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Review

Epigenetic modulation of cell fate during pancreas development

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ABSTRACT

Epigenetic modifications to DNA and its associated proteins affect cell plasticity and cell fate restrictions throughout embryonic development. Development of the vertebrate pancreas is characterized by an initial over-lapping expression of a set of transcriptional regulators in a defined region of the posterior foregut endoderm that collectively promote pancreas progenitor specification and proliferation. As development progresses, these transcription factors segregate into distinct pancreatic lineages, with some being maintained in specific subsets of terminally differentiated pancreas cell types throughout adulthood. Here we describe the progressive stages and cell fate restrictions that occur during pancreas development and the relevant known epigenetic regulatory events that drive the dynamic expression patterns of transcription factors that regulate pancreas development. In addition, we highlight how changes in epigenetic marks can affect susceptibility to pancreas diseases (such as diabetes), adult pancreas cell plasticity, and the ability to derive replacement insulin-producing β cells for the treatment of diabetes.

KEYWORDS: epigenetics, pancreas development, β cell, diabetes.

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1. Introduction

The term epigenetics was coined by Waddington in 1942 to describe how non-coding modifications can influence cell fate during embryonic development, explaining how although every cell inherits the same genetic code, each differentiated cell type expresses different genes and cellular functions [1]. Thus, through epigenetic modifications to DNA and its associated proteins, cell plasticity decreases, and cell fates become more definitive as development progresses. The process of pancreas development is characterized by an initial over-lapping expression of a set of transcriptional regulators in a defined region of the posterior foregut endoderm that collectively promote pancreas progenitor specification and proliferation. These factors later segregate into distinct pancreatic lineages and many are maintained in a specific subset(s) of terminally differentiated pancreatic cell types throughout adulthood (Figure 1). These dynamic expression patterns and cooperative functions of pancreas transcription factors are dependent on finely tuned epigenetic regulation. There is an increasing appreciation that, similar to variations and mutations in the DNA coding and non-coding sequences, defects in epigenetic processes can lead to misregulation in the timing and location of these key transcriptional regulators. Thus, epigenetic mechanisms play a critical role in the differentiation and function of specific pancreas cell types and altered expression or activity of

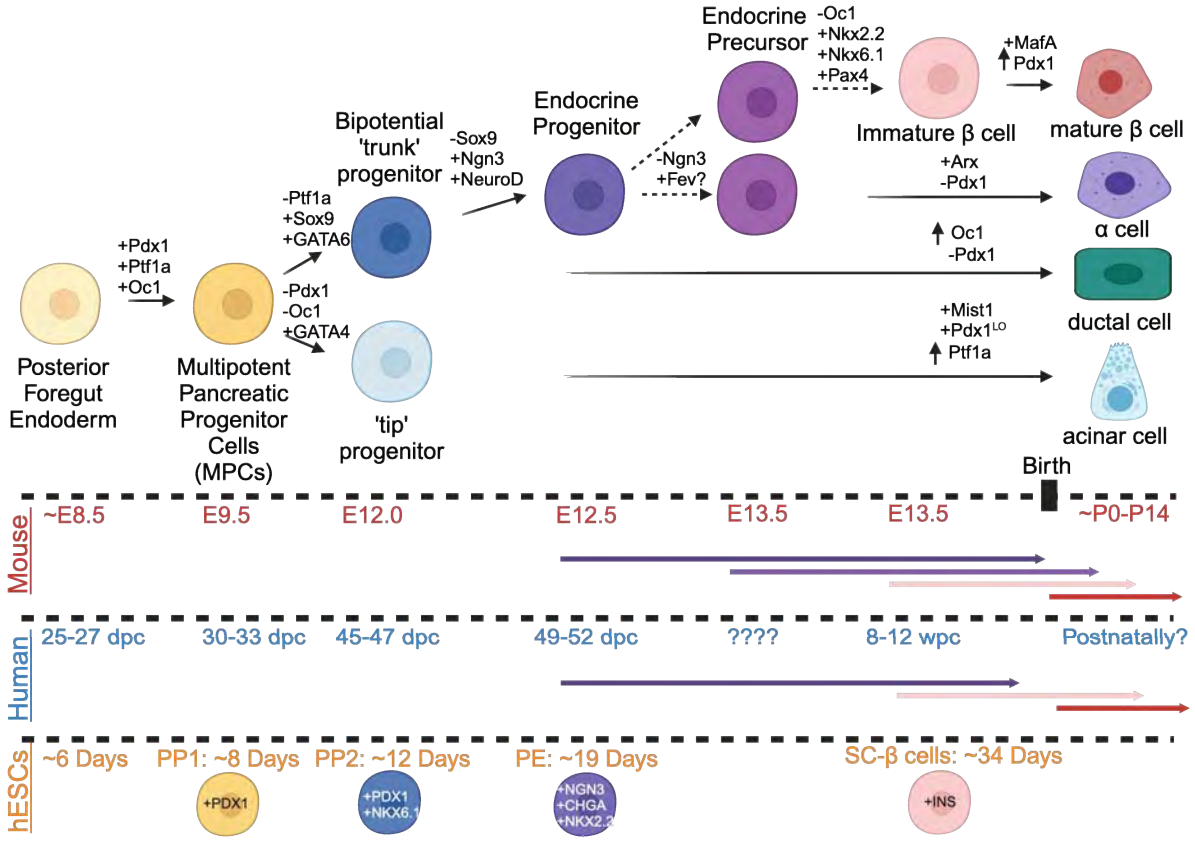

Figure 1. Comparison of pancreatic cell development across model systems. All pancreatic epithelial lineages arise from the foregut endoderm. Distinct expression of key transcription factors (shown as + or -) driving cells towards a specific pancreatic cell stage or fate are listed between each cell type. Solid black arrows indicate known cell transitions; dotted arrows represent hypothesized intermediate cell populations; question marks indicate transcription factors that have yet to be characterized. While most of the developmental stages and transcription factors are conserved between mouse and human pancreas organogenesis, there are differences in the relative time points at which each cell type arises and the length of time they persist. The different colored arrows represent the corresponding cell type and how long they persist within the pancreas throughout development. Created with Biorender.com.

epigenetic enzymes can lead to increased susceptibility to pancreatic diseases such as diabetes and cancer (reviewed in: [2-5]). Replacement or regeneration of insulin-secreting cells in the pancreas has the potential to treat diabetes; however, the availability of donor tissue for transplantation remains a challenge. Reactivation of developmental programs or transdifferentiation of non-β cells in the adult pancreas *in vivo*, as well as directed differentiation of pluripotent stem cells *ex vivo* could provide alternative sources of insulin-producing cells for the treatment of diabetes. These strategies would greatly benefit from being able to reliably manipulate the epigenome in these different cell sources. Many of the genes that have been linked to monogenic forms of diabetes or to increased diabetes risk are key pancreas development transcriptional regulators that also function in the postnatal insulin-producing cells [6, 7]. Here, we review what is known about the epigenetic regulation of pancreas development, key epigenetic enzymes that catalyze DNA or chromatin modifications in pancreas-specific genes, and the effects of *in utero* exposure to environmental insults on epigenetics at important pancreas genes. To set the stage, we first discuss important milestones and regulators of pancreas development and the basics of epigenetic modifications.

2. Summary of pancreas development

The pancreas is a bifunctional organ made up of two distinct cellular compartments: the exocrine component, which includes the acinar and ductal tissue that produce and transport digestive enzymes, respectively; and the endocrine component, which is comprised of micro-organs known as the islets of Langerhans or pancreatic islets. Pancreatic islets contain the hormone-secreting cells of the pancreas: α cells, β cells, δ cells, ε cells, and γ cells which secrete glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively. Insulin-producing β cells make up the majority (~70%) of the human pancreatic endocrine cells. Due to ethical concerns and the fact that in humans much of the pancreas develops very early (spanning approximately the first 8 weeks) during gestation, the majority of our understanding of pancreas development has come from utilizing vertebrate animal models such as zebrafish, frogs, and mice. Here we summarize what has been learned about pancreas development from vertebrate models and compare relevant developmental stages with what is known of the corresponding events in humans.

2.1. Early pancreas formation and morphogenesis

The early steps of vertebrate pancreas specification involve signals and morphogenic events within the definitive endoderm. In mouse embryos, a flat sheet of endoderm is converted over time into a primitive gut tube comprised of a foregut, midgut, and hindgut along the anterior/posterior axis [8, 9]. At embryonic day (E) 8.0 the dorsal endoderm at the region of the posterior foregut is in close proximity to the overlying mesodermally derived notochord [9, 10]. Extrinsic signals secreted by the notochord (FGF2 and Activin β2) and lateral plate mesoderm (retinoic acid) suppress sonic hedgehog (Shh) signalling in the dorsal foregut endoderm, allowing for pancreas specification [9-11]. Exclusion of Shh from the foregut endoderm is required for expression of the key homeobox transcription factor pancreatic and duodenal homeobox 1 (Pdx1) in what is termed the 'pre-pancreatic domain' [9-12]. In human embryos, SHH expression is extinguished in the foregut endoderm at 25-27 days post-conception (dpc) or Carnegie Stage 10 (CS10) with subsequent expression of PDX1 at 29-31 dpc (CS12) [11]. In the mouse, Pdx1 expression initiates at approximately E8.5 (Figure 1), with the dorsal pancreatic bud emerging as layers of stratified epithelial cells within the dorsal foregut endoderm. The dorsal pancreatic bud thickens and evaginates at E9.5 [9, 10]. Shortly after, in mice (E10) and humans (30-33 dpc or CS13), branching of the ventral pancreatic bud within a distinct region of the ventral foregut endoderm is induced through inhibition of growth factor signals, such as FGF, BMP, and TGFβ, that favor the hepatic fate [11-15]. The specific spatiotemporal expression of these parallel inductive signalling networks is critical for the determination of either the hepatic or pancreatic cell fate within the ventral foregut endoderm [16].

