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Dnmt1 activity is dispensable in δ-cells but is essential for α-cell homeostasis

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ABSTRACT

In addition to β-cells, pancreatic islets contain α- and δ-cells, which respectively produce glucagon and somatostatin. The reprogramming of these two endocrine cell types into insulin producers, as observed after a massive β-cell ablation in mice, may help restoring a functional β-cell mass in type 1 diabetes. Yet, the spontaneous α-to-β and δ-to-β conversion processes are relatively inefficient in adult animals and the underlying epigenetic mechanisms remain unclear. Several studies indicate that the conserved chromatin modifiers DNA methyltransferase 1 (Dnmt1) and Enhancer of zeste homolog 2 (Ezh2) are important for pancreas development and restrict islet cell plasticity. Here, to investigate the role of these two enzymes in α- and δ-cell development and fate maintenance, we genetically inactivated them in each of these two cell types. We found that loss of *Dnmt1* does not enhance the conversion of α- or δ-cells toward a β-like fate. In addition, while *Dnmt1* was dispensable for the development of these two cell types, we noticed a gradual loss of α-, but not δ-cells in adult mice. Finally, we found that *Ezh2* inactivation does not enhance α-cell plasticity, and, contrary to what is observed in β-cells, does not impair α-cell proliferation. Our results indicate that both Dnmt1 and Ezh2 play distinct roles in the different islet cell types.

1. Introduction

Restoration of functional β-cell mass in type 1 diabetes is a major challenge of regenerative medicine. We have previously shown that after a near-total β-cell ablation in adult mice, a fraction of pancreatic α- and δ-cells reprogram to a β-like phenotype, thus contributing to partial restoration of insulin production (Chera et al., 2014; Thorel et al., 2010). Yet, the genetic and epigenetic mechanisms underlying this direct transdifferentiation remain unclear.

Chromatin modifications contribute to cell fate decisions during development and to fate maintenance in differentiated cells, including in the pancreas (Arnes and Sussel, 2015; Chen and Dent, 2014). Consequently, alteration of chromatin marks can facilitate cell reprogramming (Hochedlinger and Plath, 2009). DNA methyltransferases and Polycomb repressive complexes (PRCs) are two of the best studied chromatin modifiers (Di Croce and Helin, 2013; Smith and Meissner, 2013). In particular, DNA methyltransferase 1 (Dnmt1) propagates DNA methylation patterns during replication (Law and Jacobsen, 2010), whereas the catalytic subunit of PRC2, named Ezh2, tri-methylates histone H3 at lysine 27 (H3K27me3), a modification associated with gene silencing (Margueron and Reinberg, 2011).

Both DNA methylation and Polycomb-mediated gene silencing play critical roles in pancreas development and β-cell function. For instance, *Dnmt1* inactivation in pancreatic progenitors impairs their survival, resulting in pancreatic hypoplasia (Georgia et al., 2013), and *de novo* DNA methylation by Dnmt3a is important for functional β-cell maturation (Dhawan et al., 2015). Polycomb group proteins play multiple roles throughout pancreas development. In foregut endoderm, Ezh2 promotes hepatic over pancreatic fate

Abbreviations: Cre, Cre recombinase; Dnmt1, DNA methyltransferase 1; Dox, doxycycline; DT, diphtheria toxin; DTR, diphtheria toxin receptor; Ezh2, enhancer of zeste homolog 2; Gcg, glucagon; IAP, intracisternal A-particle; Ins, insulin; m-o, month-old; PRC, polycomb repressive complex; RIP, rat insulin promoter; rtTA, reverse tetracyclin transactivator; Sst, somatostatin; TetO, tet operator sequence; YFP, yellow fluorescent protein.

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through selective silencing of pancreas-specific genes (Xu et al., 2011). Pro-endocrine genes exhibit repressive H3K27me3 marks in pancreatic progenitors. Consequently, Ezh2 inactivation at this stage results in increased number of $Ngn3^{+}$ endocrine progenitors, and subsequent expansion of the endocrine cell mass (Xu et al., 2014). In adult β-cells, age-dependent decline in Ezh2 expression leads to derepression of the cell cycle inhibitors $p16^{Ink4a}$ and $p19^{Arf}$, thereby limiting the proliferation of aged β-cells (Chen et al., 2011; Chen et al., 2009; Dhawan et al., 2009; Krishnamurthy et al., 2006; Zhou et al., 2013). However, the role of Dnmt1 and Ezh2 in the development and maturation of glucagon-producing α-cells and somatostatin-producing δ-cells has not been studied *in vivo*.

In addition to their role in pancreas development, both Dnmt1 and Ezh2 have been associated with cell type conversion in the endocrine pancreas. In particular, *Dnmt1* inactivation in fetal mouse β-cells causes derepression of Arx, a master regulator of the α-cell program. This results in β-to-α cell conversion, with around 35% of β-cells expressing glucagon in 8-month-old animals (Dhawan et al., 2011). Whether the reverse conversion can occur upon inactivation of *Dnmt1* in α-cells is yet unknown. On the other hand, several genes essential for β-cell development and function, such as the transcription factors Pdx1 and MafA, exhibit bivalent activating (H3K4me3) and repressing (H3K27me3) histone marks in human α-cells. Remarkably, treating human islets with a histone methyltransferase inhibitor decreased H3K27me3 enrichment at the Pdx1 locus, leading to induction of Pdx1 and the appearance of bihormonal cells (Bramswig et al., 2013). As Ezh2 is responsible for H3K27me3 deposition, inactivation of this protein in α-cells may lead to derepression of β-cell-specific genes, and thus facilitate α-cell conversion toward a β-cell fate. We thus hypothesized that combining *Ezh2* or *Dnmt1* inactivation with β-cell ablation, which induces the expression of β-cell-specific transcription factors in a subset of α-cells (Thorel et al., 2010), may enhance β-cell regeneration via reprogramming of other islet cell types.

