Genetic inducible fate mapping in larval zebrafish reveals origins of adult insulin-producing β-cells

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SUMMARY

The Notch-signaling pathway is known to be fundamental in controlling pancreas differentiation. We now report on using Cre-based fate mapping to indelibly label pancreatic Notch-responsive cells (PNCs) at larval stages and follow their fate in the adult pancreas. We show that the PNCs represent a population of progenitors that can differentiate to multiple lineages, including adult ductal cells, centroacinar cells (CACs) and endocrine cells. These endocrine cells include the insulin-producing β-cells. CACs are a functional component of the exocrine pancreas; however, our fate-mapping results indicate that CACs are more closely related to endocrine cells by lineage as they share a common progenitor. The majority of the exocrine pancreas consists of the secretory acinar cells; however, we only detect a very limited contribution of PNCs to acinar cells. To explain this observation we re-examined early events in pancreas formation. The pancreatic anlage that gives rise to the exocrine pancreas is located in the ventral gut endoderm (called the ventral bud). Ptf1a is a gene required for exocrine pancreas development and is first expressed as the ventral bud forms. We used transgenic marker lines to observe both the domain of cells expressing ptf1a and cells responding to Notch signaling. We do not detect any overlap in expression and demonstrate that the ventral bud consists of two cell populations: a ptf1a-expressing domain and a Notch-responsive progenitor core. As pancreas organogenesis continues, the ventral bud derived PNCs align along the duct, remain multipotent and later in development differentiate to form secondary islets, ducts and CACs.

KEY WORDS: β-cells, Notch-signaling, Pancreas development, Pancreatic progenitors, Zebrafish

INTRODUCTION

Embryogenesis leads to the development of the complete body plan, but not necessarily all adult cell types. Instead, pools of stem cells and progenitors are maintained to differentiate later in development or even in the adult in response to injury or other dramatic events. To identify progenitor populations and ascertain their lineage potential, long-range fate mapping is an essential tool. Fate mapping using Cre-based technologies has proved to be a powerful technique with which to indelibly label cells and their progeny (Joyner and Zervas, 2006). This method uses two transgenic elements: (1) a Cre-driver transgene that expresses Cre in cells of interest; and (2) a Cre-responder that contains sites of recombination for Cre. Following Cre-dependent recombination, the responder will continually express a marker protein, thereby labeling the cell and subsequent descendents. Temporal control of Cre is obtained by expressing a fusion of Cre to a mutant estrogen receptor with a high affinity for 4-hydroxytamoxifen (4OHT) (Feil et al., 1997). Expression of this 4OHT-dependent Cre recombinase (CreER²) has been extensively used to label embryonic pancreatic cells in the mouse and identify their ultimate fate in adult pancreas (Gu et al., 2002; Kawaguchi et al., 2002) as well as their contribution to regenerative tissues following injury (Inada et al., 2008; Solar et al., 2009).

As a model organism the zebrafish is unique in its capacity for high-throughput chemical (Zon and Peterson, 2010) and genetic screening (Driever et al., 1996). Another asset of zebrafish biology is their phenomenal capability to regenerate tissue following injury (Jopling et al., 2010; Kikuchi et al., 2010). For these reasons, the zebrafish is increasingly being used as a system to study pancreas organogenesis (Kinkel and Prince, 2009; Tiso et al., 2009) and regeneration (Curado et al., 2007; Moss et al., 2009; Pisharath et al., 2007). In common with mammals, the zebrafish pancreas is a glandular organ consisting of two main compartments: (1) the endocrine pancreas containing hormone producing cells arranged into islets; and (2) the exocrine pancreas containing acinar and ductal cells. Acinar cells are arranged into spherical structures called acini and secrete digestive enzymes (zymogens) from their apical surface. Ductal cells form a conduit to the intestine allowing digestivezymogens to gain access to the gut lumen. Analogous to mammalian pancreas formation, the zebrafish endocrine cells differentiate in two distinct phases.

In the embryonic zebrafish the first endocrine cells form in a dorsal region of the gut endoderm. These cells coalesce during the first day of development to form what is called the principal islet (Jopling et al., 2010). These early endocrine cells have limited proliferation and ultimately contribute very little to the adult endocrine mass (Hesselson et al., 2009). The ventral bud of the pancreas can first be detected as ventral group of gut endoderm cells expressing the exocrine marker ptf1a (Lin et al., 2004; Zecchin et al., 2004). These cells migrate in a dorsal and posterior direction to envelop the principal islet (see overview in Fig. 7).

