled islets over time (from one-third to one-quarter in the figure, left panel). Conversely, islet fusion would result in an increased proportion of labeled islets with age (from one-third to a half, right panel).

windows of Ngn3+ cell differentiation (Johansson et al., 2007). Yet, at the cellular level, islet cell type specification relies on the concerted activation of a precise combination of transcription factors, including Nkxs, Mafs, Pax4 and Arx (Bonal and Herrera, 2008), but the molecular mechanisms of fate allocation remain elusive. In addition, differentiation from progenitors involves broad epigenetic changes (Meissner et al., 2008). Islet cell determination, whether it occurs before or concurrent with Ngn3 expression, might rely on specific patterns of histone methylation and/or acetylation. Histone acetylase inhibition in growing pancreata has recently been shown to promote α- and PP cell fates at the expense of β- and δ-cell formation (Haumaitre et al., 2008).

The 1:1 Ngn3+-to-endocrine passage emphasizes the importance of the niche in cell fate determination. The extrinsic cues given to the Ngn3+ cell or to its immediate precursor must be tightly regulated for the correct proportions of the different cell types to be generated within a fully functional assembled islet. The MADM clonal analysis reveals that Ngn3+ cells in the developing pancreas, whether early or late in development, appear to be endowed with a restricted and

specific differentiation program. What influences the islet cell differentiation outcome triggered by Ngn3 expression remains to be determined. Where and when in the pancreatic epithelia are β- versus non-β-cells born? The fate choice process may be driven or influenced by instructive signals, morphogen gradients, from the epithelium or mesenchyme. In this context, a cellular heterogeneity during development in the tip-trunk axis of growing pancreatic epithelial cords has been reported (Zhou et al., 2007).

The number of islets remains constant during life, without islet fusion, fission or neogenesis

The persistence of Ngn3+ cells in adults, whether in islets or ducts, is a matter of current debate (Gu et al., 2002; Inada et al., 2008; Wang et al., 2009; Xu et al., 2008). Given that the number of islets remains constant, if Ngn3-dependent neogenesis contributes to the maintenance of normal islet cell mass (i.e. if recombination continued to occur with age in *Ngn3-GR/RG* healthy adult mice), then the proportion of islets containing DL cells would increase. Because this was not observed, we conclude that Ngn3-dependent formation of new islet cells during life is not crucial for normal islet homeostasis.

The constant proportion of DL cells (1%) and of labeled islets (30%) is informative with regard to the dynamics of islet formation and maintenance. If, as proposed, islets were to fuse or split with time (Seymour et al., 2004), then the proportion of islets containing DL cells should increase or decrease, accordingly, between youth and adulthood. Yet the proportion remained constant in *Ngn3-GR/RG* mice, indicating that there is little or no islet fusion or fission during the period of analysis (between 2 and 10 months of age) (Fig. 5B). These observations also imply that the total islet count per individual remains relatively constant throughout life, which we indeed observed and as reported previously by others (Dor et al., 2004).

It should be noted that the lack of subpopulations of proliferating versus non-proliferating islet cells, together with the lack of correlation between islet and cluster size, indicate that the definitive size of an islet is predetermined during pancreatic development by the number of early endocrine cells that aggregate together in a proto-islet-forming cell pool, instead of being dictated by differential islet cell proliferation during life.

Characterizing the β-cell lineage

The early genetic fate determination of islet cell lineages shown here might be a general mechanism operating in the allocation of cell fates in other developing organs. For instance, as for Ngn3+ cells in the pancreas, it was thought that precursors to granule neurons in the cerebellum were all similar. However, clonal analyses using the same approach have revealed that wiring in the cerebellum is more genetically determined than previously proposed: individual granule cell precursors are committed and restrict their axonal projections to specific sublayers of the cerebellar cortex according to the developmental stage at which they appear (Espinosa and Luo, 2008; Zong et al., 2005).

The identification of true immature islet stem or progenitor cells, i.e. at a stage prior to Ngn3 expression, might be relevant for studies aimed at promoting β-cell differentiation from either embryonic stem cells (D'Amour et al., 2006; Kroon et al., 2008) or induced pluripotent stem cells (Hochedlinger and Plath, 2009). Since Ngn3+ cells appear to be unipotent and are likely to be heterogeneous, identifying and characterizing the signature of β-committed progenitors should help to improve the chances of obtaining surrogate β-cells and of triggering intrinsic regeneration for treating diabetes.

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