Dramatic morphogenetic changes and rapid expansion of the pancreatic epithelium occurs during the primary wave of differentiation, between E9.5 – E12.5 in mice. During this period, the pool of multipotent pancreatic progenitors (MPCs; Figure 1) undergoes active proliferation to generate multiple layers of stratified epithelium. Within the layers of stratified epithelium, scattered cells undergo polarization forming *de novo* microlumens that connect at the central luminal duct to create a complex primary ductal plexus [17-19]. As this plexus forms, the epithelium segregates into distinct domains that later give rise to the three main pancreatic cell types (acinar, ductal, endocrine) [20]. The first wave of endocrine cell differentiation gives rise to hormone-expressing cells (mainly glucagon-producing α cells) in the dorsal pancreatic bud, although these early endocrine cells do not contribute to the mature islets [21]. Rotation of the gut tube positions the dorsal and ventral buds close together, where they eventually fuse to create a single connected organ [9, 10]. Importantly, the number of MPCs that arise during the primary wave determines the overall size of the mature pancreas [22]. A similar process has yet to be identified during human pancreas development; however, growth of the pancreas and proliferation of the pool of MPCs has been reported to occur between 33-45 dpc (CS14-CS18) [11].

The second wave of differentiation occurs between E13.5-E17.5 in mice, and results in differentiation of all three pancreatic lineages [9, 10]. In mice, the ductal plexus formed during the primary wave undergoes remodelling into a tree-like branched

ductal system [17-19, 23]; the parallels in human pancreas development remain unknown. During the secondary wave of differentiation, expansion of the acinar cell pool occurs at the tips of the epithelial branches. Meanwhile, a bipotential pool of cells in the trunks of the branches gives rise to both endocrine cells and ductal cells. Similar types of progenitor cells have been described during human pancreas development, beginning at 30-33 dpc (CS13) for MPCs and 45-47 dpc (CS19) for the trunk and tip progenitors [11, 20] (Figure 1).

Following specification to the endocrine lineage, endocrine precursors delaminate from the primitive duct and migrate to form islet clusters in the pancreas parenchyma [24]. There are a few possible mechanisms regulating endocrine cell delamination from the primitive duct including epithelial-to-mesenchymal transition (EMT) or asymmetric cell division. Prior studies have suggested that delamination of endocrine cells from the epithelium occurs *via* EMT, since the transcription factor Snail 2, a known regulator of EMT, is upregulated during this process [25]. However, further study is needed to confirm this mechanism. Beginning at late gestation (E18.5-E19.5) and continuing for some time after birth, the hormone-expressing endocrine cells proliferate, while neogenesis from bipotent progenitors declines and ultimately ceases. Taken together, pancreas development relies on perfectly timed and distinct steps/waves of proliferation, differentiation, and remodelling (Figure 1).

2.2. Endocrine differentiation

Endocrine progenitor specification from bipotential cells in the trunk epithelium during the secondary wave of differentiation is dependent on transient, high levels of the transcription factor Neurogenin 3 (Ngn3 or Neurog3) [10, 24] (Figure 1). In the mouse, the transcription factors Pdx1 and Onecut 1 (Oc1; also known as hepatic nuclear factor 6/Hnf6), are co-expressed in MPCs (Figure 1) and cooperate to directly activate *Ngn3* expression within the bipotential progenitor cells scattered throughout the dense trunk epithelium [26-28]. These cells initially express Ngn3 at low levels ($Ngn3^{LO}$) and represent a transient, endocrine-biased progenitor cell population that undergoes asymmetric cell division, giving rise to one endocrine-committed $Ngn3^{HI}$ daughter cell and one $Ngn3^{LO}$ progenitor cell that can undergo more rounds of division [24]. Each $Ngn3^{HI}$ cells is unipotent, post-mitotic, and gives rise to a single endocrine cell type [29]. High Ngn3 expression within the endocrine-committed $Ngn3^{HI}$ daughter cells is thought to activate expression of the general endocrine transcription factors (*NeuroD1* and *Isl1*) leading to a putative intermediate endocrine precursor stage [24, 30] (Figure 1). Activation of lineage-specific transcription factors, such as *Pax4*, *Nkx2.2*, and *Arx*, in endocrine precursors promotes their progression to hormone-expressing endocrine cells, including α and β cells [10, 24, 30] (Figure 1). More studies are needed to confirm if an intermediate endocrine precursor population exists, and to characterize stage-specific markers that differentiate the different cell stages leading to endocrine differentiation. Recent work suggests the transcription factor Fev is a strong candidate for a marker of this intermediate endocrine differentiation state between a $Ngn3^{HI}$ cell and a hormone expressing cell [31] (Figure 1).

Endocrine differentiation in humans is thought to coincide with the first $NGN3^{HI}$ expressing cells, which are detected at 8 weeks post conception (wpc) and remain high until around 17 wpc, before becoming undetected by 35 wpc [11, 32, 33] (Figure 1). While expression of NGN3 in humans is also thought to be transient, there has been little description of distinct $NGN3^{LO}$ and $NGN3^{HI}$ cells populations or their respective cell fates in humans.

2.3. Exocrine differentiation

The exocrine compartment of the pancreas is made up of two distinct cell types: ductal cells and acinar cells. In mice, acinar cells are first detected at ~E13.5 when expression of important MPC markers such as sex-determining region Y-box 9 (Sox9), Pdx1, Oc1, and FoxA2 decreases in cells located at the 'tips' of epithelial branches. At the same time, Pancreas-specific transcription factor 1a (Ptf1a), which is already expressed in MPCs, and Mist1, are upregulated and maintained in mature acinar cells [10, 34] (Figure 1). In humans, the expression pattern of PTF1A resembles that in rodents; however, the timing of acinar cell differentiation remains unclear [11]. While pathology studies from the early 1980s have shown that zymogen granules, a marker of mature acinar cells, are detected at

around 14 wpc, there is still much unknown about the timing and process of human acinar differentiation [35, 36]. In both rodents and humans, ductal cells arise from the pool of bipotential endocrine-ductal cells located in the trunk of the pancreatic epithelium [10, 11, 20]. Ductal cell differentiation occurs in mice as early as E14.5 during the secondary transition when Pdx1 becomes downregulated in the bipotential 'trunk' cells [10]. Oc1 expression is maintained at high levels within the ductal epithelium and at low levels within the acinar cells throughout life [27, 37, 38] (Figure 1). These differentiated ductal cells form the epithelium that lines the branches of the complex tree-like network of the adult pancreas and function to drain the exocrine fluid and acinar-derived enzymes into the duodenum of the small intestine.

3. Transcription factors that regulate stages of pancreas development

The combined expression of several transcription factors including PDX1, PTF1A, SOX9, GATA binding proteins 4/6 (GATA4/6), and OC1, creates a pancreas-specific transcriptional landscape within the posterior foregut endoderm and marks the pool of MPCs in both humans and mice [10, 11, 32, 35] (Figure 1). Although initially co-expressed in MPCs, their expression patterns diverge as development proceeds such that they show cell-type-restricted expression in the postnatal pancreas (Figure 1).

3.1. Regulators of pancreas and endocrine specification

PDX1: Pdx1 is one of the first transcription factors expressed in the earliest pre-pancreatic endoderm at approximately E8.5, and its expression is maintained in MPCs throughout development. Likewise, in humans, PDX1 is expressed in the early pancreatic endoderm as early as 29 dpc (CS12) and is maintained in all subsequent MPCs. Pdx1 function is specifically required between E11.5 and E13.5 for proper differentiation of endocrine and exocrine cellular compartments; lineage tracing studies in mice have shown that Pdx1-positive MPCs give rise to all pancreatic epithelial cell lineages (acinar, ductal, and endocrine) [39]. In concert with Oc1 (see below), Pdx1 directly regulates the *Ngn3* promoter to initiate specification of endocrine progenitors [26, 28]. Expression of Pdx1 is dynamic; in late gestation and after birth it becomes restricted to β cells and subsets of acinar cells [40] (Figure 1). Complete *Pdx1* deficiency in either mice or humans results in pancreatic agenesis [41-43], while *Pdx1* heterozygosity in mice results in impaired glucose tolerance with age [44]. Similarly, in humans, single nucleotide polymorphisms (SNPs) or point mutations within *PDX1* have been linked to decreased β-cell function, Type 2 diabetes (T2D), and maturity-onset diabetes of the young (MODY) [45-48]. In adults, Pdx1 maintains β-cell identity by activating genes essential for β-cell function (*Mafa*, *Nkx6.1*, *Insulin*) and in turn repressing those associated with α-cell identity (*Mafb* and *Glucagon*) [49].

