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Notch-mediated post-translational control of Ngn3 protein stability regulates pancreatic patterning and cell fate commitment

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ABSTRACT

Ngn3 is recognized as a regulator of pancreatic endocrine formation, and Notch signaling as an important negative regulator *Ngn3* gene expression. By conditionally controlling expression of Ngn3 in the pancreas, we find that these two signaling components are dynamically linked. This connection involves transcriptional repression as previously shown, but also incorporates a novel post-translational mechanism. In addition to its ability to promote endocrine fate, we provide evidence of a competing ability of Ngn3 in the patterning of multipotent progenitor cells in turn controlling the formation of ducts. On one hand, Ngn3 cell-intrinsically activates endocrine target genes; on the other, Ngn3 cell-extrinsically promotes lateral signaling via the Dll1 > Notch > Hes1 pathway which substantially limits its ability to sustain endocrine formation. Prior to endocrine commitment, the Ngn3-mediated activation of the Notch > Hes1 pathway impacts formation of the trunk domain in the pancreas causing multipotent progenitors to lose acinar, while gaining endocrine and ductal, competence. The subsequent selection of fate from such bipotential progenitors is then governed by lateral inhibition, where Notch > Hes1-mediated Ngn3 protein destabilization serves to limit endocrine differentiation by reducing cellular levels of Ngn3. This system thus allows for rapid dynamic changes between opposing bHLH proteins in cells approaching a terminal differentiation event. Inhibition of Notch signaling leads to Ngn3 protein stabilization in the normal mouse pancreas explants. We conclude that the mutually exclusive expression pattern of Ngn3/Hes1 proteins in the mammalian pancreas is partially controlled through Notch-mediated post-translational regulation and we demonstrate that the formation of insulin-producing beta-cells can be significantly enhanced upon induction of a pro-endocrine drive combined with the inhibition of Notch processing.

Introduction

Diabetes results from insufficient insulin signaling in response to blood glucose. Islet cell replacement therapy promises a future restoration of normal glycemic control in diabetic patients. Although cadaveric islet cell transplantation is currently possible, the scarcity of human donors has highlighted the need of an unlimited islet cell source. Promising findings highlight the possibility of converting adult cells into islet cells via somatic cell reprogramming (Zhou et al., 2008), or alternatively directing the differentiation of pluripotent embryonic stem (ES) cells to islet cells (D'Amour et al., 2006). Whether the objective is to create replacement cells *in-vivo* or *ex-vivo*, the methodology relies on our current understanding of the formation and patterning of the pancreas during embryogenesis.

Genetic studies in mice have provided evidence that all endocrine cells in the pancreas are formed from a progenitor cell that has expressed the gene *Ngn3* (Gradwohl et al., 2000). *Ngn3* in the mouse has a temporal expression pattern that increases from embryonic day 9.5 (E9.5) and culminates at E13.5–14.5 where after expression decreases and approaches undetectable levels at birth (Gradwohl et al., 2000; Schwitzgebel et al., 2000). A number of experimental evidence attests to the pro-endocrine effects of Ngn3. During directed differentiation of human embryonic stem cells, NGN3 is expressed prior to the detection of

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hormone-positive cells (D'Amour et al., 2006) and introduction of Ngn3 in mouse ES cells enhanced endocrine cell formation (Treff et al., 2006). *Ngn3* expression can be reactivated following partial pancreatectomy (Li et al., 2010) and in duct-ligated adult mouse pancreas. In the duct-ligated model, Ngn3-expressing cells can be traced from adult pancreatic epithelial cells, purified, and observed to adopt an endocrine cell fate including insulin-producing beta-cells (Xu et al., 2008). Additional pro-endocrine effects of Ngn3 was observed in virus-mediated gene delivery to pancreatic ductal cells (Noguchi et al., 2006; Heremans et al., 2002), and in the liver when Ngn3 is coordinately expressed with Pdx1 (Yoon, 2007). Ngn3 is therefore capable of partially inducing an endocrine program in hepatic progenitors (Yechoor et al., 2009).

While Ngn3 has been recognized as a 'master regulator' of endocrine cell formation (Rukstalis and Habener, 2009), its expression per se is not sufficient for endocrine fate specification, rather the right dosage of *Ngn3* is critical to secure the endocrine fate (Wang et al., 2010). Lineage tracing of *Ngn3*-expressing WT cells have been shown to give rise to few acinar and ductal cells in addition to the predominantly endocrine lineage contribution (Gu et al., 2002; Schonhoff et al., 2004). Recent lineage tracing of hypomorphic *Ngn3* cells suggest that these non-endocrine *Ngn3*-expressing descendants may be the result of cells that failed to achieve the required threshold of Ngn3 for the endocrine fate, as *Ngn3* heterozygous, or *Ngn3*-deficient, cells have an increased propensity to adopt acinar and ductal fates (Wang et al., 2010; Beucher et al., 2011).