In the heart and soul (has) mutation, particular morphological movements are blocked, resulting in a phenotype that includes the failure of the ventral bud to fuse with the principal islet; hence, ventral tissue differentiates in isolation. In has mutant fish, endocrine cells differentiate in the isolated ventral pancreas (Field et al., 2003). This result implies the existence of endocrine progenitors within the ventral bud. Differentiation of these endocrine cells is equivalent to the secondary transition in
mammals and these cells ultimately contribute to the majority of the endocrine mass (Hesselson et al., 2009). This second wave of differentiation gives rise to endocrine cells that contribute to the principal islet between 48 and 120 hours post fertilization (hpf) (Pisharath et al., 2007). Following the envelopment of the principal islet, the exocrine part of the pancreas grows in a posterior direction to form the pancreatic tail. After 5-6 days of development, more endocrine cells appear along the pancreatic duct, which is located down the centre of the tail. The progressive differentiation of endocrine cells during pancreogenesis suggested the presence of a progenitor population.

The Notch-signaling pathway is used reiteratively during embryogenesis and is often instrumental in regulating cellular differentiation and fate specification. Studies in both mouse and zebrafish have shown that Notch signaling regulates the fate of pancreatic progenitors (Apelqvist et al., 1999; Esni et al., 2004; Hald et al., 2003; Jensen et al., 2000a; Jensen et al., 2000b; Zecchin et al., 2007). Using Notch-responsive transgenic elements, we recently described a population of pancreatic Notch-responsive cells (PNCs) (Parsons et al., 2009). PNCs are associated with the ducts of the pancreas, a region suggested to be the source of pancreatic progenitors (Inada et al., 2008). Blocking Notch-signaling with DAPT (a $\gamma$-secretase inhibitor) caused concomitant loss of PNCs and appearance of endocrine cells of the secondary islets. These observations led us to hypothesize that the PNCs are in fact pancreatic progenitor cells. To test this hypothesis, we adapted genetic inducible fate mapping (GIFM) to report on Notch-responsive cells and their progeny. For the first time, we report on long-range GIFM in zebrafish, labeling embryonic/larval cells and ascertaining their fate in adult tissues.

**MATERIALS AND METHODS**

**Transgenic lines used**

All new transgenic lines were generated using constructs consisting of expression cassettes cloned into the backbone of T2KXIGAN and Tol2-mediated transgenesis (Kawakami, 2004). The construct used to create the Cre-driver line (T2KTP1glob:creERT2)$^{622}$ (abbreviated to Tp1:creERT2) contains six concatamerized Notch-responsive elements from the Epstein Barr Virus terminal protein 1 (TP1) gene, which in total contains 12 Rbp-Jk binding sites (Grossman et al., 1994; Henkel et al., 1994), upstream of the rabbit $\beta$-globin minimal promoter (Minoguchi et al., 1997; Parsons et al., 2009). The gene encoding the optimized tamoxifen inducible Cre recombinase was cloned from pCre-ERT2 (Feil et al., 1997). The promoter/enhancer sequences in Tg(T2Kbactin:loxP-stop-loxP-hmgb1-mCherry)$^{913}$ (abbreviated to bactin:lox-stop-lox-hmgb1-mCherry) were cloned from pSE2-bactin (Kwan et al., 2007). The same element was used in Tg(T2Kbactin:GFP-F2A-creERT2)$^{69}$ to drive expression of GFP-F2A-creERT2, a cassette that includes sequence encoding the F2A peptide that leads to equimolar production of GFP and CreERT2 (a gift from the Meffert laboratory, Johns Hopkins University, Baltimore, MD, USA). Tg(T2Ksts12:eGFP)$^{120}$ (abbreviated to stst2:eGFP) was created using a 2.5 kb genomic fragment upstream of the ATG of st2 driving expression of GFP. Other lines used in this report are: Tg(T2Kmkins:kaede)$^{92}$ (abbreviated to ins:kaede), which labels $\beta$-cells with photoconvertible fluorescent protein (Pisharath et al., 2007); Tg(T2Kmkins:tm:GFP)$^{123}$ (abbreviated to ins:tm:GFP), which labels $\beta$-cells nuclei (Parsons et al., 2009); Tg(ggax:eGFP)$^{14}$ (abbreviated to ggax:eGFP), which labels $\alpha$-cells (Pauls et al., 2007); Tg(pfs12a:eGFP)$^{124}$ (abbreviated to pfs12a:eGFP), which labels acinar cells of the exocrine pancreas (Park et al., 2008); Tg(–3.5kbnsx2.2a:GFP)$^{14}$ (abbreviated to nsx2.2a:GFP), which labels larval duct cells (Pauls et al., 2007); and Tg(Tp1:beta-globin:eGFP)$^{14}$ (abbreviated to Tp1:beta-globin:eGFP), which labels acinar cells of the exocrine pancreas (Park et al., 2008); Tg(–3.5kbnsx2.2a:GFP)$^{14}$ (abbreviated to nsx2.2a:GFP), which labels larval duct cells (Pauls et al., 2007); and Tg(Tp1:beta-globin:eGFP)$^{14}$ (abbreviated to Tp1:beta-globin:eGFP), which labels acinar cells of the exocrine pancreas (Park et al., 2008). Kaede labeling and induction of secondary islets