PTF1A: In the mouse, Ptf1a is expressed in the pancreatic buds at E9.5, shortly after Pdx1 [50, 51]. Ptf1a is expressed in early MPCs in a transcriptional complex with the nuclear mediator of Notch signalling, RBPJ [20, 52]. As MPCs differentiate into tip and trunk progenitors within the epithelium, Ptf1a expression becomes restricted to the tip epithelium progenitors destined for an acinar cell fate [20] (Figure 1). Thus, at birth, Ptf1a expression is restricted to acinar cells [53]. *Ptf1a*-deficient mice lack the ventral pancreatic bud while the dorsal bud is severely arrested, with a lack of exocrine cells and a dramatic reduction in endocrine cells [50]. Lineage tracing experiments in *Ptf1a*-deficient mice revealed that in the absence of functional Ptf1a, MPCs become redirected to a duodenal cell fate [51]. Similar phenotypes are seen in humans, where a mutation that leads to a truncation of PTF1A results in pancreatic agenesis [54]. These data demonstrate that *Ptf1a* is important in specification of both the MPCs within the early pancreatic buds and in exocrine cells later in development.

SOX9: The earliest expression of Sox9 is seen at E9.5 within the pool of MPCs of the ventral and dorsal pancreatic buds [55] (Figure 1). By E15.5 and throughout the secondary wave, Sox9 expression is restricted to the bipotential trunk progenitors that give rise to the endocrine and ductal cell lineages [55, 56] (Figure 1). Following the secondary wave, Sox9 expression is progressively downregulated, becoming restricted to ductal and centroacinar cells (potential long-lived stem-like cells) in adult mice and humans [55, 57, 58]. In

mice, *Sox9* deficiency leads to cystic pancreatic hypoplasia with a poorly branched ductal system, and little to no endocrine cells [55]. In humans, weak expression of SOX9 is seen in pancreatic endoderm as early as 29 dpc (CS12) before being strongly expressed in both pancreatic buds between 30-40 dpc (CS13-CS16) [11, 32] (Figure 1). Loss or haploinsufficiency of *SOX9* results in pancreatic hypoplasia with small and disordered islets in humans [59].

GATA4 and GATA6: GATA4 and GATA6 are members of a family of zinc-finger transcription factors involved in the specification and differentiation of multiple endoderm- and mesoderm-derived cell types [60]. During early development in the mouse, GATA4 and GATA6 are largely co-expressed in the foregut endoderm and in the pancreatic epithelium; however, as embryonic development progresses, expression of GATA4 and GATA6 becomes mutually exclusive, where GATA4 is restricted to acinar cells and GATA6 to endocrine cells of the islet [61, 62]. While global deletion of either *GATA4* or *GATA6* results in early embryonic lethality [63-66], pancreas-specific deletion of either factor results in mild pancreatic defects which resolve postnatally. Deletion of both *GATA4/6* in the pancreas primordium leads to pancreatic agenesis – suggesting a functionally redundant role of these transcription factors during the stages of pancreas outgrowth [62]. In *GATA4/6* double mutant mice, Pdx1 expression is reduced and cellular commitment towards both endocrine and exocrine lineages is impaired [67]. GATA4 and GATA6 bind directly to a region (Area III) within the *Pdx1* proximal enhancer [67]. Mutations of *GATA4* and *GATA6* that lead to haploinsufficiency in humans are associated with permanent neonatal diabetes mellitus (PNDM), pancreas agenesis, and hypoplasia [11, 32, 68, 69].

OC1/HNF6: Oc1 acts upstream of Pdx1 and directly activates expression of *Pdx1* in MPCs within both pancreatic buds [70]. Similar to Ptf1a, Oc1 expression diverges as pancreas development progresses: expression decreases in differentiating acinar cells and becomes elevated in bipotential progenitors of the trunk epithelium, ultimately being maintained at low levels in acinar cells and high levels in ductal cells throughout life [27, 37] (Figure 1). Importantly, while Oc1 is expressed in bipotential trunk progenitors and plays a role in endocrine cell specification, its expression is rapidly decreased in committed endocrine cells and it is not expressed in differentiated (hormone-positive) endocrine cells [27] (Figure 1). Oc1 and Pdx1 both directly activate *Ngn3* and work cooperatively to promote *Ngn3* expression [26, 28, 71]. Oc1 is required to generate the correct number of endocrine progenitors during embryonic development; inactivation results in pancreas hypoplasia and a decrease in *Ngn3* expression [26]. Transgenic maintenance of Oc1 in hormone-positive cells leads to impaired separation of endocrine cells from the ducts, abnormal islet architecture, and loss of β-cell identity as indicated by dramatic reductions in the glucose transporter, Glut2, and the β-cell transcription factor, MafA [72, 73]. Several *OC1* mutations and variants are associated with neonatal syndromic diabetes, early onset diabetes, increased risk of adult-onset diabetes, and T2D in humans [74]. *OC1* null and diabetes-causing mutations/variants identified in human patients have been modelled using directed differentiation of induced pluripotent stem cells (iPSC), which revealed defective formation of pancreatic progenitors and decreased endocrine differentiation [74]. Transcriptome-based approaches revealed that these *OC1* variants fail to bind putative enhancers of genes essential for endocrine differentiation [74].

3.2. Factors that regulate endocrine differentiation

NGN3: Ngn3 is critical for specification and differentiation of the pancreatic endocrine cell lineage. It is detected in the pancreatic epithelium of mice as early as E8.5 [75], which precedes dorsal bud formation. Expression declines as development proceeds, with little to no expression in adult endocrine cells [39]. Interestingly, distinct temporal expression of Ngn3 during development dictates specific allocation of endocrine progenitor cells to the different endocrine cell lineages [76]. Early Ngn3-expressing cells give rise to glucagon-positive α cells, while later expression of Ngn3 leads to production of insulin-positive β cells, somatostatin-positive δ cells, and pancreatic polypeptide-positive γ cells [76]. *Ngn3* deficiency in mice results in the complete loss of all endocrine cell lineages in the pancreas and all enteroendocrine

cells in the intestine [30]. In humans, NGN3 is not detected until late embryogenesis, between 47-50 dpc (CS20-21) (Figure 1), with peak expression at the end of the first trimester; expression is gone by 35wpc [77]. Mutations in human *NGN3* are associated with intestinal malabsorption, due to the loss of enteroendocrine cells, and insulin-dependent diabetes mellitus (IDDM) [78-81]. Evidence in humans suggests that endocrine specification is not entirely dependent on NGN3, and that there are likely redundant pathways for β-cell development [79].

PAX6: Pax6 is expressed in the mouse pancreas at E9.0 within both pancreatic buds; however, Pax6 expression is restricted to committed endocrine cells later in development [82]. Ectopic expression of *Ngn3* within chicken embryos *in ovo* triggers *Pax6* expression suggesting that Pax6 acts downstream of Ngn3 during endocrine differentiation [83]. Pax6 is mainly required for the differentiation of α cells, but also regulates expression of other endocrine hormones and is maintained in all endocrine lineages throughout life in the mouse [82]. Global loss of *Pax6* results in loss of α cells and decreased numbers of all other endocrine cell lineages [82]. In humans, PAX6 is not detected until 10 wpc in hormone-positive endocrine cells [11]. Heterozygous mutations in PAX6 result in impaired glucose tolerance and permanent neonatal diabetes mellitus [84, 85].

MAFA and MAFB: MafA and MafB belong to the large Maf family of transcription factors. MafB is expressed as early as E10.5 in glucagon- and insulin-expressing cells [86-88]. This expression is critical for α- and β-cell development and maturation [86, 89, 90], and *MafB* deficiency in mice results in a reduction in both α and β cells [90]. In the absence of *MafB*, the emergence of α and β cells is delayed until the onset of MafA expression that occurs later during the secondary wave [89]. MafB becomes restricted to α cells soon after birth in mice [88]. In contrast, MAFB is expressed in human α cells and a large subset of β cells [91, 92]. MafA is considered a β-cell-specific transcription factor in mice and humans [93], although one group has reported that MAFA is also expressed in human α cells at low levels [92]. The earliest expression of MafA in the mouse pancreas is observed at E13.5 in insulin-producing cells and is maintained in β cells throughout adulthood [94] (Figure 1). MafA directly interacts with Pdx1 in β cells to activate *insulin* gene transcription [95, 96]. Surprisingly, *MafA* is not required developmentally in mice; deficiency has no effect on the number of differentiated β cells at birth. However, mice lacking *MafA* become glucose intolerant due to impaired postnatal glucose-stimulated insulin secretion [97]. MAFA nuclear localization and protein stability in β cells is impaired in mice and humans with T2D, and mutations in the *MAFA* gene in humans are associated with both T1D and T2D [91, 98, 99].