To examine the role of Dnmt1 in α- and δ-cell development and plasticity, we generated transgenic mice in which we can lineage-trace α- or δ-cells and inactivate *Dnmt1*, as well as induce massive β-cell ablation. We then took a similar approach to determine if loss of *Ezh2* could foster α-to-β cell conversion.

2. Material and methods

2.1. Mice

RIP-DTR (Thorel et al., 2010), *Glucagon-rtTA* (Thorel et al., 2010), *TetO-Cre* (Perl et al., 2002), *Somatostatin-Cre* (Chera et al., 2014), *R26-YFP* (Srinivas et al., 2001), $Dnmt1^{fl/fl}$ (Jackson-Grusby et al., 2001), and $Ezh2^{fl/fl}$ (Su et al., 2003) transgenic animals were previously described. Both males and females were used for experiments. Mice were housed in 12 h light/dark cycles with ad libitum access to standard chow and water. They were cared for and treated in accordance with the guidelines of the Direction Générale de la Santé, state of Geneva (license number GE/103/14).

2.2. Diphtheria toxin (DT) and doxycycline (Dox) treatments

For β-cell ablation, DT (D0564; Sigma, St. Louis, MO) was injected i.p. in 10-week-old *RIP-DTR* mice (on days 0, 3, and 4). Each of the three injections consisted of 125 ng DT diluted in 200 μl NaCl 0.9%. For rtTA-mediated induction of Cre recombinase in α-cells, Dox (D9891; Sigma) was added to the drinking water of breeding cages at a concentration of 1 mg/ml.

2.3. Glycemia monitoring and insulin administration

After β-cell ablation, glycemia was measured from tail-tip blood using a handheld glucometer. Diabetic animals were implanted on average every 4 weeks with a subcutaneous insulin pellet (Linbit; LinShin Canada Inc., Canada).

2.4. Immunofluorescence

Following euthanasia, collected pancreata were fixed 1h30 in cold 4% paraformaldehyde, washed in PBS, and incubated overnight in a 30% sucrose solution. After embedding in OCT compound (Sakura Finetek, Netherlands), pancreata were cut into 10 μm sections. Immunostaining was performed as described (Desgraz and Herrera, 2009). Primary antibodies were: guinea pig anti-insulin (1:400; Dako, Denmark); chicken anti-insulin (1:750; Sigma); mouse anti-glucagon (1:1000; Sigma); rabbit anti-somatostatin (1:200; Dako); mouse anti-somatostatin (1:200, Novo Nordisk, Denmark); rabbit anti-GFP (1:300; Molecular Probes Inc., Eugene, OR); and chicken anti-GFP (1:200; Abcam, UK). For fluorescent detection, secondary antibodies were coupled to Alexa Fluor dyes 488, 568, or 647 (1:500; Molecular Probes Inc.); or to FITC, Cy3, or Cy5 (1:500; Jackson ImmunoResearch, West Grove, PA). Images were acquired on a confocal microscope (TCS SPE; Leica Microsystems, Germany). Immunomorphometry: the number of islet sections and cells counted in each experiment is indicated in Suppl. Table S1.

2.5. Islet isolation and cell sorting

Islet isolation was performed as described (Strom et al., 2007). In brief, pancreata were perfused with a collagenase solution, dissected, and incubated for 15′ in 37 °C collagenase. Islets were purified on a Histopaque density gradient and dissociated in a trypsin solution to generate a single-cell suspension. Lineage-traced α- or δ-cells were sorted based on YFP fluorescence on a Moflo Astrios (Beckman Coulter, Brea, CA) or a S3e (Bio-Rad, Hercules, CA) cell sorter.

2.6. Nucleic acid extraction

RNA from purified α- or δ-cells was extracted with the AllPrep DNA/RNA Micro Kit (Qiagen, Germany).

2.7. Reverse transcription and quantitative PCR

For RNA isolated from *αDnmt1* and *δDnmt1* cells, reverse transcription and pre-amplification were performed using the RT^2 first strand cDNA synthesis kit and a custom PreAmp primer mix (Qiagen), according to the manufacturer's instructions. qPCR reactions were completed on a RotorGene 6000 cycler (Corbett) using a custom RT^2 PCR array and the RT^2 SYBR Green ROX FAST mastermix (Qiagen). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), β-actin (Actb), Beta-glucuronidase (Gusb), and Cyclophilin A (Ppia) were used for normalization. Data analysis was done as described (Thorel et al., 2010). The complete list of genes in the custom array is available in Suppl. Table S2. The expression of mouse endogenous retroviruses was measured using the same primer pairs as in (Sharif et al., 2016). RNA isolated from *αEzh2* cells was reverse-transcribed using the QuantiTect RT kit (Qiagen). qPCR reactions and analyses were performed as described (Thorel et al., 2010). Ppia and Gapdh were used for normalization. Primers sequences are listed in Suppl. Table S3.

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