To photoconvert kaede protein in the $\beta$-cells of 3 days post fertilization (dpf) ins:kaede, larval zebrafish were exposed for 30 seconds UV using a DAPI filter ($\lambda _{ex}$=375nm) on an Axiovert inverted scope (Pisharath et al., 2007). To induce secondary islets, larvae were incubated in 100 $\mu$M N-[3,5-difluorophenacyl]-L-alanyl]-S-phenylglycine t-buty1 ester (DAPT, D 5942, Sigma) at 3 dpf until 5 dpf as described by Parsons et al. (Parsons et al., 2009). Vehicle alone (1% DMSO in E3) was used as a negative control. All the larvae were incubated at 28°C in the dark. Larvae were fixed overnight in 4% PFA and micro-dissected to remove the pancreata at 5 dpf.

**Embryo dissection and confocal microscopy**

Embryo dissection and confocal microscopy were performed as described previously (Parsons et al., 2009).

**Temporal control of CreERT2 activity**

4-Hydroxytamoxifen (4OHT, T176, Sigma) was dissolved in 100% ethanol to create a stock solution of 10 mM. A working concentration of 5 $\mu$M 4OHT was demonstrated to optimally cause Cre-mediated recombination without causing deleterious development defects. Embryos were placed in 5 $\mu$M solution of 4OHT in E3 and kept in dark at 28°C for 24 hours (unless otherwise stated in text), then washed in fresh E3.

**Semi-automated cell counts using ImageJ software**

To quantify labeled cells during fate-mapping experiments and control experiments, we counted the recombined cells labeled with nuclear mCherry, $\beta$-cells with nuclear eGFP and total pancreatic cells stained with DAPI. ImageJ software (NIH) was used to separate the color channels to quantify numbers of red, green and blue cells.

Standard box plot used to display results (i.e. drawn boxes demarcate central 50% of data with the median shown by horizontal line). Limits of all data indicated by bracketed lines.

**Section and immunofluorescence**

Embryos were fixed overnight in 4% paraformaldehyde at 4°C. Adult fish were fixed overnight in 10% formalin (at 4°C) followed by viscera dissection. Samples were equilibrated in 30% sucrose, either overnight or until non-buoyant at 4°C, before being embedded in OCT compound (Tissue-Tek). Cryostat sections (5 $\mu$m) were cut and stained with DAPI. When fluorescence was weak, as in the case in adult Tp1:beta-globin:eGFP fish, sections were processed using standard immunofluorescent procedures (Abcam) to detect GFP (rabbit polyclonal, anti-eGFP from Invitrogen, 1:400) and mCherry (mouse monoclonal, anti-DsRed from Clontech, 1:400). For co-labeling adult ducts and the lineage tracer mCherry, immunofluorescent staining for Cytookeratin 18 (mouse monoclonal, anti-Keratin 18 from Progen; 1:10) and mCherry (rabbit polyclonal, anti-DsRed from Clontech, 1:400) was carried out.

