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Review

Transcriptional control of pancreatic development: bHLH factors at the crossroads of exocrine and endocrine specification and differentiation

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ABSTRACT

Pancreatic development is a complex and highly coordinated process in which two distinct histological and functional tissues (the exocrine and endocrine cell compartments) must arise from a common cell progenitor within a simple epithelium. The morphological events, the specification and differentiation of the different cell types (ductal, centro-acinar, acinar and endocrine) are under the control of a transcriptional factor network which needs to be tightly regulated in order to ensure timely and precise activation of the alternative gene expression programs that lead to formation of the different pancreatic lineages. Among the transcription factors involved in this regulatory cascade are members of the bHLH (basic helix-loop-helix) family, which play relevant roles during multiple processes of organogenesis. In this review we summarize the current knowledge on a specific set of pancreatic bHLH factors that regulate early pancreatic specification and later, initiate the differentiation program of the endocrine and exocrine cell types by governing cell fate decisions. The information about how these bHLH regulators dictate commitment to a pancreatic fate and modulate lineage choices should be valuable to manipulate both *in vitro* and *in vivo* the plasticity of adult cells or the ability of pluripotent stem cells to acquire specific phenotypes. Ultimately, this knowledge will help to improve cell replacement-based therapies aimed at targeting important and devastating diseases such as diabetes, pancreatitis or pancreatic adenocarcinoma.

KEYWORDS: bHLH transcription factors, Neurogenin3, Ptf1a, NeuroD1, Mist1, pancreas development

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INTRODUCTION

The pancreas is a complex organ composed by an exocrine and an endocrine compartment that play central roles in digestion and glucose homeostasis respectively. The exocrine portion comprises 95-99% of the pancreas and includes the acinar cells devoted to the synthesis and secretion of digestive enzymes, the ductal cells that secrete mucins, chloride and bicarbonate and compose the complex tubular system that drains acinar secretions to the gastrointestinal tract, and the centroacinar cells, located at the terminal compartment of ducts and thought to be important in pancreatic homeostasis. The endocrine pancreas represents 1-2% of the total pancreatic volume and is organized in functional units called the islets of Langerhans, which are clusters of

endocrine cells scattered throughout the exocrine tissue. Islets are composed of five distinct hormone-expressing cell types: β-cell, α-cells, pancreatic polypeptide (PP) cells, δ-cells and ε-cells that produce insulin, glucagon, PP, somatostatin and ghrelin, respectively.

Pathologies associated with the pancreas include severe diseases affecting both the exocrine (chronic pancreatitis, pancreatic cancer) and the endocrine (diabetes) compartments. In chronic pancreatitis, long-standing inflammation results in permanent alterations in pancreatic structure and function. During this process, acinar cells are progressively replaced by ductal complexes, which results in loss of digestive enzymes and nutritional malabsorption. Importantly, this pathology constitutes an increased risk factor for development of pancreatic adenocarcinoma, the pancreatic cancer with the poorest prognosis and highest frequency these days. While this type of tumor displays a ductal-like phenotype, the origin of the pancreatic cells (ductal, acinar or centroacinar) that contribute to tumorigenesis remains largely controversial. However, it has become apparent that pathways active during embryogenesis may be reactivated during pancreatic tumorigenesis. On the other hand, in diabetes, a reduction in β-cell function and mass results in hyperglycemia which is associated with debilitating long-term secondary macrovascular (coronary artery disease, peripheral arterial disease and stroke) and microvascular (diabetic nephropathy, neuropathy and retinopathy) complications.

In recent years, with the advent of regenerative medicine, research on the molecular basis of pancreatic development has raised considerable interest among the scientific community. Indeed, understanding how pancreatic cells are normally formed during embryogenesis may provide important cues not only for illuminating mechanisms underlying the etiology of pancreatic and exocrine diseases, but also for devising strategies to manipulate specific epithelial phenotypes or replace damaged and lost cells to, ultimately, cure these diseases.