4. Basics of epigenetic modifications

Epigenetic changes are stable, but reversible, modifications of DNA and histone proteins, which can affect gene expression and thus, cell fate and function. We are just beginning to understand how these modifications and their catalytic enzymes affect pancreas specification, endocrine differentiation, and longer term β-cell identity and function (Figure 2A). There are several classes of epigenetic modifications that either positively or negatively influence the cellular transcriptional machinery to achieve a particular transcriptional state. Due to space limitations, only some of these will be covered here.

4.1. DNA methylation

DNA methylation is one of the most extensively studied epigenetic modifications [100, 101]. During this process, a methyl group is covalently transferred from S-Adenosylmethionine to cytosine nucleotides, creating 5-methylcytosine. The process is catalyzed by DNA methyltransferase enzymes (DNMTs), and normally occurs at CpG islands, clustered regions of cytosine-guanine dinucleotides [102, 103] (Figure 2B). The overall concentration of CpG methylation or total methylation levels at a genetic locus correlates to a distinct chromatin accessibility pattern [102, 103]. Thus, regulation of DNMTs plays an important role in repression or activation of genes. High levels of CpG methylation at gene promoters often leads to gene silencing [102, 103], whereas low CpG methylation at gene promoters leads to an increase in chromatin accessibility and thereby facilitates an increase in gene transcription [102, 104]. Differences in genomic methylation

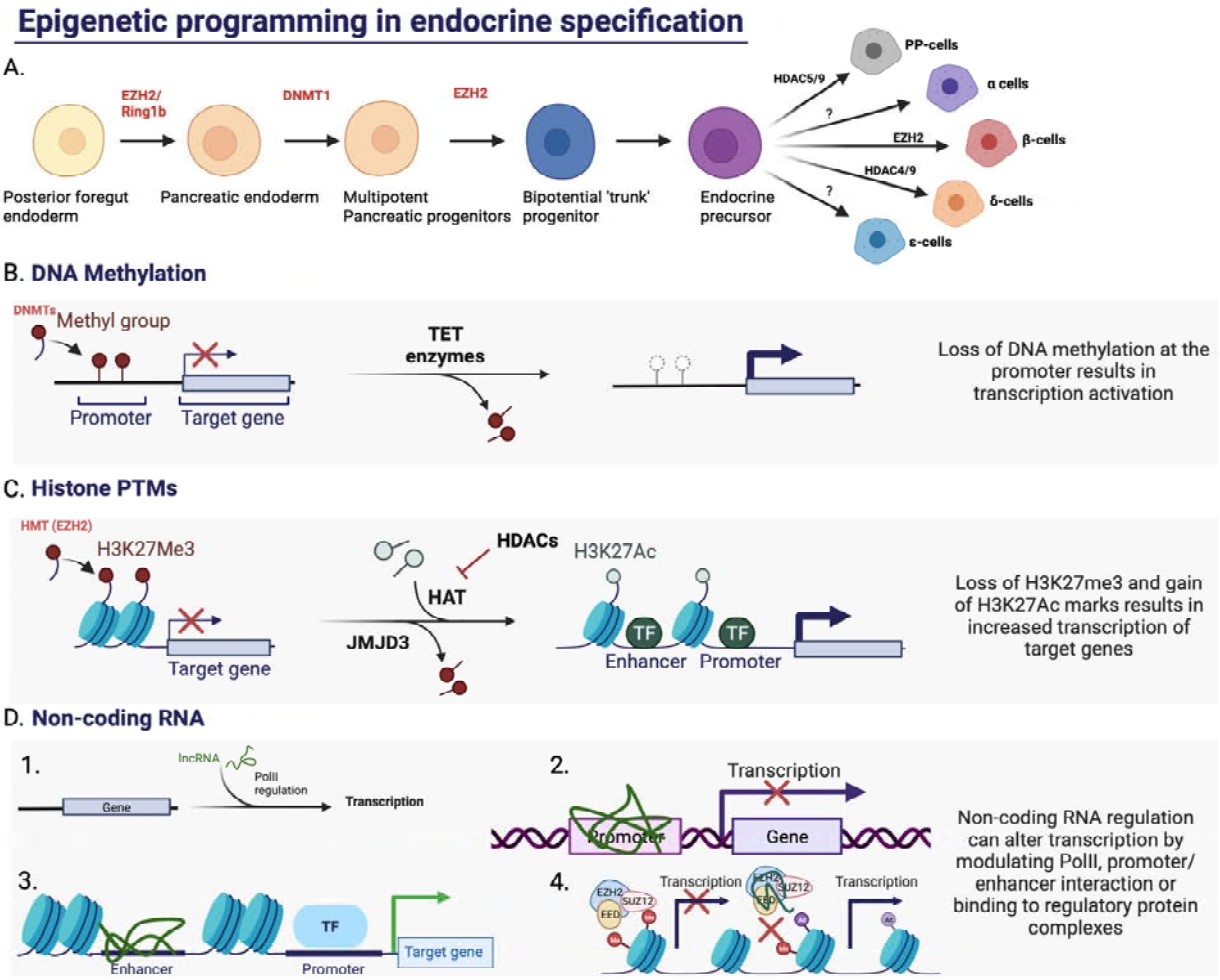

Figure 2. Epigenetic programming during endocrine lineage specification. (A) Pancreas developmental stages are specifically affected by different epigenetic modifications (and their enzyme catalysts) such as DNA methylation (DNMT), histone methylation (EZH2), and histone deacetylation (HDAC). (B) Histone PTMs: Histone methyl transferases (HMTs) add methyl groups, which can inhibit gene transcription; demethylases (JMJD3) reverse histone methylation. Histone acetyl transferases (HATs) add acetyl groups to histones leading to increased transcription; histone deacetylases (HDACs) remove acetylation marks. (C) DNMTs add methyl groups to mainly cytosine bases in DNA at the promoter region, inhibiting transcription; TET enzymes remove methyl groups, leading to derepression. (D) Long noncoding RNAs (lncRNAs) regulate transcription through epigenetic modification by acting on 1. RNA Polymerase (Pol) II; 2. binding to gene promoters or 3. enhancers, or 4. binding to transcription regulator complexes. Created with Biorender.com.

status that occur between different cell types or cellular states are often called 'differentially methylated regions' or DMRs and are considered to be regions crucial for gene regulation [105].

The DNMT family is composed of five members: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3l [106, 107]. Among these, DNMT1, DNMT3a, and DNMT3b are the most relevant here. DNMT1 is constitutively expressed and responsible for maintenance of existing methylation patterns on the newly synthesized DNA strand after DNA replication, preferring hemi-methylated DNA [108]. In mouse embryonic stem cells (ESCs), DNMT1 facilitates transcriptional repression and is required to maintain cells in an undifferentiated state [109]. DNMT3a and DNMT3b are responsible for generating new patterns of DNA methylation in mammals [110] and can also introduce non-CpG

methylation, primarily on CpA, as observed in oocytes, ESCs, and neural cells [111-114]. Both DNMT3a and DNMT3b are strongly expressed in early embryos and ESCs, and are markedly downregulated during ESC differentiation and in terminally differentiated somatic cells [110, 115, 116]. Since complex patterns of DNA methylation control the expression of key genes during development, regulation of CpG de-methylation, catalyzed by TET (Ten-eleven translocation) enzymes [117, 118], is also critical.

4.2. Histone modifications

Histones, a class of DNA-interacting proteins found in eukaryotes, provide the basis for DNA organization by regulating chromatin accessibility. DNA-histone interactions affect how readily replication machinery and transcriptional regulators can access the DNA. DNA in heterochromatin is tightly compacted around histones and thus less accessible, while regions of euchromatin, which are less compacted, are more accessible. Post-translational modifications (PTMs) of histones alter their interaction with DNA, thereby changing accessibility of gene regulatory elements, which leads to changes in gene transcription [119] (Figure 2C and Table 1). Acetylation and methylation are the two most well-studied histone PTMs, but histones can also be ubiquitinated and phosphorylated [120]. One class of histone modifying enzymes, the 'writers', add acetyl or methyl groups to histones and are termed histone acetyltransferases (HATs) or histone methyltransferases (HMTs), respectively [121]. The other class known as 'erasers' remove previously existing PTMs from histones and are termed histone deacetylases (HDACs) and histone demethylases (HDMs) [121] (Figure 2C). Gene regulatory elements that simultaneously possess both repressive (for example H3K27me3) and active (for example H3K9/K14ac or H3K4me3) histone marks are described as being bivalently marked or in a 'poised' state [122] (see Figure 3). Generally, genes that remain in a poised state, similar to those with only repressive marks, are transcriptionally inactive or expressed at very low levels [123]. In stem or progenitor cells, promoters of important developmental regulators are observed to possess bivalent marks and remain in a 'poised' state [123-127].