**RESULTS**

**Secondary islet $\beta$-cells do not originate from pre-existing principal islet $\beta$-cells**

Two models have been proposed for the formation of secondary islets. Either the secondary islets are ‘seeded’ from pre-existing endocrine cells of the principal islet that migrate down the duct and colonize new islets or, as is the case during mammalian neogenesis, progenitors within the ductal epithelia differentiate into new endocrine tissue (Tiso et al., 2009). To determine which of these two models represents what occurs during secondary islet formation, we took advantage of the transgenic line ins:kaede in which photoconvertible Kaede protein is produced in the $\beta$-cells. UV light permanently converts green fluorescent kaede protein into a red fluorescent protein that labels these cells and their progeny for several days (Pisharath et al., 2007). By converting Kaede in $\beta$-cells into red before secondary islet formation, we sought to determine whether the secondary islets that appeared were derived from principal islet cells labeled both red and
In cells of the Cre-responder fish, Cre activity removes a stop green fluorescent kaede protein in (white arrowheads) but is not detected in DAPT-induced secondary islets (white arrows), including differentiated β-cells that are marked by kaede protein. (E,G) Photoconversion at 72 hpf labeled all β-cells present at that time point with red fluorescence. By 120 hpf, photoconverted kaede is still apparent in principal islet cells labeled all (photoconverted to red fluorescent protein by UV exposure at 72 hpf. By 120 hpf, photoconverted kaede is still apparent in principal islet cells labeled all (photoconverted to red fluorescent protein by UV exposure at 72 hpf. (B,F) DAPT treatment from 72-120 hpf induces secondary islets (white arrows), including differentiated β-cells that are marked by kaede protein. (E,G) Photoconversion at 72 hpf labeled all β-cells present at that time point with red fluorescence.

**Genetic inducible fate mapping of Notch-responsive cells of the pancreas**

Studies in mouse (reviewed by Kim et al., 2010) and fish (reviewed by Tiso et al., 2009) have implicated Notch signaling in regulating differentiation of pancreatic progenitors. We have previously identified a population of pancreatic Notch-responsive cells (PNCs) within the pancreatic duct (Parsons et al., 2009). To ascertain whether PNCs are multi-lineage pancreatic progenitors, we developed a transgenic system to carry out genetic inducible fate mapping of the Notch-responsive cells.

We generated a Cre-driver line of fish from a transgene called Tp1:creER\(^{12}\) (Fig. 2A). In these transgenic fish, expression of 4OHT inducible Cre-recombinase (creER\(^{12}\)) is transcribed under the control of a Notch-responsive element (Tp1). To detect Cre-activity, we also generated a Cre-responder line called βactin:lox-stop-lox-hmgb1-mCherry (Fig. 2B). In cells of the Cre-responder fish, Cre activity removes a stop cassette leading to mCherry being expressed from the β-actin promoter/enhancer. Crossing the Cre-driver to the Cre-responder line creates double transgenic fish (Tp1:creER\(^{12}\); βactin:lox-stop-lox-hmgb1-mCherry) in which genetic inducible fate mapping of Notch-responsive cells can be carried out. For simplification, these compound transgensics are called GIFM fish. To demonstrate that the Tp1:creER\(^{12}\) expresses creER\(^{12}\) in the predicted expression pattern, we out-crossed GIFM fish to the Notch-responsive marker line Tp1:eGFP (Lorent et al., 2010; Parsons et al., 2009). Progeny of this cross (Tp1:eGFP; GIFM) were treated with 4OHT at various points during development. Within 6 hours of treatment, the lineage tracer hmgb1-mCherry could be detected by immunofluorescence and compared with the eGFP marker of Notch responsivity. All cells labeled with by hmgb1-mCherry expression were also marked with eGFP (see Figs S1 and S2 in the supplementary material for examples). This demonstrates that Tp1:creER\(^{12}\) transgene is directing creER\(^{12}\) expression to the intended target Notch-responsive cells.

To further facilitate interpretation of fate-mapping results, we also established the highest level of 4OHT independent Cre-activity (‘leakage’) that can occur in our GIFM fish. To do this GIFM/ins:hmgb1-eGFP larvae were treated with 4OHT (n=26) at 56 hpf for 24 hours. These larvae were raised along with untreated controls (n=20). At 55 dpf, the fish were euthanized, sectioned and the number of lineage traced cells contributing to the juvenile pancreas counted. Eighteen out of 26 4OHT treated and five out of 20 untreated controls showed evidence of at least some red cells, indicating evidence of Cre activity. As we were interested in calculating the highest levels of leakage possible, we examined pancreata derived from the five untreated control fish showing leakage. Examination of multiple sections indicated leakage occurred in 1.2% of cells (432/30,935). Lineage traced cells with red fluorescent cells were found scattered throughout the pancreas parenchyma (Fig. 2D,J). This compares with an average of 19% red cells (8558/41,981) in pancreata from the group treated with 4OHT as larvae (five fish counted from 18, Fig. 2E,J). Using the green fluorescence as a marker for β-cells, no ‘leakage’ was detected in control β-cells (0/1149; Fig. 2F,J), but lineage tracing of this cell-type was evident (564/2561, 22%; Fig. 2G,J) in adult sections derived from 4OHT-treated larvae.