Furthermore, while the endocrine inducing properties of Ngn3 makes it a promising molecular factor for the creation of insulin producing cells, ectopic expression of Ngn3 in the mouse pancreas and in chick endoderm has been shown to cause a predominant increased glucagon cell formation but not the other endocrine cell types (Schwitzgebel et al., 2000; Apelqvist et al., 1999; Grapin-Botton et al., 2001). This appears to be rooted in a change in competence of Ngn3+ cells in a time-dependent manner. Utilizing a tamoxifen-controlled Ngn3 gain-of-function approach competence for the beta cell fate was only observed at, and beyond the E12.5 time point, thus coinciding with the onset of the secondary transition (Johansson et al., 2007).

As a means to investigate the ability of Ngn3 to promote pancreatic insulin-type cells, and address the molecular mechanisms that may explain the change in competence of the Ngn3-expressing cells, we created a conditional model of Ngn3 over-expression using a tetracycline transactivator-regulatory system. Utilizing the Pdx1-tTA TetOFF driver, we find that in absence of doxycycline, this transgenic model system is sufficiently powerful to induce a widespread endocrine cell conversion within the distal foregut segment effectively depleting the pancreatic/anterior duodenal segment progenitors. Bypassing this early effect through gestational timed intra-peritoneal doxycycline administrations we here describe the phenotypes of delayed activation of Ngn3 in pancreas. Unexpectedly, we found that conditionally activating Ngn3 prior to the secondary transition does not lead to increased beta cell formation, but the induction of pancreatic ductal cells with interspersed, glucagon-producing endocrine cells.

Mechanistically, we show that this experimental outcome resulted from a Ngn3-mediated change in the patterning of multipotent pancreatic progenitor cells (MPCs). Ectopic activation of Ngn3 leads to activation of *Dll1*, thus leading to cell extrinsic activation of Notch within the MPC population. These data are consistent with recent observations clarifying a role of Notch during tip/trunk patterning of the MPC population (Afelik et al., 2012; Cras-Meneur et al., 2009; Horn et al., 2012; Schaffer et al., 2010; Shih et al., 2012), where Notch signaling is needed for TrPC (Trunk-Progenitor Cell) formation directly via *Nkx6.1* activation (Afelik et al., 2012). Our findings reveal that the previously observed gestational change in competence of *Ngn3*-expressing cells is a result of the pancreas undergoing TipPC/TrPC patterning as a prelude to the secondary transition and that Notch signaling establishes competence for the beta cell fate. Importantly, our data also uncovers a novel involvement of Notch signaling in the control of Ngn3 protein dynamics. We demonstrate the Notch, via Hes1, controls the stability of Ngn3 protein. This additional post-translational control of Ngn3 decay helps explain the observed preservation of a mutually exclusive Hes1/Ngn3 expression pattern in both the Tet-regulated transgenic model as well as in the WT pancreas. These findings provides a conceptual platform in which rapid-change dynamics of an oscillating bHLH pair consisting of Ngn3 and Hes1 is linked to the possible simultaneous allocation of endocrine and ductal fates from a more homogenous Notch-active TrPC population.

Materials and methods

Animals

A linearized fragment containing *pTRE2-Ngn3FLAG* was used for oocyte injection, and founder lines were established. Functional testing was performed by intercrossing to the *Pdx1-tTA* transgenic knock-in line, allowing identification of four lines all of which provided similar phenotypes.

Embryonic studies and conditional gene activation

Gestational day 0.5 (E0.5) was defined as the day of vaginal plug detection. To temporarily allow for suppression of transgenic (TG) expression, doxycycline (Dox) was administered (0.05 μg/g body weight) as once-daily intraperitoneal (i.p.) injections at times noted. Throughout, control littermates either of WT, or single transgenic (STG) for the *pTRE2-Ngn3* genotype were used for comparative analysis. In all studies, these developed like WT embryos. Embryonic genotypes were defined by PCR on limb/paw tissue.

Histology, immunohistochemistry and microscopy

Mouse tissue: for each analysis, at least $n=3$ double transgenic (DTG) type pancreatic, or dissected distal foregut/midgut regions were compared to at least 3 littermate controls (for specific numbers, see supp. Table S1). Endodermal tissues were fixed in 4% paraformaldehyde (PFA) at 4 °C for 12–24 h. PFA-fixed specimens were equilibrated in 30% sucrose in PBS at 4 °C overnight, transferred to a 1:1 mixture of 30% sucrose in PBS:OCT for 4 h, hereafter transferred to OCT overnight and subsequently embedded in OCT by slow freezing. For immunofluorescence staining, sections were dried at 37 °C and microwave treated followed by washing and blocking. Primary antibodies were applied overnight at 4 °C. Next day, secondary antibodies were applied for 1 h at room temperature. Following washing, slides were mounted.

Pancreatic explants and DAPT treatment

The dorsal pancreatic rudiment of E12.5 mouse embryos was removed in cold PBS and transferred to a tissue culture filter insert system. Pancreatic explants were treated with *N*-(*S*)-phenyl-glycine-*t*-butyl ester (DAPT) (Sigma) in DMSO diluted in medium to final concentrations of 5–100 μM. Cultures were incubated at 37 °C for 4 days and medium was changed daily.