Acetylation: HATs transfer an acetyl group from acetyl Co-A to lysine residues on histone proteins to form ε-N-acetyl lysine [121]. This modification loosens the interaction between histones and negatively-charged DNA, resulting in enhancement of chromatin accessibility and allowing for binding of coactivating or corepressing transcription factors and RNA polymerase II [128]. This process is reversible by HDACs. Thus, the acetylation/ deacetylation state regulates gene expression through alterations in chromatin accessibility [129]. Acetylation can occur in the core and tail regions of all histones, although HATs typically modify the exposed N-terminal tails of histones H3 and H4 [129]. Histone H3 is commonly acetylated at lysine residues K9, K14, and K27 [129]. Acetylation at both H3K9 and H3K14 is associated with open chromatin at gene promotor regions [128, 130]; acetylation at H3K27 is associated with open chromatin at gene enhancer regions [128, 130]. Commonly acetylated lysine residues on histone H4 include K5, K8, K12, and K16, and these are most often associated with gene activation [129].

Table 1. List of common histone PTMs found during pancreas development, their location, and how they impact gene transcription.

Histone Modification	Function	Location
H3K4me1	Activator	Enhancers
H3K4me3	Activator	Promoters/poised state
H3K27Ac	Activator	Enhancers and promoters
H3K27me3	Repressor	Promoters, developmental regulators and poised state
H3K9me3	Repressor	telomers, pericentromeres, satellite repeats
H3K9Ac	Activator	Promoter
H3K14Ac	Activator	Promoter

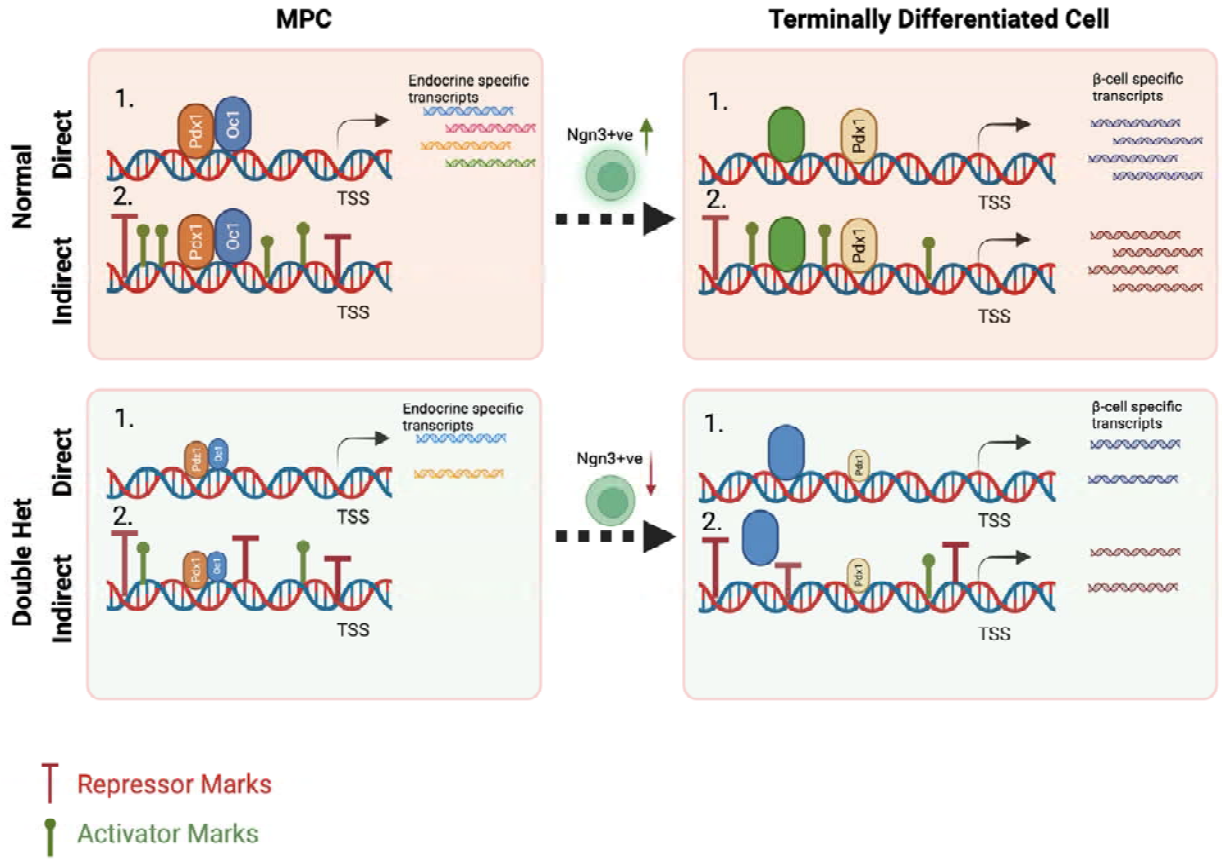

Figure 3. Working models for Pdx1 and Oc1 cooperative function in β cell development. Top row: In normal development, Pdx1 and Oc1 cooperate to promote endocrine development in two potential ways: 1. Direct: By binding to and activating promoters of downstream transcriptional regulators in MPCs (left) that promote endocrine development (represented by different colored transcripts). In terminally differentiated β cells (right), those downstream transcriptional regulators (colored ovals) activate expression of functional genes (represented by black transcripts); 2. Indirect: In MPCs (left) Pdx1 and Oc1 establish a permissive epigenetic environment by adding activating histone modifications (green symbols) to existing repressive histone modifications (red symbols) at key endocrine genes. This poised, permissive epigenetic environment allows for later expression of functional genes in differentiated β cells (right). Bottom row: In the context of Pdx1/Oc1 double heterozygosity, reduced levels of Pdx1 and Oc1 result in: 1. Reduced expression in MPCs (left) of downstream transcription factors required for endocrine differentiation and thus reduced expression of functional genes in differentiated β cells (right); 2. Lower levels of Pdx1 and Oc1 prevent the establishment of a permissive epigenetic landscape at functional genes in MPCs (left), resulting in inaccessibility of these promoters later in differentiated β cells (right). Created with Biorender.com.

Acetylated histone lysine residues are recognized by "readers", which recruit regulatory proteins that facilitate transcription of the "marked" gene [131]. Additionally, these "readers" can recruit nucleosome remodelling complexes to alter overall chromatin structure. Dynamically expressed genes are thus ultimately controlled by the balance of HAT and HDAC activity [132].

Methylation: Methylation of histones can occur on arginine or lysine residues [133]. The histone methylation code is complex: arginine residues can be mono- or di-methylated; lysine residues can be mono-, di-, or tri-methylated [133]. Methylation of arginine residues 2, 8, 17, and 26 typically occurs on histones H3 and H4, and is catalyzed by members of the arginine methyltransferase (PRMT) enzyme family [134]; this modification is associated with gene silencing. Methylation of histone H3 at lysine residues 4, 36, and 79 is typically linked to active chromatin, whereas methylation at H3 lysine residues 9, 20 and 27 is

typically linked to inactive or repressed chromatin [134, 135]. Because methylation of histones does not change their charge, it is more likely that addition of the methyl group either serves as steric hindrance to its interaction with DNA or as an attractant for effector proteins to certain chromatin regions [134]. Numerous developmental abnormalities have been linked to mutations in histone methyltransferases and demethylases [136, 137], highlighting the need for a deeper understanding of DNA methylation regulation at the molecular level. There are additional chromatin-modifying proteins/transcription factors, such as pioneer factors and polycomb groups that are also able to bind to DNA and recruit histone-modifying enzymes [138, 139].

4.3. Chromatin remodelling complexes

Chromatin remodelling complexes are also able to directly regulate the transcriptional accessibility and activity of many dynamically expressed genes [119]. Distinct from the addition of PTMs, remodelling complexes use the energy from ATP hydrolysis to alter the contacts between DNA and the nucleosome thereby influencing the overall chromatin accessibility around genes [119]. One of the most well studied chromatin remodelling complexes is the switch defective/sucrose non-fermentable (SWI/SNF) complexes. In mammalian cells, SWI/SNF complexes are large evolutionarily conserved complexes that each contain ~15 subunits, with the ATPase subunits being Brg1 or Brm [140]. SWI/SNF complexes participate in chromatin remodelling around important gene promoter and enhancer regions [141, 142]. They interact with the HAT, p300, at enhancer regions to modulate H3K27ac, which in turn promotes an increase in chromatin accessibility and transcriptional activation of associated genes [141].

4.4. lncRNAs

Long non-coding RNAs (lncRNAs) are non-translated RNAs greater than 200 nucleotides long [143]. There are several distinct categories of lncRNAs: intergenic, bidirectional, intronic, and antisense [144]. lncRNAs regulate the expression of genes both transcriptionally and post-transcriptionally by interacting with DNA, RNA, or proteins [144] (Figure 2D). lncRNAs can interact with chromatin modifiers and their recruitment to gene promoters can impact gene expression [144-147]. They can function as decoys for particular chromatin modifiers, effectively isolating them from the promoters of target genes, and can also alter recruitment of accessory transcription factors, leading to changes in histone PTMs [148, 149] and chromatin accessibility [150, 151]. lncRNAs can also form RNA-DNA hybrids called R-loops during transcription [152-154]. R-loops can be detected by transcription factors or by chromatin modifiers, which can either activate or inhibit the transcription of target genes [152-154]. By interfering with the transcriptional machinery, such as RNA polymerase, lncRNAs can also decrease gene expression [148].