Next, to test the efficiency of fate mapping in our GIFM fish, we again used the transgenic line where the PNCs are labeled with GFP (Tp1:eGFP) and asked what proportion of the Notch-responsive cells can be also lineage traced with our Notch-responsive Cre-driver. GIFM/Tp1:eGFP larvae were treated with 4OHT at either 12 hpf (n=10) or 56 hpf (n=6) for 24 hours then fixed and imaged at 5 days. Assuming the rate of differentiation and proliferation of labeled and non-labeled PNCs is the same, the percentage of lineage traced PNCs at 5 dpf gives an indication of the efficiency to label the target cells in our GIFM fish. Regardless of whether 4OHT was added at 12 or 56 hpf, by 5 days the majority of PNCs were labeled by the lineage tracer (76.5% and 75.4%, respectively; example results shown in Fig. 2I,J). This frequency is not 100%, which implies 4OHT, Cre-driver or Cre-responder activity (or combination) is not optimal in all PNCs. As is the case with fate mapping in other systems, our results indicate that we can follow the identity of traced cells and show their contribution to specific cell types; however, it is harder to interpret when no contribution is detected (Kawaguchi et al., 2010). Having tested the background levels and efficiency of Cre-activity in the GIFM fish, we preceded to fate map the Notch-responsive cells of the larval pancreas.
Early Notch-responsive cells contribute to the principal islet of larval zebrafish

By incubating GIFM embryos/larvae in 4OHT at different stages of development and then following the fate of labeled cells, we can ask whether and at what stages do PNCs contribute to different cell populations of the pancreas. 4OHT was added either early (12-36 hpf) during pancreas formation or late (56-80 hpf). Contributions of Notch responsive cells were assessed at 5 dpf, a time point when most of the pancreas architecture is already formed.

The first pancreatic cell types to form during embryogenesis are endocrine cells of the principal islet. Adding 4OHT early (12-36 hpf) leads to traced cells contributing to 21.33% of principal islet \( \beta \)-cells (36/158 lineage traced \( \beta \)-cells out from five embryos; Fig. 3B,C) as well as to \( \alpha \)- and \( \delta \)-cells (see Fig. S3 in the supplementary material). From our fate mapping it is clear that early Notch-responsive cells represent a population of progenitors that can differentiate to multiple lineages. These results are consistent with work of Zecchin and colleagues, who showed that Notch-inhibition via DAPT between 5 and 13 hpf influenced both the composition and cell number of the principal islet (Zecchin et al., 2007). It is clear, therefore, that Notch-responsive progenitors exist in nascent endoderm and give rise to the endocrine cells of the principal islet.

Although ventral-derived progenitors do contribute to a small number of endocrine cells of the principal islet (Field et al., 2003; Hesselson et al., 2009; Pisharath et al., 2007), later addition of 4OHT (56-80 hpf) led to only very limited contribution of lineage traced cells in the principal islet (Fig. 3F,G). Of six fate-mapped GIFM larvae examined, only one larval pancreas showed any principal islet contribution (one \( \beta \)-cell out of 45). It is possible that the exact timing of 4OHT addition is too late to effectively lineage trace progenitors that are differentiating and contributing to the principal islet. Alternatively, as the number of \( \beta \)-cell precursors that differentiate and add to the principal islet during secondary transition is small, it is possible that the 4OHT-dependent Cre-activity in our GIFM fish is just not sensitive enough to efficiently label and allow detection of such a small population of cells.

The ventral bud of the developing pancreas can first be detected by the expression of the exocrine marker ptf1a in a ventral/anterior group of gut endodermal cells (Lin et al., 2004; Zecchin et al., 2004). We asked whether and when do PNCs contribute to exocrine pancreas. As before, we used early (12-36 hpf) or late (56-80 hpf) time points to add 4OHT and counted lineage traced cells at 5 dpf. Both early and late 4OHT incubation led to some traced cells contributing to ptf1a-positive exocrine tissue, although this labeling was only seen in a very small fraction of cells. Out of six early treated fish, three fish showed some contribution giving an

