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REST represses a subset of the pancreatic endocrine differentiation program

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ABSTRACT

To contribute to devise successful beta-cell differentiation strategies for the cure of Type 1 diabetes we sought to uncover barriers that restrict endocrine fate acquisition by studying the role of the transcriptional repressor REST in the developing pancreas. *Rest* expression is prevented in neurons and in endocrine cells, which is necessary for their normal function. During development, REST represses a subset of genes in the neuronal differentiation program and *Rest* is down-regulated as neurons differentiate. Here, we investigate the role of REST in the differentiation of pancreatic endocrine cells, which are molecularly close to neurons. We show that *Rest* is widely expressed in pancreas progenitors and that it is down-regulated in differentiated endocrine cells. Sustained expression of REST in Pdx1$^+$ progenitors impairs the differentiation of endocrine-committed Neurog3$^+$ progenitors, decreases beta and alpha cell mass by E18.5, and triggers diabetes in adulthood. Conditional inactivation of *Rest* in Pdx1$^+$ progenitors is not sufficient to trigger endocrine differentiation but up-regulates a subset of differentiation genes. Our results show that the transcriptional repressor REST is active in pancreas progenitors where it gates the activation of part of the beta cell differentiation program.

1. Introduction

Type 1 diabetes (T1D) is caused by an absolute deficiency of insulin secretion due to autoimmune destruction of insulin-secreting beta cells and can be treated by islet cell replacement therapy. However, the lack of high quality donor cells has prompted the search for alternative sources of beta cells to try generating an unlimited supply of beta cells for transplantation. There are promising findings demonstrating *in vivo* transdifferentiation of acinar, alpha or delta cells into beta cells (Chera et al., 2014; Collombat et al., 2009; Zhou et al., 2008) or differentiation of human embryonic stem cells (ESC) to islet cells (Kroon et al., 2008; Pagliuca et al., 2014; Rezania et al., 2014). However, the differentiation of these cells into beta cells is often partial, a problem which can potentially be solved by better understanding how beta cells differentiate during pancreas embryogenesis.

During development, endocrine cells originate from progenitors in two differentiation waves. Between E8.5 and E12.5, pancreas progenitors are multipotent and can give rise to acinar, ductal and endocrine cells, most of which express glucagon (Kopp et al., 2011; Pan et al., 2013; Solar et al., 2009). After E13.5, the progenitors have become polarized, part of their transcriptional program has changed and they become bi-potent, giving rise to ductal and endocrine cells, while the acinar compartment becomes segregated. Beta cells are essentially born at these stages and until shortly after birth (Johansson et al., 2007; Kopp et al., 2011). We know that a transient *Neurog3* expression in progenitors is necessary to direct endocrine differentiation (Gradwohl et al., 2000; Gu et al., 2002) and that the pro-endocrine commitment only proceeds when *Neurog3* expression levels reach a threshold (Wang et al., 2008, 2010), triggering direct activation by NEUROG3 of several pro-endocrine transcription factors (Gittes, 2009; Pan and Wright, 2011).

Cell fate decision is, however, the result of inputs from positive as well as negative signals. It is therefore essential to take into account the interplay between the positive drive established by pro-endocrine genes such as *Neurog3* and restrictive signals. One such antagonistic signal comes from the Notch effector HES1 which constrains endocrine cell formation by negatively

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regulating *Neurog3* expression (Apelqvist et al., 1999; Jensen et al., 2000). Another factor that might be considered as an attractive new player in this repressive function is the RE-1 Silencing Transcription Factor (REST). This zinc finger transcription factor binds to a 21 bp motif called Repressor Element-1 (RE-1) and recruits several chromatin modifiers to block the expression of its target genes (Ooi and Wood, 2007). Given that the first identified REST targets were associated to terminal function of neurons and because REST is mainly absent from mature neuronal cells, REST has originally been considered as a master repressor of neuronal traits outside of the central nervous system (Chong et al., 1995; Schoenherr and Anderson, 1995). However, a number of new findings have challenged this assertion. First, genome wide analyses of the REST regulon have revealed the existence of a wider than originally thought set of RE-1 containing genes, some of them bearing a non-canonical motif (Otto et al., 2007). Hundreds of new RE-1-bearing genes have been identified and shown to be bound by REST in diverse cell types and contexts (Johnson et al., 2007, 2008; Otto et al., 2007). Importantly, these reports also emphasized that several subsets of REST target genes were associated to non-neuronal functions, showing that REST is not merely a repressor of neuronal traits (Bruce et al., 2004; Johnson et al., 2007, 2008; Mortazavi et al., 2006; Otto et al., 2007; Wu and Xie, 2006). Second, many studies have linked modulations of REST levels in non-neuronal cells to non-neuronal pathologies like colon cancer (Westbrook et al., 2005), cardiac hypertrophy (Kuwahara et al., 2003) or smooth muscle cell neointimal hyperplasia (Cheong et al., 2005); for reviews, see Coulson (2005), Majumder (2006), Thiel et al. (2014).