5. Epigenetic regulation of early pancreas development

Epigenetic mechanisms, including DNA methylation and histone PTMs, play a crucial role in the early stages of pancreas specification (Figure 2A). Regulatory elements of pancreas-specific genes are in a poised state at the definitive endoderm stage [155]. To initiate pancreatic development, these poised regulatory elements can be activated in coordination within bipotential duodenal and pancreas foregut endoderm progenitor cells simply by the removal of the repressive H3K27me3 marks [156]. The default outcome of the ventral foregut endoderm is the pancreatic program, and epigenetic changes in response to local inductive factors play an important role in promoting alternative endoderm fates such as the liver [14, 156]. For example, increased histone acetylation, which is linked to gene activation and is driven by the HAT p300, leads to an expansion of the liver domain at the expense of pancreas specification [156]. Similarly, the Enhancer of Zeste Homolog 2 (EZH2), which is part of the Polycomb Repressive Complex 2 (PRC2) complex, catalyzes H3K27me3 at *Pdx1* gene regulatory elements in response to FGF signaling from the adjacent cardiac mesoderm [156] resulting in repression of *Pdx1* and shifting the cellular fate within the ventral foregut endoderm from a pancreatic program towards a hepatic fate [156]. Within committed pancreas cells, enrichment of H3K27me3 is maintained at genes that specify alternative endoderm fates [156]. Knowledge regarding the epigenetic alterations regulating

dorsal bud evagination and growth is still very limited; EZH2 does not appear to impact this process [156]. Additionally, the role of EZH2 during MPC expansion remains unknown.

SWI/SNF functions to modulate H3K27ac at gene enhancer regions that increase the specification of MPCs within the pancreatic buds [157]. A pancreas-wide knockout of the *Brg1* component of the SWI/SNF complex during embryonic development results in reduced proliferation of MPCs and subsequent pancreas hypoplasia [157]. In other cell types, SWI/SNF activity antagonizes PRC2 [141]. Thus, during the process of early MPC expansion, absence of SWI/SNF function *via* loss of *Brg1* would be predicted to result in increased PRC2 activity. Increased PRC2 would lead to maintenance of methylation at regulatory regions in important pancreatic genes such as *Pdx1,* and thus decreased MPC expansion and subsequent pancreatic hypoplasia.

The state of histone acetylation within the pool of MPCs directly determines commitment to an acinar cell fate or endocrine/ductal fate [158]. Specifically, high HDAC activity promotes acinar cell differentiation, as HDAC inhibition results in an increase in the pool of Ngn3-positive endocrine progenitors within the ductal epithelium [158]. The epigenetic mechanism(s) that influence ductal versus endocrine differentiation from the bipotent trunk progenitors and the role that EZH2 might play in this process are currently unknown.

Pioneer factors such as GATA6, which, as described above, is crucial for early pancreas development, are able to bind directly to chromatin regardless of its state and alter accessibility to other factors through multiple mechanisms, including histone modifications, methylation, or nucleosome remodelling [159, 160]. In iPSCs, GATA6 interacts with SWI/SNF, FOXA2, and HNF1β to modulate accessibly around a definitive endoderm enhancer region, allowing for recruitment of secondary transcription factors and progression towards MPC specification [159]. Whether this mechanism occurs *in vi*vo to promote pancreas fate in posterior foregut endoderm is currently unknown.

6. Epigenetic regulation of pancreatic endocrine fate

As cell lineages become specified and restricted during the course of pancreas organogenesis, whole gene programs become repressed in certain cell types. Blocking the activity of repressive epigenetic regulators could allow for certain cell fates to remain accessible for an extended developmental period. In mice, inactivation of *EZH2* throughout the pancreatic epithelium very early in development (using *Pdx1-Cre*) results in an increase in Ngn3-positive endocrine progenitors [161]. Similarly, treatment of mouse pancreas explants and human embryonic stem cells with an EZH2 inhibitor also resulted in an increase in the formation of Ngn3-positive endocrine progenitors [161]. Later in development, inactivation of *EZH2* in β cells results in increased expression of the cell cycle inhibitors p16 and p19, leading to decreased β-cell proliferation [162]. Thus PRC2, and specifically EZH2, play distinct roles throughout pancreas organogenesis, acting on different gene sets at different stages.

Single-cell RNA-sequencing has revealed distinct sub-populations of Ngn3-positive endocrine progenitor cells that are epigenetically biased towards either an α-cell or β-cell fate [163]. These sub-populations exhibit differential expression of *Dnmt1* and display different DNA methylation patterns at enhancers of genes known to promote the β-cell (*Myt1*) or α-cell (*Arx*) fate [163]. Overexpression of *Dnmt1* or targeted methylation of the *Arx* enhancer region within Ngn3-positive endocrine progenitors can both promote β-cell production and alter cell fate [163].

Pancreatic endocrine cell specification is also driven in part by changes in histone PTMs, such as H3K27me3, at regulatory elements of key transcription factors for endocrine development [164]. The transition from $Ngn3^{LO}$ cells to $Ngn3^{HI}$ definitive endocrine progenitor cells is accompanied by H3K27 demethylation by the histone demethylase JMJD3 [164]. In addition, a decline in HDAC expression as pancreas development proceeds is essential for the increase in Ngn3-positive endocrine progenitor cells, and *ex vivo* inhibition of HDAC activity in mouse embryo

pancreatic bud explants promotes Ngn3 expression [158]. HDACs also play a role in endocrine cell lineage allocation. For example, following small molecule-mediated HDAC inhibition in mouse embryonic pancreas explants, there is an increase in δ and γ cells at the expense of other endocrine cell types, suggesting HDAC activity hinders the specification of δ and γ cells from endocrine progenitors [158, 165]. Likewise, global knockout of *HDAC4* or *HDAC9* led to a greater number of δ cells *in vivo,* while mice with a global knockout of *HDAC5* or *HDAC9* exhibited an increase in β cells [165].

Our group previously discovered that Pdx1 and Oc1 cooperate to promote endocrine differentiation. *Pdx1/Oc1* double heterozygotes show a decrease in the number of Ngn3-positive cells at E15.5 and a concomitant decrease in hormone-expressing cells later in embryogenesis [166]. This phenotype is distinct from each of the *Pdx1* and *Oc1* single heterozygotes [166]. Surprisingly, although endocrine mass and lineage allocation are normalized by birth in *Pdx1/Oc1* double heterozygotes due to increased proliferation at late gestation, β cells in adult double heterozygotes fail to respond to proliferative stimuli such as placental lactogen and high fat diet [71]. Since Pdx1 and Oc1 are only transiently co-expressed in MPCs and endocrine progenitors, we suggest that they work cooperatively to modify the epigenetic landscape within MPCs at genes that regulate endocrine specification, differentiation, and proliferation later in development and postnatally (Figure 3). However, since differentiation and proliferation are often thought to be disparate cellular activities, future studies are needed to elucidate the mechanism(s) by which Pdx1 and Oc1 cooperate to regulate genes that promote differentiation or proliferation.

Activation of β-cell-specific genes is crucial for acquisition of mature β-cell function during postnatal development. The transcription factor MafA directly interacts with components of the MLL3/4 methyltransferase complex [167], which catalyzes methylation of H3K4 at gene enhancers and promoters, leading to gene activation [168]. β-cell-specific knockout of a key component of the MLL3/MLL4 complex, *NCOA6*, leads to decreases in MafA target gene expression and impaired β-cell function *in vivo* [167]. While the interaction of MafA with MLL3/4 is critical to activate key genes involved in β-cell maturation and function, it is unclear whether the MLL3/4 complex in this context is altering histone methylation at MafA target genes. Therefore, more studies are needed to uncover the precise mechanism by which the MafA:MLL3/4 complex regulates β-cell maturation *in vivo*. MafB is also capable of interacting directly with the MLL3/4 complex, at least in the immortalized human ENDOC-βH1 β-cell line [167]. In adult mouse β cells, repressive chromatin marks (e.g. H3K9me3) are abundant on *MafB*, while during embryonic development, when *MafB* is expressed in both glucagon- and insulin-expressing cells, both cell types have activating marks (H3K4me3, H3K9/14Ac) at the *MafB* locus [169].

Various studies have shown the impact of lncRNAs in endocrine cell differentiation, β-cell identity, and β-cell function [170, 171]. lncRNAs modulate the epigenetic landscape and are involved in cell-specific gene regulation in pancreatic β cells. For example, the pancreas-specific lncRNA *Meg3* physically interacts with EZH2, resulting in H3K27me3 at the promoter region of transcription factors (*Rad21*, *Smc3*, and *Sin3α*) that bind to the *MafA* promoter region and inhibit its expression [172]. Therefore, the lncRNA *Meg3* promotes expression of MafA [172]. A study in EndoC-βH1 cells highlights the impact of the lncRNA *PLUTO* on cis-regulation of *PDX1* gene expression [173]. Quantitative chromatin conformation capture (3C) analysis of the *PDX1* locus demonstrated altered chromatin structure following *PLUTO* knockdown, with decreased interactions between critical enhancers and the *PDX1* promoter [173]. Similar to *PLUTO*, the cis-regulatory lncRNA *βlinc1*, and its human ortholog *HI-LNC15,* alters the expression of *Nkx2.2* and a large subset of *Nkx2.2* β-cell gene targets [174]. Knockdown of *βlinc1* in immortalized MIN6 mouse β cells or EndoC-βH1 cells resulted in a decline in expression of *Nkx2.2* [174].