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Fig. 2. Regulation of Cre-recombinase activity in the pancreas. (A) Schematic of Notch responsive Cre-driver transgene Tp1:creER\(^{T2}\) and (B) the Cre-responder transgene \( \beta \)-actin:loxP-stop-loxP-hmgb1-mCherry. (A) Tol2 arms (open triangles) flank 12 RBP-J\( \kappa \)-binding sites (gray circles), the \( \beta \)-globin minimal promoter (arrow) and the gene encoding the 4OHT-regulated Cre recombinase (CreER\(^{T2}\)). (B) The \( \beta \)-actin enhancer/promoter upstream of a loxP-flanked (black triangles) transational stop. (C) Following Cre-dependent recombination, the stop cassette is removed, allowing transcription of a gene encoding nuclear Hmgb1-mCherry (nuc-mCherry). (D-G) Sections through adult pancreas (55 dpf) from GIFM fish. (D) Limited Cre-activity is evident without 4OHT. (E) Treatment with 4OHT at 56 hpf results in widespread labeling in adult (55 dpf) pancreatic tissue. (F,G) \( \beta \)-Cells are marked green in GIFM/ins:hmgb1-eGFP fish. (F) Without larval exposure to 4OHT, \( \beta \)-cells are not labeled with red fluorescent Hmgb1-mCherry. (G) With larval 4OHT treatment (at 56 hpf), adult \( \beta \)-cells are labeled. (H,I) Micro-dissected pancreata from 5 dpf GIFM/Tp1:eGFP larvae. PNCs are marked by GFP fluorescence. (I) 4OHT incubation from 56-80 hpf results in Cre-dependent labeling of 75% PNCs by 5 dpf. DAPI used to counterstain nuclei (D-I). (J) Box plots quantifying results shown in D-I. Scale bars: 100 \( \mu \)m in E,G,I.
average of eight lineage traced cells per pancreas (n=6, Fig. 3J,K). Five out of 6 of the late-treated fish showed no contribution (Fig. 3N,O); however, one fish had two labeled cells giving an average of 0.33 cells per pancreas. These results are likely to represent a very small percentage as the exocrine pancreas of a 5 dpf larvae consist of over 260 cells (Jiang et al., 2008). To ensure the apparent lack of PNC contribution to exocrine pancreas was not an artifact of the Cre-responder, we tested the responder line against a different Cre-driver line (namely β-actin:gfp-F2A-creERT2). Adding 4OHT at our ‘late’ developmental stage readily labeled the β-cells as detected by the expression of nuclear GFP (Fig. 5A). One hundred and nineteen of these cells (51%) also expressed the ptf1a-positive cells and a core of PNCs. As with earlier stages, no colocalization of ptf1a expression and Notch-responsive progenitors is formed from both a dorsal bud-derived principal islet and a ventral bud (Field et al., 2003). This latter anlage forms from the ventral surface of the gut tube, in a position that is anterior to the principal bud (Field et al., 2003). This previous observation taken together with our present results, using ins:kaede fish, lead us to hypothesize that it is ductal PNCs that are differentiating into endocrine cells and giving rise to the secondary islets. To test this hypothesis, we used GIFM/ins:hmgb1-eGFP fish (n=18) so we could simultaneously observe β-cells and fate map PNCs. These fish were treated first with 4OHT, from 56-72 hpf, to indelibly label and allow lineage tracing of the PNCs. After changing the media, the larvae were incubated in DAPT (72-120 hpf) to induce inhibition (with DAPT) causes PNCs to lose their Notch responsivity, concurrent with the appearance of secondary islets (Parsons et al., 2009). This previous observation taken together with our present results, using ins:kaede fish, lead us to hypothesize that it is ductal PNCs that are differentiating into endocrine cells and giving rise to the secondary islets. To test this hypothesis, we used GIFM/ins:hmgb1-eGFP fish (n=18) so we could simultaneously observe β-cells and fate map PNCs. These fish were treated first with 4OHT, from 56-72 hpf, to indelibly label and allow lineage tracing of the PNCs. After changing the media, the larvae were incubated in DAPT (72-120 hpf) to induce secondary islets. At 120 hpf, these fish were fixed, microdissected and the pancreas imaged by confocal microscopy (Fig. 5D). From the 18 pancreata imaged, we counted 234 induced secondary islet cells (Parsons et al., 2009). This observation is consistent with our fate-mapping result where we see no contribution of PNCs to the exocrine pancreas between 56 and 80 hpf.