In the context of pancreatic endocrine cells, in which REST is excluded (Atouf et al., 1997; Martin et al., 2008), we have previously shown using RIP-REST transgenic animals, that RE-1-containing genes are essential for glucose homeostasis. Indeed, we have demonstrated that *Rest* ectopic expression in pancreatic insulin-producing cells impairs their function and survival by specifically down-regulating the expression of important exocytotic members as well as pro-survival genes (Martin et al., 2008, 2012). As specified for a subset of other genes (Pullen et al., 2010; Quintens et al., 2008), REST is thus "disallowed" in beta cells, as it is in neurons (Atouf et al., 1997). The observation made by ChIP seq analysis that REST binds to the chromatin of drivers of islet cell development (Johnson et al., 2007), together with the fact that REST clearance in neural progenitors has been evoked as a trigger for neural differentiation (Ballas et al., 2005), prompted us to assess the role of REST in the developing pancreas.

In the present study, we show that *Rest* is expressed in progenitors, and is down-regulated in differentiating endocrine cells during development. We have also generated a transgenic model of REST gain-of-function in pancreatic progenitors to show that REST is sufficient to impair the formation of NEUROG3$^+$ precursors and of differentiated endocrine cells. Finally, using a model of *Rest* loss-of-function specifically directed to pancreatic progenitors we show that REST inhibits the expression of important factors of endocrine differentiation.

2. Materials and methods

2.1. Mouse strains

The mouse lines (Pdx1-tTA, Pdx1-Cre and REST Fl/Fl) were previously described and are referenced in the result section. The Swiss Veterinary Office and competent Danish authorities approved all animal experiments. Embryos were collected at indicated times; midday on the day of vaginal plug appearance was considered E0.5.

To generate mice with inducible REST expression (TetO-REST mice), an XbaI fragment containing the rabbit β-globin intronic sequence and human Rest cDNA, which served for the generation of RIP-REST mice (Martin et al., 2008), was sub-cloned into a pUHD 10-3 plasmid downstream of a tetracycline operator (TetO) minimal promoter sequence. The resulting XhoI/KasI fragment was used for oocytes injection. These mice were used in the absence of Doxycycline or tetracycline and thus had continuous expression of the transgene in Pdx1-expressing cells, comprising the epithelial cells of the pancreas during development as well as beta cells and a subset of delta cells in the adult.

2.2. Embryonic pancreas preparation

Pancreatic buds were fixed overnight at 4 °C in 4% paraformaldehyde (PFA). Samples were then washed, and immersed in phosphate buffer containing 15% sucrose for 24 h for cryo-preservation. Pancreas were finally incubated in phosphate buffer containing 7% gelatin and 15% sucrose at 37 °C for 1 h, solidified at RT and quickly frozen in methylbutane kept at −65 °C by immersion in dry ice-/EtOH 100%. Blocks were kept at −80 °C until sectioning.

2.3. Mouse islet isolation and cell lines

The rat insulinoma cell lines INS-1E (Merglen et al., 2004) were maintained in complete RPMI 1640 medium as previously described (Martin et al., 2003). Human carcinoma HeLa cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin.

Islets of Langerhans were isolated from adult mice bearing REST gain-of-function, Rest loss-of-function and control littermates that were anaesthetized by inhalation of 5% halothane (Arovet) and sacrificed. The pancreas was perfused by collagenase injection into the common bile duct. After excision, the pancreas was digested at 37 °C. After filtration on a 100 μm cell strainer (BD Biosciences), the islets were washed in a Hank's balanced saline solution (HBSS) and cultured in RPMI 1640 containing 11.1 mmol/l glucose supplemented with 10% FCS, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin.

2.4. Western blotting

Western blots were performed as previously described (Martin et al., 2012). Specific protein levels were revealed with polyclonal rabbit antibodies against human REST (Martin et al., 2003), or with polyclonal rabbit antibodies against murine REST (07-579, Merck Millipore). Monoclonal antibodies against α-tubulin (Sigma-Aldrich) were used to normalize the signals.

2.5. Immunofluorescence and EdU staining

Frozen sections (8 μm) were rehydrated in PBS before 30 min blocking in phosphate buffer saline (PBS), Bovine serum albumin (BSA) 1%, Triton 0.2%. Primary antibodies, anti-Glucagon (rabbit; 1:100; Zymed and guinea pig; 1/400; Linco), anti-Insulin (guinea pig; 1/100; Dako), anti-NEUROG3 (rabbit; 1/500; Beta Cell Biology Consortium), anti-SOX9 (rabbit; 1/500; Chemicon), anti-PDX1 (goat; 1/2000; Beta Cell Biology Consortium), and anti-Amylase (rabbit; 1/500; Calbiochem) were incubated overnight at 4 °C.

Secondary antibodies were incubated 1 h at room temperature. Alexa Fluor secondary antibodies (all from Molecular Probes-Invitrogen) were used for multicolor detection. Cell proliferation was assessed using the EdU Click-iT kit, according to the