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An insulin signaling feedback loop regulates pancreas progenitor cell differentiation during islet development and regeneration

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ABSTRACT

As one of the key nutrient sensors, insulin signaling plays an important role in integrating environmental energy cues with organism growth. In adult organisms, relative insufficiency of insulin signaling induces compensatory expansion of insulin-secreting pancreatic beta (β) cells. However, little is known about how insulin signaling feedback might influence neogenesis of β cells during embryonic development. Using genetic approaches and a unique cell transplantation system in developing zebrafish, we have uncovered a novel role for insulin signaling in the negative regulation of pancreatic progenitor cell differentiation. Blocking insulin signaling in the pancreatic progenitors hastened the expression of the essential β cell genes *insulin* and *pdx1*, and promoted β cell fate at the expense of alpha cell fate. In addition, loss of insulin signaling promoted β cell regeneration and destabilization of alpha cell character. These data indicate that insulin signaling constitutes a tunable mechanism for β cell compensatory plasticity during early development. Moreover, using a novel blastomere-to-larva transplantation strategy, we found that loss of insulin signaling in endoderm-committed blastomeres drove their differentiation into β cells. Furthermore, the extent of this differentiation was dependent on the function of the β cell mass in the host. Altogether, our results indicate that modulation of insulin signaling will be crucial for the development of β cell restoration therapies for diabetics; further clarification of the mechanisms of insulin signaling in β cell progenitors will reveal therapeutic targets for both *in vivo* and *in vitro* β cell generation.

1. Introduction

Insulin is a crucial gluco-regulatory peptide hormone that is produced by pancreatic beta (β) cells and released in proportion to levels of circulating glucose. Under conditions of fluctuating metabolic demands and energy availability, the effective functional β cell mass (insulin releasing capacity of the pancreas) is regulated to match physiological demands through β cell compensation. The failure of β cell compensation to meet insulin demand results in diabetes mellitus, a metabolic disease of insufficient insulin signaling that is characterized by uncontrolled hyperglycemia and its associated morbid complications. While compensation can be transiently mediated in part via increased insulin production and release from existing β cells, long term β cell compensation involves the expansion of β cell mass by multiple mechanisms (Asghar et al., 2006; Wang et al., 2015; Weir and Bonner-Weir, 2004). For instance, in mice physiological stresses like over-nutrition and pregnancy can accelerate β cell replication (Kim et al., 2010; Lee and Nielsen, 2009; Tanaka and Wands, 1996). However, the replicative capacity of β cells varies widely among vertebrates by species and age. The capacity of human β cells is lower than that of most model organisms used to study replication (Kulkarni et al., 2012). Furthermore, β cell replicative capacity diminishes sharply after adolescence (Kulkarni et al., 2012; Linnemann et al., 2014). In addition, using model organisms, β cells have been shown to arise via neogenesis from non-β cell sources, including differentiation from facultative progenitor cells and conversion from other pancreatic endocrine cells (Bonner-Weir et al., 2010; Chera et al., 2014; Chung et al., 2010; Thorel et al., 2010; Xu et al., 2008; Ye et al., 2015). Stimulating β cell neogenesis and replication in humans will be crucial for restoring β cell function and ultimately curing diabetes. Thus, a comprehensive understanding of the molecular mechanisms that sense insulin insufficiency and translate it into β cell compensatory responses will impact the

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design of better diabetes therapies.

Several growth factors and cytokines have been shown to regulate β cell replication in response to metabolic demand. In the adult islet, insulin secreted by β cells has been shown to feedback upon β cells to regulate islet size and β cell mass (Withers et al., 1999). Activation of the insulin receptor triggers its autophosphorylation, which is followed by downstream signal propagation via the key effector Insulin Receptor Substrate 1/2 (IRS1/2)) to the Akt and Mitogen Activated Protein Kinase (MAPK) pathways; these pathways mediate many growth and metabolic responses (Siddle, 2011). Deletion of the insulin receptors in β cells (βIRKO) abolished compensatory β cell mass expansion in adult mice and resulted in hyperglycemia (Okada et al., 2007). Further, this suggests that reported influences of glucose on β cell replication (Otani et al., 2004) may be due in part to the indirect effects of augmenting insulin secretion. Although much is known about replication-mediated β cell mass compensation, little is known regarding the cellular and molecular mechanisms of *de novo* neogenesis-mediated β cell mass compensation.

It is likely that some mechanisms regulating β cell compensation via neogenesis are common to both the mature and developing pancreas, and the embryo is an especially amenable system in which to study β cell formation. However, while the intrinsic developmental programs regulating endocrine differentiation have been very well characterized (Pan and Wright, 2011), the extrinsic signals that control induction and differentiation of β cells, as well as those signals that match β cell mass to the needs of the embryo are less well understood. Among the pathways studied are fibroblast growth factor and Notch signaling, which suppress differentiation of pancreas progenitors (Apelqvist et al., 1999; Jensen et al., 2000; Norgaard et al., 2003) and epithelial growth factor signaling, which influences β cell neogenesis (Cras-Méneur et al., 2001; Miettinen et al., 2008; Suarez-Pinzon et al., 2005). Surprisingly, the roles of the pancreatic hormones have not been extensively studied during islet development. While glucagon signaling has been shown to regulate alpha (α) cell mass by proliferation, neogenesis, and cell fate switching mechanisms (Ye et al., 2015; Gelling et al., 2003; Hayashi et al., 2009; Prasadan et al., 2002), it is not clear whether other islet hormones like insulin have a significant role in the acquisition and stability of cell fates in the developing islet. Even though the insulin signaling pathway has been studied using mouse knockout models, the results from previous developmental studies appear contradictory. Mice lacking the insulin receptor exhibit severe hyperglycemia at birth despite the presence of normal islets (Accili et al., 1996; Joshi et al., 1996; Kitamura et al., 2003). However, deletion of either or both of the mouse *insulin* orthologues (Duvillié et al., 1997) or downstream effectors such as Akt lead to marked islet hyperplasia (Buzzi et al., 2010). Therefore, further investigation is required to resolve how insulin signaling regulates β cell neogenesis during development as well as in pathologies like diabetes.

