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Retinoic acid plays an evolutionarily conserved and biphasic role in pancreas development

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ABSTRACT

As the developing zebrafish pancreas matures, hormone-producing endocrine cells differentiate from pancreatic Notch-responsive cells (PNCs) that reside within the ducts. These new endocrine cells form small clusters known as secondary (2°) islets. We use the formation of 2° islets in the pancreatic tail of the larval zebrafish as a model of β-cell neogenesis. Pharmacological inhibition of Notch signaling leads to precocious endocrine differentiation and the early appearance of 2° islets in the tail of the pancreas. Following a chemical screen, we discovered that blocking the retinoic acid (RA)-signaling pathway also leads to the induction of 2° islets. Conversely, the addition of exogenous RA blocks the differentiation caused by Notch inhibition. In this report we characterize the interaction of these two pathways. We first verified that signaling via both RA and Notch ligands act together to regulate pancreatic progenitor differentiation. We produced a transgenic RA reporter, which demonstrated that PNCs directly respond to RA signaling through the canonical transcriptional pathway. Next, using a genetic lineage tracing approach, we demonstrated these progenitors produce endocrine cells following inhibition of RA signaling. Lastly, inhibition of RA signaling using a cell-type specific inducible cre/lox system revealed that RA signaling acts cell-autonomously in PNCs to regulate their differentiation. Importantly, the action of RA inhibition on endocrine formation is evolutionarily conserved, as shown by the differentiation of human embryonic stem cells in a model of human pancreas development. Together, these results revealed a biphasic function for RA in pancreatogenesis. As previously shown by others, RA initially plays an essential role during embryogenesis as it patterns the endoderm and specifies the pancreatic field. We reveal here that later in development RA is involved in negatively regulating the further differentiation of pancreatic progenitors and expands upon the developmental mechanisms by which this occurs.

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Introduction

Type I diabetes is a chronic disease caused by the loss of insulin-expressing β cells, leading to elevated blood glucose levels (hyperglycemia) and tissue damage. Ultimately, type I diabetics require insulin therapy. Long-term insulin therapy can be associated with serious complications such as inadvertent hypoglycemia and insulin resistance (Donga et al., 2013). β-cell replacement therapy is a promising way to cure type I diabetes, but its use is limited by both the paucity of donor islet tissue for transplantation and problems associated with continuous immunosuppression (Robertson, 2004). Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) possess the ability to differentiate into many derivatives of the three primary germ layers: ectoderm, mesoderm, and endoderm (Murry and Keller, 2008). Progress has been made in generating β cells *in vitro* from hESCs and iPSCs (Nostro and Keller, 2012); however, because this method is still relatively inefficient and has accompanying safety concerns, this technique is still some way from becoming a cure. Elucidating mechanisms regulating β-cell development in normal pancreas helps identify crucial signals that improve the efficiency of generating mature β cells *in vitro* and could potentially point to ways of inducing endogenous pancreatic progenitors to differentiate in diabetic patients.

The development of the zebrafish pancreas has been well studied and is closely conserved with that of the mammalian pancreas (Kinkel and Prince, 2009; Tiso et al., 2009). The first step of pancreatogenesis is the specification of the pancreatic field from nascent foregut endoderm, which in zebrafish occurs in the first day of development. The retinoic acid (RA)-signaling pathway is

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crucial in specifying the pancreatic field (Kinkel et al., 2009; Stafford and Prince, 2002; Stafford et al., 2006). RA is derived from vitamin A and acts as a ligand for nuclear RA receptors (RARs) that directly regulate the transcription of downstream target genes important for development (Rhinn and Dolle, 2012). The distribution and levels of RA in the embryo are tightly controlled by synthesis enzymes (aldehyde dehydrogenases, Aldhs) and specific degradation enzymes of the cytochrome P450 subfamily (CYP26A1, CYP26B1 and CYP26C1), allowing RA to function like a morphogen to control the differentiation and patterning of different stem and progenitor cell populations (Rhinn and Dolle, 2012). *Neckless* (*nls*) mutant fish lack Aldh1a2 activity and, as a consequence, RA production is compromised. In *nls* mutants there is a dramatic reduction in the number of pancreatic cells formed (Stafford and Prince, 2002). Conversely, increasing RA-signaling activity (either by exogenous RA supplement or the removal of RA-degradation enzymes) leads to an expansion of the pancreatic field (Kinkel et al., 2009; Stafford and Prince, 2002; Stafford et al., 2006).

By 24 h post fertilization (hpf), dorsal pancreatic endoderm has coalesced at the midline of the zebrafish embryo to form the principal islet. In the majority of fish before 5 days post fertilization (dpf), this islet represents the sole location of the pancreatic endocrine cells (Biemar et al., 2001). These first-transition endocrine cells of the principal islet possess a low proliferative capacity and contribute little to the future adult endocrine system (Hesselson et al., 2009; Wang et al., 2011). Around 32 hpf, ventral endoderm cells start to express the transcription factor Ptf1a (Lin et al., 2004; Zecchin et al., 2004) and migrate in a posterior and dorsal direction to meet and envelop the principal islet and to create a recognizable pancreas. Around 80 hpf a second wave of endocrine differentiation (or secondary transition) occurs as hormone-producing cells differentiate from the extra-pancreatic duct and contribute to the principal islet (Dong et al., 2007, 2008). By 5 dpf, the pancreas is elongated and mostly exocrine tissue derived from the ventral cells, structured with an anterior 'head' containing the principal islet and a 'tail' containing intrapancreatic ducts. The ducts contain pancreatic Notch-responsive cells (PNCs). These PNCs are larval progenitors that differentiate during later stages of development to form the 2° islets along the duct in the pancreatic tail (Ninov et al., 2012; Wang et al., 2011). The formation of such 2° islets in the larval zebrafish pancreas is analogous to endocrine formation in mammalian pancreas. Both events involve the differentiation of Notch-responsive ductal-associated progenitors. For these reasons studying the formation of endocrine cells of the 2° islets is a promising way to discover new strategies for β-cell recovery in type I diabetes.

