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MECHANISMS OF DEVELOPMENT

Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas

Michael J. Parsons[a,b,*], *Harshan Pisharath*[c], *Shamila Yusuff*[a], *John C. Moore*[d], *Arndt F. Siekmann*[d], *Nathan Lawson*[d], *Steven D. Leach*[a,b,e]

[a]*Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*
[b]*McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*
[c]*Department of Comparative Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*
[d]*Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, MA 01605, USA*
[e]*Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*

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ABSTRACT

Zebrafish provide a highly versatile model in which to study vertebrate development. Many recent studies have elucidated early events in the organogenesis of the zebrafish pancreas; however, several aspects of early endocrine pancreas formation in the zebrafish are not homologous to the mammalian system. To better identify mechanisms of islet formation in the zebrafish, with true homology to those observed in mammals, we have temporally and spatially characterized zebrafish secondary islet formation. As is the case in the mouse, we show that Notch inhibition leads to precocious differentiation of endocrine tissues. Furthermore, we have used transgenic fish expressing fluorescent markers under the control of a Notch-responsive element to observe the precursors of these induced endocrine cells. These pancreatic Notch-responsive cells represent a novel population of putative progenitors that are associated with larval pancreatic ductal epithelium, suggesting functional homology between secondary islet formation in zebrafish and the secondary transition in mammals. We also show that Notch-responsive cells persist in the adult pancreas and possess the classical characteristics of centroacinar cells, a cell type believed to be a multipotent progenitor cell in adult mammalian pancreas.

1. Introduction

Eukaryotic organisms have evolved multiple strategies for achieving reliable glucose homeostasis, manifested by a variety of cell types capable of producing insulin- and/or other glucoregulatory peptide hormones. In vertebrates, these include: (a) the dispersed enteroendocrine system, present in all bilaterian organisms (Drucker, 2007), (b) the Brockmann body, a foregut-associated collection of endocrine cells observed in larval lamprey and other jawless fish (Youson and Al-Mahrouki, 1999), (c) immature endocrine cells observed in the early pancreatic bud of mammals (Herrera, 2000; Teitelman et al., 1993), and (d) mature α-, β-, δ-, PP and ghrelin-producing cells located within the pancreatic islets of cartilaginous and bony fish, as well as in all tetrapods (Burlison et al., 2008; Chiang and Melton, 2003; Kawaguchi et al., 2002; Offield et al., 1996; Prado et al., 2004). In mammals, the stages of pancreas development corresponding to active morphogenesis and formation of early endocrine cells (E9.5–E12.5) are often referred to as the "primary transition", while the

* *Corresponding author.* Address: Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA. Tel.: +1 410 502 2982; fax: +1 410 502 1853.
E-mail address: mparson3@jhmi.edu (M.J. Parsons).
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later appearance of mature α-, β-, ε-, δ-, and PP-producing cells is often referred to as the "secondary transition" (Kemp et al., 1972; Wang et al., 2005). During the secondary transition, endocrine cells are formed by delamination of dedicated endocrine progenitor cells from the nascent branching ductal epithelium, followed by their differentiation, migration and consolidation into islet clusters (Jensen, 2004; Murtaugh and Melton, 2003). Several observations have indicated that initiation of islet neogenesis occurs in association with ductal epithelium in a variety of settings (Bonner-Weir et al., 2008; Gu and Sarvetnick, 1993; Hayashi et al., 2003; Rosenberg et al., 1983; Song et al., 1999; Xu et al., 2008; Zhang et al., 2002). Furthermore, recent work using lineage tracing in the mouse has demonstrated that endocrine progenitors, responsible for compensatory regeneration following tissue injury, are also ductal in nature (Inada et al., 2008).

Type 1 diabetes mellitus is characterized by the autoimmune destruction of pancreatic β-cells, resulting in insulin deficiency. The path to a cure for diabetes will rely on achieving a cessation of β-cell destruction, followed by restoration of β-cell mass, either by replacement with exogenous cells or by regeneration from endogenous progenitors. For these reasons, there continues to be considerable interest in understanding how β-cell specification and differentiation are regulated in both adult and embryonic pancreas.