β-Cells of the secondary islets are formed from Notch-responsive progenitors

We have previously shown that PNCs stay associated with the pancreatic duct throughout development; however, Notch-inhibition (with DAPT) causes PNCs to lose their Notch responsivity, concurrent with the appearance of secondary islets (Parsons et al., 2009). This previous observation taken together with our present results, using ins:kaede fish, lead us to hypothesize that it is ductal PNCs that are differentiating into endocrine cells and giving rise to the secondary islets. To test this hypothesis, we used GIFM/ins:hmgb1-eGFP fish (n=18) so we could simultaneously observe β-cells and fate map PNCs. These fish were treated first with 4OHT, from 56-72 hpf, to indelibly label and allow lineage tracing of the PNCs. After changing the media, the larvae were incubated in DAPT (72-120 hpf) to induce secondary islets. At 120 hpf, these fish were fixed, microdissected and the pancreas imaged by confocal microscopy (Fig. 5D). From the 18 pancreata imaged, we counted 234 induced secondary islet β-cells as detected by the expression of nuclear GFP (Fig. 5A). One hundred and nineteen of these cells (51%) also expressed the
As a guide, traced cells were frequently located surrounded by the apical surface of acinar cells, in a position that predicts centroacinar cell (CAC) identity (Fig. 6O). Contribution of larval PNCs to the CACs of the adult was confirmed by using Tp1:eGFP transgenic fish that express GFP in the CACs of the adult pancreas (Fig. 6S). We sectioned pancreata from these lineage-traced, Tp1:eGFP/GIFM fish (n=6) and observed that 74% (381/514) of the GFP marked cells also expressed the mCherry lineage tracer. Finally, contribution of larval PNCs to the adult duct was demonstrated by the immunofluorescent detection of the ductal marker cytokeratin 18 in fate-mapped cells (Fig. 6W). In conclusion, we have shown that larval PNCs represent a progenitor population with multi-lineage potential that can differentiate into all tested endocrine lineages, as well as into ductal and centroacinar cells.

**DISCUSSION**

To summarize this report, we have demonstrated the following: (1) that genetic inducible fate mapping (GIFM) can be used in the zebrafish over long periods of time, enabling the adult fate of larval cells to be determined; (2) that previously described populations of pancreatic Notch-responsive cells are the progenitors of adult α, β, δ, ductal and centroacinar cells; and (3) that PNCs can be distinguished as a ventral bud-derived population that is separate from the ptf1a-expressing pancreatic progenitor pool (see overview in Fig. 7).
More specifically, we use two different fate-mapping techniques to elucidate the origins of the early endocrine cells of the principal islet and the later forming secondary islets. As in the mouse, the zebrafish endocrine system forms in two waves. In the fish, Notch-responsive progenitors are involved in both processes. Early labeling of Notch-responsive cells with 4OHT application (12-36 hpf) demonstrates contribution of these progenitors to the dorsal bud-derived principal islet. Our results are consistent with the results of Zecchin and colleagues, who observed that inhibiting Notch function affects the composition of the principal islet. Application of DAPT between 5 and 13 hpf leads to an increased number of $\beta$ and $\delta$-cells at the expense of later differentiating $\alpha$-cells and exocrine pancreas (Zecchin et al., 2007). It is clear, therefore, that there is a population of Notch-responsive progenitors within the developing gut endoderm that is fated to become pancreatic endocrine cells. Furthermore, these cells are using the Notch-signaling pathway to resolve specific endocrine cell-fate decisions.

As summarized in Fig. 7, we have shown for the first time that the ventral bud of the developing zebrafish pancreas initially consists of two separate domains: a so-called dorsal bud that forms early (around 20 hpf); and the later-forming ventral bud. Six stages of pancreas formation, following the appearance and contribution of the ventral bud derived tissue, are shown. The schematic represents dorsal views of the developing pancreas. The position of the gut epithelium is represented in yellow. By 30 hpf, a cluster of PNCs is first observed (blue circles) that is located anterior and ventral to the principal islet (red circles). Slightly later in development, Ptf1a expression is turned on in cells (green circles) that are adjacent to the PNCs, and anterior and ventral to the principal islet. The Ptf1a cells actively migrate to engulf PNCs (see Movie 1 in the supplementary material) and move along ventral surface of gut endoderm and cross the midline. As a single entity the Ptf1a/PNCs ventral bud moves in a dorsal direction to contact and engulf the principal islet to create the head of the pancreas (head). The Ptf1a cells proliferate and differentiate into acinar cells (larger green circles) and the pancreas elongates to create the pancreatic tail (tail). The PNCs surround the principal islet and line the central lumen of the pancreas. The PNCs differentiate to add new endocrine cells (magenta circles) to the principal islet and secondary islets located in the tail.
ventral bud does contact the principal islet, the Notch-responsive cells localize around the early endocrine cells (Fig. 7). As the pancreatic tail is formed, PNCs align along the central pancreatic duct (Parsons et al., 2009). These PNCs in the duct form a population of progenitors that form secondary islets during development. This result is significant as it both identifies the precursors of the insulin producing β-cells of the secondary islets and demonstrates that the Tp1 Notch-responsive element can be used as a molecular tool to manipulate and study β-cell progenitors. It is hoped that a better understanding of pancreatic progenitor biology will ultimately facilitate discovery of therapies to resolve the β-cell paucity in individuals with diabetes (Limbert et al., 2008).