Several different signaling pathways are essential for pancreas development (Kimmel and Meyer, 2010; Serup, 2012). For instance, Notch signaling has long been known as central to both mammalian (Apelqvist et al., 1999; Esni et al., 2004; Hald et al., 2003; Jensen et al., 2000; Murtaugh et al., 2003) and zebrafish pancreas development (Esni et al., 2004; Lorent et al., 2004; Ninov et al., 2012; Parsons et al., 2009; Zecchin et al., 2007). Inhibition of Notch signaling leads to precocious differentiation of PNCs and the early appearance of endocrine cell types in the 2° islet position within the pancreatic tail (Parsons et al., 2009). To find pathways other than Notch signaling that regulate 2° islet formation, we performed a chemical screen in larval zebrafish from 3 to 5 dpf. One of the hit compounds we identified was Tetraethylthiuram disulfide (Disulfiram, DSF) and this drug was shown to induce precocious 2° islets by inhibiting the Aldh-dependent production of RA (Rovira et al., 2011). Conversely, increasing levels of RA rescued the effects of Notch inhibition by blocking precocious endocrine differentiation, suggesting RA signaling is also involved in the regulation of endocrine differentiation. Together, these results suggested that RA plays a role in endocrine pancreas differentiation; hence, a pancreatic source of RA should exist. Previously we showed that the developing larval exocrine pancreas (6 dpf) has Aldh enzymatic activity, consistent with RA production (Rovira et al., 2011). As the larval fish mature (from 15 dpf), a second potential RA source appears as Aldh1 positive cells differentiate along the duct from the PNCs. These Aldh1+ cells are rare at first but increase in number with age (Matsuda et al., 2013).

In this present manuscript, we further characterize the pancreatic function of the RA-signaling pathway, and show that RA signaling cell-autonomously regulates the differentiation of pancreatic progenitors during the secondary transition of endocrine development. Together with the results of other groups, our results suggest that RA signaling has a biphasic function in pancreatogenesis. RA is both required for the specification of the embryonic pancreas and later in regulating pancreatic progenitor differentiation. Using hESC differentiation protocols to model human pancreas development (Rezania et al., 2012), we demonstrate that the action of RA inhibition on endocrine progenitor differentiation is a conserved phenomenon.

Material and methods

Transgenic lines

All transgenic lines used are listed in Supplementary Table S1. All new transgenic lines were generated as described (Wang et al., 2011) using the T2KXIGΔIN backbone and Tol2-mediated transgenesis (Kawakami, 2004). Two cre drivers were used in this report that express 4-hydroxytamoxifen (4OHT) inducible cre (creER^{T2}) and are described in Wang et al. (2011). *Tg*(*Tp1glob*:*creER*^{T2})^{jh12} drives creER^{T2} in cells undergoing Notch signaling and is called '*Notch-responsive creER*^{T2} *driver*'. *Tg*(*β-actin*:*GFP-F2A-creER*^{T2})^{jh29} drives creER^{T2} ubiquitously and is called '*ubiquitous creER*^{T2} *driver*'. Two cre-responder lines were utilized: (1) *Tg*(*β-actin*:*loxP-stop-loxP-hmgb1-mCherry*)^{jh15}, which is used in lineage tracing and is referred to as '*nuclear-red cre responder*' (Wang et al., 2011); and (2) *Tg*(*ubb*:*loxP-eCFP-loxP-dnRAR-GFP*)^{jh39}, a new cre-responder line utilizing the ubiquitous promoter/enhancer element from *ubiquitin B* (*ubb*) (Mosimann et al., 2011) and called '*dnRAR cre responder*'. This transgene was generated using a gene cassette encoding a fusion of dominant-negative zebrafish retinoic acid receptor α (dnRAR) fused with GFP (gift from K. Poss (Kikuchi et al., 2011)). Several transgenic lines were used to label specific cell types with fluorescent protein expression. *Tg*(*Tp1bglob*:*hmgb1-mCherry*)^{jh11} (*Notch reporter*) labels Notch-responsive cells with nuclear mCherry. *Tg*(*pax6b*:*GFP*)^{ulg515} (Delporte et al., 2008) labels all endocrine cells; *Tg*(*neuroD*:*GFP*)^{nl1} labels committed endocrine progenitors (Obholzer et al., 2008). The construct used to label cells responding to RA signaling, *Tg*(*RARE-cFos*:*QF; QUAS*:*GFP*), here called '*RA reporter*', was made using an oligo containing four copies of 5′-ggttca(n5)agttca-3′, each copy separated by 11 nucleotides. This RA-responsive element (RARE) was cloned upstream of the *cFos* minimal promoter and sequence encoding the QF transcriptional activator. Included in the same transgene were the QF-binding upstream activating sequence (QUAS) and the *cFos* minimal promoter, which together regulate transcription of *gfp* (Subedi et al., 2014).

In situ hybridization and immunofluorescence

5 dpf larvae were fixed in 4% PFA overnight and *in situ* hybridization was performed on dissected larval pancreata essentially as in (Xu et al., 2002). The primer sequences used to generate the

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