7. β-cell replacement from non-β-cell origins

The understanding of pancreas development through studies in model organisms has been very

useful to develop a framework for *in vitro*-directed differentiation protocols to generate pancreatic progenitors and β-like cells from human ESCs or iPSCs [175-179], and to consider strategies to enhance *in vivo* neogenesis or transdifferentiation in the adult pancreas as a treatment for diabetes. Because of their close lineal relationships, glucagon-producing α cells and pancreatic exocrine cells (ductal epithelial cells and acinar cells) are particularly appealing candidates for *in vivo* β-cell replacement. Additionally, activation of endocrine cell neogenesis from adult pancreatic ductal cells and differentiation of centroacinar cells suggest that these cells can act as facultative stem cells for islet regeneration under certain circumstances.

7.1. Human embryonic stem cells

Protocols to differentiate human ESCs towards a β-cell-like fate have been reiteratively revised, resulting in great improvements in the generation of functionally mature human β cells [175]. Although existing protocols to generate stem-cell-derived β cells (SC-β cells) *in vitro* vary slightly, they each strive to recreate key stages of pancreatic organogenesis and lineage specification (Figure 1) through the temporal application of small molecules and growth factors (i.e. retinoic acid, Activin A, and BMP inhibitors) to pluripotent human ESCs [175, 180]. These SC-β cells express insulin and C-peptide, as well as many key β-cell transcription factors, including PDX1 and NKX6.1, similar to endogenously developing β cells. However, transcriptomic and epigenomic analyses have revealed that gene expression and chromatin accessibility profiles of SC-β cells are not identical to primary adult human β cells [181-183] Specifically, expression of key markers of β-cell maturation such as *MAFA*, *INS,* and *MNX1* is low or absent [184]. Additionally, SC-β cells are functionally immature and lack appropriate glucose-stimulated insulin secretion *in vitro* [184, 185]. During directed differentiation of human ESCs, many initially bivalently marked lineage-specific developmental genes resolve; H3K27me3 repressive marks are lost, and H3K4me3 activating marks are maintained. However, many key endocrine functional genes retain the repressive H3K27me3 mark after differentiation to β-like cells [183], which may explain in part why they are less functional. Engraftment of pancreatic endoderm stage cells or SC-β cells *in vivo* promotes the formation of mature, functional β cells from human ESCs [183, 184]. However, the chromatin landscape even in these cells does not fully replicate endogenous adult β cells [183]. The *in vivo* factors promoting terminal β-cell differentiation have not been identified and it is unclear if these signals are similar to the postnatal signals that normally drive functional maturation of β cells [182, 186, 187]. Future studies are needed to understand the differences between *in vitro*-generated β-like cells and their endogenous counterparts to develop SC-β cells that more closely mimic the functionality of primary β cells.

Although imperfect, these models have led the way for developing islet mini-organs that can be transplanted *in vivo* in humans in an attempt to cure disease [188]. For example, during a recent phase 1/2 clinical trial, two patients with T1D were given a single infusion of SC-derived differentiated pancreatic islets into the hepatic portal vein as a replacement therapy, in conjunction with immunosuppressive therapy [188]. While these initial findings have not yet been peer reviewed, the cells were reported to successfully engraft, improve glycemic control, and decrease exogenous insulin requirements by day 90 [188].

7.2. Induced pluripotent stem cells

Although there are some developmental advantages to using human ESCs as a starting point for deriving β-like cells, ethical concerns remain, since these cells are derived from the inner cell mass or blastocysts of early human embryos, resulting in the death of those embryos. Thus, an alternative source for developing mature β cells comes from the directed differentiation of human iPSCs. Somatic cells from any tissue, commonly fibroblasts, can be reprogrammed or 'induced' into a state of pluripotency by ectopically expressing four pluripotency transcription factors: *OCT4*, *SOX2*, *KLF*, and *C-MYC* [189]. iPSCs resemble ESCs in morphology and can be differentiated into most somatic cell types, including β cells. Several groups have successfully differentiated somatic cell-derived iPSCs from human, mouse,

and macaque into β-like cells following similar protocols used to differentiate human ESCs [176, 190-192]. Similar to ESC-derived β-like cells, iPSC-derived β cells are functionally immature with limited glucose responsiveness *in vitro*, but show increased C-peptide release and can improve glucose tolerance in diabetic mice when transplanted *in vivo* [193].

At first, iPSCs were assumed to be epigenetically, functionally, and developmentally equivalent to ESCs [194, 195]. However, many groups have shown that there are clear differences at the molecular and epigenetic level, and that iPSCs retain an epigenetic memory of the original tissue or cell type from which they were derived, which could predispose them to differentiate preferentially to the same or a similar cell type [196, 197]. In iPSCs generated from primary human pancreatic β cells, the chromatin around key β-cell genes such as *PDX1* and *insulin* remained accessible throughout the reprogramming and β-cell directed differentiation process [198]. Thus, β-cell-derived iPSCs had an increased ability to differentiate into insulin-producing β cells compared to non-β-cell derived iPSCs [198]. While this might indicate that primary β cells are the best cellular source from which to derive iPSCs due to their epigenetic memory, there was still decreased chromatin accessibility around the *MAFA* gene in induced β-like cells, indicating these cells still have a more immature phenotype [198]. For both ESC-derived and iPSC-derived β cells, the concern remains that, in the setting of T1D, the autoimmune response will destroy the transplanted β cells in the absence of some form of immunosuppression or encapsulation [199].

7.3. Alpha cells

Several studies suggest that α cells retain plasticity and are more able to transdifferentiate into β cells than are other endocrine cell types. Although α, β, and δ cells exhibit similar DNA methylation patterns at the promoters of the *glucagon* and *insulin* genes, the enhancers of these genes exhibit distinct cell-type-specific methylation patterns [200]. Demethylation of the *glucagon* and *insulin* promoters occurs during the differentiation of Ngn3-positive endocrine progenitor cells towards an endocrine cell fate [200]. Recent studies in mice have indicated that in certain extreme circumstances, such as when Pax4 or Pdx1 are artificially increased or when a large majority of β cells are ablated, α cells can transdifferentiate into β cells [201-203]. In a separate study, bile duct infusion of a virus in which the human *glucagon* promoter drives expression of *Pdx1* and *MafA* restored blood glucose levels in mice made diabetic by alloxan treatment [204], suggesting the conversion of α cells into functional insulin-producing β-like cells. Studies with human islets have revealed that α cells have more bivalently tagged (poised) genes compared to β cells or exocrine cells [205]. Many bivalently marked genes in α cells encode proteins crucial for β-cell maturation and function (*MAFA*, *PCSK1*, and *PDX1*) [205]. Thus, α cells may be inherently more plastic than other pancreatic cell types. Manipulation of these bivalent marks at key β-cell genes can drive transdifferentiation of α cells towards a β-cell fate.

7.4. Acinar cells

Acinar cells develop from MPCs and are thus closely related to β cells. Transdifferentiation of acinar cells into β cells was thought to first involve the reprogramming of these cells to a less specialized, pancreas progenitor-like state [206]. However, in some circumstances, this cell fate switch may be direct and not require a progenitor intermediate, similar to transversing a hill in a Waddington landscape [207]. In one study, adenoviral delivery of three crucial islet transcription factors, *Ngn3, Pdx1,* and *MafA*, to the adult mouse pancreas *in vivo* resulted in a high level of infected acinar cells and an induction of scattered, insulin-expressing β-like cells with similar ultrastructure and morphology to endogenous β cells [208]. These results were interpreted to indicate direct transdifferentiation of acinar cells into functional β-like cells [208], although this was not definitively shown with lineage tracing or careful temporal analysis. A transgenic mouse model of inducible, acinar-specific *Ngn3, Pdx1, and MafA* expression confirmed that adult acinar cells can indeed give rise to β cells [209]. A lineage tracing strategy using Cre-recombinase-expressing adenoviruses delivered to acinar cells of R26R-ECFP reporter mice also confirmed that acinar cells can transdifferentiate into

insulin-producing β-like cells, but these cells had low secretory capacity compared to native β cells [210]. To determine the epigenetic remodeling that accompanies reprogramming of acinar cells to β-like cells, a time course study was performed using adenoviral overexpression of *Ngn3, Mafa*, or *Pdx1* [211]. By day 10, acinar cells had transdifferentiated into insulin-expressing β-like cells; however these cells were not glucose-responsive [211]. These β-like cells underwent gradual DNA methylome and transcriptional remodeling, resulting in glucose responsiveness by two months [211]. This same group determined that both *p53* and *Dnmt3a* act as barriers to successful acinar to β-cell reprogramming [212]. Thus, moderate to low expression of *Dnmt3a* promotes transdifferentiation of acinar cells to β-like cells.