During mouse pancreatogenesis, nearly all acinar, ductal and islet cells are derived from early Ptf1a-expressing multi-potent progenitor cells (Kawaguchi et al., 2002). Our results suggest a different mechanism occurs in the developing zebrafish. At the level of transgenic marker analysis, we show the existence of a population of PNCs that do not express ptf1a. These PNCs give rise to both cells of the pancreatic duct and endocrine system. The relationship between ptf1a expression and PNCs warrants further investigation for two reasons. First, by fate mapping PNCs in our GIFM fish, we do not label all α, β, δ, ductal or centroacinar cells. The most likely reason being a combination of incomplete 4OHT function and Cre-driver/Cre-responder expression. However, it cannot be ruled out that endocrine cells also differentiate from a non-PNC progenitor. It is a formal possibility that the ptf1a-expressing region of the ventral bud also contributes to the endocrine and ductal system in an analogous way to that reported in the mouse. To test this hypothesis, a ptf1a Cre-driver line would be desirable. Second, such a ptf1a Cre-driver could be used to ascertain whether PNCs express ptf1a transiently, in a manner that could be missed at the level of transgenic marker analysis.

One cell type that remains Notch responsive in the adult pancreas are the centroacinar cells (CACs) (Miymamoto et al., 2003; Parsons et al., 2009). As shown in this report, at least 76% of CACs share a common lineage with the endocrine system as both cell types are derived from larval PNCs. Either all CACs are derived from PNCs and the lineage tracing is incomplete, or CACs are derived from multiple cell types. Detailed investigation of CAC's shows they share several characteristics with their PNC predecessors. First, both PNCs and CACs are closely associated with the pancreatic ducts. PNCs are aligned along the length of the developing pancreatic duct and CACs are located within the adult pancreatic acinus at the end of the terminal ducts. Second, both PNCs and CACs are morphologically similar, both possessing long cellular extensions that contact each other and form a network throughout the pancreatic tissue (Leeson and Leeson, 1986; Parsons et al., 2009). Whether CACs retain the capacity for differentiation in a similar fashion to their predecessors, the PNCs, is an intriguing question. Several studies in the mouse imply that CACs maybe a multi-lineage pancreatic progenitor: (1) they are a cell type that undergoes active Notch signaling (Miymamoto et al., 2003; Parsons et al., 2009); (2) CACs proliferate following pancreas injury and prior to regeneration (Gasslander et al., 1992; Hayashi et al., 2003; Nagasao et al., 2005; Seymour et al., 2007); (3) expansion of the CAC population is integral to the development of metaplasia caused by pancreas-specific knockout of the tumor suppressor PTEN (Stanger et al., 2005); and (4) a CAC/terminal ductal cell population, with high levels of ALDH activity, has recently been isolated. These cells can be kept in tissue culture and display all characteristics of a multi-potent pancreatic progenitors (Rovira et al., 2010).

Our GIFM fish can now be used to elucidate the role of CACs in pancreas biology. As with murine experiments, it has recently been shown that the function of CreER<sup>T2</sup> can be temporally regulated in adult fish by injection of 4OHT (Jopling et al., 2010; Kikuchi et al., 2010). The ability to temporally regulate CreER<sup>T2</sup> in adult Notch-responsive cells will allow us to ascertain whether the CACs of adult fish are a population of progenitors and whether these cells are involved in the remarkable ability of the fish pancreas to regenerate (Moss et al., 2009). Besides elucidating the biology of CACs, the coupling of Notch-responsive elements with genetic inducible lineage fate mapping creates a unique resource that can facilitate understanding the function of progenitors in many other areas of biology.

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