Over the past decade, the zebrafish (*Danio rerio*) has been increasingly utilized as complementary system in which to study β-cell differentiation, both during normal development (reviewed in Gnugge et al., 2004; Kinkel and Prince, 2009) and in the context of regeneration following injury (Curado et al., 2007; Moss et al., 2009; Pisharath et al., 2007). Zebrafish pancreas development shares many cellular and molecular events in common with pancreas development in mammals (reviewed in Gnugge et al., 2004; Kinkel and Prince, 2009). As in the mouse, the zebrafish pancreas is formed from both dorsal and ventral anlagen (Field et al., 2003). Cells of the dorsal pancreas appear to exclusively form endocrine cells, which by 24 h post fertilization (hpf) have coalesced to form the 'principal islet'. In contrast, cells in the ventral anlage serve as progenitors for all acinar and ductal cells of the future pancreas, as well as an additional population of early endocrine cells (Dong et al., 2007, 2008; Field et al., 2003; Lin et al., 2004). While the embryonic and larval zebrafish pancreas consist of a single principal islet, the adult pancreas contains many secondary islets, similar to the situation observed in mammals (Chen et al., 2007). Because these cells do not commonly appear during the first 6 days of development, it seems likely that a population of endocrine progenitors must exist in larval zebrafish pancreas (Biemar et al., 2001). However, the location and identity of these cells has not yet been determined.

Among the different cell populations in zebrafish endocrine pancreas, prior studies of β-cell differentiation have been largely limited to cells in the principal islet, as the early differentiation of these cells facilitates transient genetic manipulations including gene knockdown using antisense morpholinos. However, the mechanism and timing of principal islet development differs considerably from islet formation in mammals, as the zebrafish principal islet forms prior to formation of an actual gut tube and in absence of exocrine elements. This discrepancy leads to uncertainty regarding the relationship between principal islet formation in zebrafish and formation of endocrine cells during either the primary or secondary transition in mammals. In order to better identify mechanisms of islet formation in zebrafish with true homology to those observed in mammals, we have characterized secondary islet formation in zebrafish pancreas. In so doing, we have defined a novel population of pancreatic Notch-responsive cells that reside within the larval pancreatic ductal epithelium. These cells differentiate concomitantly with the appearance of new endocrine cells of the secondary islets, suggesting functional homology between secondary islet formation in zebrafish and the secondary transition in mammals.

2. Results

2.1. Occurrence and localization of secondary islets

In order to characterize a secondary transition in islet formation in the zebrafish, we analyzed both the timing and localization of forming secondary islets during larval development. Secondary islets form within the tail of the pancreas, posterior and distinct from the principal islet. We examined, using a dissecting stereoscope, transgenic larvae for both *Tg(T2Kins:mCherry)*^{jh2} (abbreviated to *ins:mCherry*) (Pisharath et al., 2007) and *Tg(P0-pax6b:GFP)*^{ulg515} (abbreviated to *pax6b:GFP*) (Delporte et al., 2008) through larval development. Larvae were raised in petri dishes as normal until 5 dpf. After 5 dpf, these larvae were placed in tanks and fed in our fish facility in the usual manner. A dissecting stereoscope was used to observe the presence of fluorescent islets in the pancreatic tail of living larvae. By counting the numbers of larvae displaying fluorescence in the tail of the pancreas over time, we established the temporal pattern of secondary islet formation. The two lines used mark either early pan-endocrine (*pax6b:GFP*) or a mature endocrine cell type; namely, β-cells (*ins:mCherry*). At the level of detection afforded by a dissecting scope on living larvae, only a minority of larvae possessed detectable secondary islets at 5 dpf, in either line (Fig. 1A); *pax6b:GFP* (average 17.7%, $n = 36$) or *ins:mCherry* (average 9.2%, $n = 86$). Conversely, by 20 dpf the majority have developed secondary islets as seen in both lines; *pax6b:GFP* (average 96%, $n = 54$) or *ins:mCherry* (average 89%, $n = 101$). At all time points more larvae display *pax6b* driven expression in a secondary islet position than express *ins* driven mCherry, consistent with *pax6b* marking both nascent and mature endocrine tissue.

Next we used a combination of transgenic analysis and immunofluorescent detection to characterize secondary islets in juvenile fish. Our results show that in juvenile fish (28 dpf) secondary islets are located near the center of the pancreas trunk/tail, in close proximity to a morphologically distinct and *ptf1a* negative, main pancreatic duct (Fig. 1B). The secondary islets maintain this position in adults (Fig. 1C), an observation also made in other teleosts (Morrison et al., 2004). At high magnification it can be seen that these secondary islets are in direct contact with branch points along the main duct (Fig. 1D–F). This observation is consistent with islets forming from progenitors located within or immediately