7.5. Ductal cells

There has been an ongoing controversy as to whether adult pancreatic duct cells can act as a source of β-cell neogenesis [21, 58, 213]. The evidence supporting the ability of adult ductal cells to generate new β cells includes lineage tracing, 3D imaging, and single-cell RNA sequencing techniques [214, 215]. For example, inducible global deletion of the E3 ubiquitin ligase *Fbw7* resulted in direct conversion of some pancreatic ductal cells into cells that expressed genes crucial for β-cell function, and secreted insulin in response to glucose [215]. Absence of Fbw7 seems to reactivate the endocrine neogenic pathway in adult pancreatic ductal cells. In another study using primary adult human ductal cells grown as organoids in culture, infection with an adenovirus expressing *Ngn3* resulted in elevated expression of key regulators of islet endocrine fate including *NEUROD1, Nkx2.2, INSM1,* and *RFX6* [216]; however, these cells never progressed to insulin- or glucagon-positive cells. Similar to studies of acinar to β-cell transdifferentiation, a combination of *Neurog3, MafA,* and *Pdx1* can transform adult duct cells into cells with characteristic properties of mature β cells [216]. Certain peptides, such as GLP-1 and gastrin, may also have the potential to promote transdifferentiation of ductal cells to β-like cells [217, 218]. These studies highlight the potential of ductal cells to generate new β cells in the mature adult pancreas but did not elucidate the epigenetic changes that occur during the transdifferentiation process. More recently, it was shown in both immortalized human duct cells and primary human ductal epithelium from T1D donors that treatment with an EZH2 inhibitor results in activation of genes associated with β-cell differentiation (e.g. *NEUROD1, PTF1A, MAFA, INS,* and *PDX1*) [219, 220]. For example, the presence of bivalent activating (H3K27me3) and silencing (H3K4me3) marks within the *PDX1* and *INS* gene regulatory regions was resolved such that only the H3K4me3 mark remained, leading to gene activation [219, 220]. Thus, an actively repressed β-cell neogenic program in adult duct cells may be reactivated by blocking the activity of EZH2.

8. Effects of environmental factors on the epigenome during pancreas development

Environmental factors such as maternal pre-pregnancy nutrition status, pregnancy diet, placental function, stress, exposure to toxins, or pharmacological treatments are known to alter the epigenetic landscape during embryonic development [221]. Each of these have the potential to influence the availability of substrates required for epigenetic marks as well as the activity of enzymes that regulate histone and DNA modifications.

8.1. Nutrient access

There is evidence in multiple animal models that exposure to either undernutrition or overnutrition during development results in altered endocrine pancreas development [222-224]. For example, administration of a low-calorie or low-protein diet to pregnant Wistar rats resulted in decreased expression of *Ngn3* and *Pdx1* at E15 and a reduction in β-cell mass at birth [224]. Exposure of zebrafish embryos to overnutrition increases β-cell number [225]; however, in mice, the effects of exposure to overnutrition during embryonic development on β-cell mass are inconclusive, depending on the diet composition, duration of diet, and strain of mouse used (reviewed in [226]). Interestingly, in non-human primate fetuses exposed to maternal western-style diet during development, β-cell mass was unchanged, but α-cell mass was reduced [227].

Although effects of maternal diet on islet and β-cell development have been documented in different model systems, relatively few studies

have addressed how maternal diet alters the offspring epigenetic landscape during pancreas development. Nutrient availability affects the levels of metabolites (such as acetyl-CoA, S-adenosylmethionine) used for some epigenetic modifications and thus can result in changes in histone PTMs [228]. For example, in a rat model of maternal undernutrition, expression of *Hnf4α*, a T2D susceptibility gene that is also mutated in some forms of monogenenic diabetes, was decreased and its islet promoter showed increased H3K27me3 [229]. Models of intrauterine growth restriction (IUGR) reducing blood flow or nutrient access to the fetus have also been used and have shown changes in several epigenetic marks such as H3K4me3, H3K27me3, and H3K27Ac at promoter or enhancer regions of critical transcription factors like *Sox9* and *Mitf* [230]. IUGR also leads to changes in the 'poised' state of many bivalently marked islet genes, a 50% decrease in *Pdx1* mRNA, long-term inhibition of *Pdx1,* and a significant reduction in β-cell mass in adult rats [230, 231].

8.2. Environmental toxins

Developmental exposure to environmental toxicants (pesticides, dioxins, heavy metals, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), and bisphenol A (BPA)) can have a negative impact on postnatal islet health and function and can influence transgenerational disease susceptibility by causing changes in epigenetic patterns. [232].

Dioxin (2,3,7,8-Tetrachlorodibenzo-p-dioxin) is an organic pollutant associated with increased risk of diabetes and impaired β-cell function [233, 234]. In directed differentiation protocols, hESCs exposed to dioxin showed a decreased ability to differentiate into pancreatic lineages and showed alterations in patterns of DNA methylation with four crucial transcription factors (*PRKAG1, CAPN10, HNF1B,* and *MAFA*) exhibiting hypermethylation [235]. *In vivo*, treatment of pregnant mice with dioxin resulted in decreased β-cell area, but only in female offspring [236].

BPA is a recognized xenoestrogen that triggers programmed cell death in β cells by activating the estrogen receptors ERα, ERβ, and GPER [237]. Exposure of dams to BPA during pregnancy and lactation can result in multi-generational disturbances in global DNA methylation including in the liver-specific promoter for *glucokinase (Gck)*, a key gene in glucose metabolism and glucose-stimulated insulin secretion, leading to impaired glucose homeostasis in the male offspring [238]. This study did not investigate or report the impact of BPA exposure on the DNA methylation status of the *Gck* promoter in the pancreas. In a rat model, exposure to BPA during development resulted in a reduction in β-cell mass at birth and dysglycemia in adulthood [239]. This was attributed to a decrease in the number of *Pdx1*-positive cells and a decrease in *Pdx1* mRNA as early as E15.5 [239]. Correspondingly, the *Pdx1* promoter saw increased methylation, suggesting that BPA has a negative impact on epigenetic modulation during pancreas development [239]. Paternal BPA exposure earlier in life is associated with DNA hypermethylation at the *Igf2* locus and reduced Igf2 expression in the islets from male F2 offspring and induces pancreatic impairment in rat offspring, suggesting that the male germ line may be the pathway by which BPA-induced glucose intolerance is passed down through generations [240].

8.3. Pharmacological treatments

Some drugs or pharmaceuticals consumed during pregnancy can cross the placenta and exert an influence on the growing fetus. For example, prenatal exposure to the corticosteroid dexamethasone, which is administered during preterm labor for fetal lung maturation [241], may contribute to increased risk for diabetes in some children and adolescents. Exposure of pregnant rats to dexamethasone in the middle or late stages of pregnancy significantly altered the function of the pancreatic islets and glucose metabolism in male progeny [242]. Another drug more commonly used in women of child-bearing years is the diabetes medication metformin. Metformin can be prescribed during pregnancy for pre-gestational diabetes, gestational diabetes, polycystic ovarian syndrome, and pre-eclampsia [243, 244]. We previously reviewed the mechanisms of action of metformin and the developmental effects of *in utero* exposure to metformin [245]. The impact of metformin on pancreas and islet development and function has been investigated in multiple model systems including zebrafish, mice, and human

stem cells [246-248]. In mice, offspring exposed to metformin during gestation displayed an increase in overall pancreas size, and in the number of Pdx1-positive pancreatic progenitors and Ngn3-positive endocrine progenitors at E14.0 [248]. In zebrafish embryos, exposure to metformin also resulted in an increased number of β cells, but no increase in *insulin* mRNA expression was observed [247]. Interestingly, metformin-treated zebrafish embryos exhibited increased mRNA expression of *somatostatin*, which is known to counterregulate insulin secretion [247]. Additionally, exposure of hESCs to metformin during directed differentiation resulted in downregulation of key islet genes such as *Isl1, Pcsk2, chromogranin A, and insulin,* and upregulation of genes involved in mitochondrial respiration [246], which could impact β-cell function. Metformin has been shown to impact epigenetics *via* its effects on the production of citric acid cycle intermediates involved in histone PTMs in human ovarian tumors [249]; however it remains to be seen if this is also true in β cells.

9. Conclusions and future directions

The *in utero* and early postnatal environment have the potential to modify biological processes that impact epigenetic systems, such as DNA methylation, histone PTMs, and lncRNA expression. Alterations in the epigenetic code influence gene expression, and can impact the risk of developing metabolic diseases such as diabetes, compounding the effects of genetic variants that modify disease risk in conjunction with the adult envirome. Advancements in single cell technologies and computational methods allow for comprehensive simultaneous multi-omics analysis of gene expression, chromatin modifications, DNA methylation, and metabolomics, allowing us to generate a more complete picture of the β-cell epigenome and transcriptome from developmental stages through adulthood, as well as in the setting of environmental insults and disease. However, most omics studies are still mainly correlative and descriptive. New methodologies are needed to elucidate the mechanisms whereby environmental exposures and nutrients affect proteins and non-coding RNAs that control epigenetic gene regulation and thus expression of crucial endocrine lineage-specific genes.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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