Zebrafish are a relevant and powerful system for the study of β cell formation and homeostasis: they share key features of both carbohydrate metabolism and their β cell differentiation program with mammalian systems (Kinkel and Prince, 2009) while also affording many experimental advantages (Grunwald and Eisen, 2002). As in mice and humans, the zebrafish pancreas arises from two discrete endodermal progenitor domains that fuse to establish the architecture of the pancreas (Field et al., 2003; Jørgensen et al., 2007; Pauls et al., 2007). In zebrafish, the dorsal bud appears at approximately 14 hours post fertilization (hpf) and gives rise exclusively to differentiated endocrine cell types, which then cluster to form the principal islet by 24 hpf. Emerging around 34 hpf, the ventral bud engulfs the principal islet while differentiating into both exocrine and endocrine cell lineages. In this study, we have used zebrafish to explore the role of insulin signaling during embryonic β cell formation. Using genetic approaches in zebrafish that either inhibit insulin production or impair transduction through the insulin signaling pathway, we have shown that insulin signaling has an inhibitory role during early pancreas development: loss of insulin signaling drove the precocious differentiation of pancreatic progenitors into β cells. Using chimera analysis we found that insulin signaling within the endoderm itself suppresses β cell differentiation. Moreover, using a novel blastomere-to-larva transplantation strategy, we found that loss of insulin signaling in endoderm-committed blastomeres fostered their differentiation into β cells, and that the extent of this differentiation was dependent on the function of the host β cell mass. Taken together, our data suggest that manipulation of the insulin signaling pathway will be crucial for regenerative medicine approaches to diabetes therapies, including β cell differentiation from *in situ* progenitors during regeneration, and from stem cells *in vitro*.

2. Results

2.1. Intracellular blockade of insulin signaling promotes embryonic β cell formation

To determine whether pancreatic progenitor cells are competent to receive insulin signals, we performed whole mount *in situ* hybridization and quantitative PCR to evaluate the expression of insulin receptors at key time points during pancreas development. There are two isoforms of the zebrafish insulin receptor, insulin receptor a (*insra*) and *insulin receptor b* (*insrb*) (Toyoshima et al., 2008). Both were strongly expressed in zygotes, indicating that maternal contribution may affect early embryonic development (Fig. 1A, Fig. S1A–B). Only *insrb* was expressed in the embryonic pancreatic endoderm during early pancreas development, as visualized by co-localization with endoderm marker *sox17* at 48 hours post fertilization (hpf) (Fig. 1B–C). In 108 hpf larvae, both *insra* and *insrb* were expressed in the pancreas, liver and intestine, which may reflect a metabolic role for insulin signaling during later developmental stages (Fig. 1A, Fig. S1C–D′).

To investigate the influence of insulin signaling on β cell differentiation, we generated a truncated mutant form of zebrafish IRS2 in which GFP is substituted for the C-terminal SH2-binding domains, while retaining the N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains (Fig. 2A; Fig. S2A). As reported with similar constructs (Tanaka and Wands, 1996), this mutant protein (hereafter dnIRS2-GFP) is expected to non-productively bind to the insulin receptor and act as a dominant negative regulator of insulin signaling. Indeed, when *dnIRS2-GFP* mRNA was injected into zygotes, we observed that dnIRS2-GFP protein was enriched at the plasma membrane in 5 hpf embryos (Fig. 2B,C) and remained expressed in the embryos through 24 hpf (Fig. S2B). To determine whether this mode of insulin signaling blockade affected the formation of β cells, we counted cells expressing insulin in *dnIRS2-GFP* mRNA-injected embryos at 24 hpf. Relative to controls, we observed a 32% increase in the number of *ins*+ β cells marked by dsRed in *Tg*(*insa:dsRed*) embryos (Fig. 2D–F). Previous studies have shown that homeodomain transcription factor Pdx1 is essential for β cell differentiation and fate maintenance (Ahlgren et al., 1998; Jonsson et al., 1994; Offield et al., 1996). These roles of Pdx1 are conserved in zebrafish during β cell formation (Kimmel et al., 2011; Yee et al., 2001). We therefore checked the expression of *pdx1* in dnIRS2-GFP injected embryos; consistent with the increased β cell quantity, we observed a marked increase in *pdx1* expression in the pancreatic region at 24 hpf (Fig. S2C,D). Concordantly, blockade of the Akt branch of insulin signaling via treatment with the PI3 kinase inhibitor wortmannin (Standaert et al., 1996) also resulted in increased β

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