During embryonic development, pancreatic exocrine and endocrine cells arise from a common pool of progenitors present in the gut endoderm. This process, which entails the stepwise progression from a pluripotent to various distinct finally-differentiated cellular states, is under the control of a transcriptional code regulated by lineage-specific transcription factors that drive an intrinsic program to specify new cell fate identities and by another one, in which ubiquitous transcription factors are regulated by extrinsic signals in an integrated fashion. Over the past 20 years, gene inactivation in mice together with lineage tracing studies and classical molecular biology assays have helped identify and establish the hierarchy of developmental transcription factors that regulate pancreatic differentiation (for extended reviews, see [1-3]). These factors belong to various protein families and may serve single or multiple distinct roles at different stages of pancreatic development, during early organ specification and later during cellular differentiation and maintenance of mature pancreatic cell functions. Among them, proteins of the basic-helix-loop-helix (bHLH) family occupy a prominent role in the regulation of cell fate determination and differentiation. In this review, we have centered on four bHLH proteins, namely Ptf1a, Neurog3, Mist1 and NeuroD1, for which knockout strategies have demonstrated major non-redundant roles in the formation of both exocrine and endocrine lineages of the pancreas. Importantly, their ability to modulate early cell fate decisions and differentiation of progenitor cells makes these proteins promising candidates to promote differentiated phenotypes from both *in vitro* and *in vivo* systems in regenerative medicine protocols.

1. Basic developmental biology of the pancreas

The pancreas originates from the gut endoderm that begins as a flat sheet of cells that becomes specified during gastrulation. In the mouse, pancreatic specification is induced around embryonic day (E) 8.5 with the expression of the transcription factor Pancreatic Duodenal Homeobox 1 (Pdx1) in two ventral domains first, and in a dorsal domain later at around E8.5-E8.75 [4, 5]. Through coordinated tissue interactions with adjacent mesodermal tissues, including the notochord, aorta and cardiac mesoderm, permissive and instructive signaling pathways are activated that induce a series of morphological events that lead to pancreatic

organogenesis. The earliest recognizable pancreatic anlagen are the dorsal and ventral pancreatic buds which begin as evaginations of the endoderm into the surrounding mesenchyme. The dorsal bud appears first at around E9.5, followed closely by the emergence of the ventral bud. Once evaginated, buds elongate and elaborate into a complex branching epithelial tree. Both buds fuse later in development (around E13-E14), in concert with rotation of the gut (for extensive reviews, see [1, 6-8]).

After the establishment of the dorsal and ventral pancreatic buds there is a period of extensive growth and cytodifferentiation. At early stages (E9-E12), differentiation begins with the emergence of endocrine cells from the undifferentiated pancreatic epithelium, mostly glucagon expressing-cells, which will not contribute to mature islets. This period is called the first transition. True endocrine progenitors start to rapidly amplify in number around E13.5, at a new stage called the secondary transition, characterized by concurrent major waves of differentiation of both endocrine (particularly β-cells) and exocrine lineages. Indeed, rapid branching morphogenesis and acinar differentiation occurs, accompanied by exponential rises in acinar gene expression and the formation of acinar cell clusters, which become histo-logically distinguishable from ducts as acini around E15.5. Meanwhile, endocrine cells delaminate from the primitive duct-like epithelium and coalesce into clusters that represent the first islets of Langerhans, which are found interspersed in the exocrine tissue. Until birth, additional maturation and growth of the differentiated cells occur in both pancreatic compartments. The major steps of pancreatic embryonic development in rodents appear to be highly conserved in other species, including chicken, zebrafish and humans.

2. General overview of the bHLH family of transcription factors

The bHLH proteins form a large family of transcription factors that play critical roles in many developmental events including cellular differentiation, lineage commitment and sex determination. Their common trait, the bHLH domain, is approximately 60 aminoacids in length and is comprised of a basic domain for binding to DNA adjacent to a HLH domain constituted by 2 amphipathic α-helixes, each 15-20 aminoacids in length, and separated by a variable loop region. The HLH domain allows dimerization with other family members to form homo- or heterodimeric complexes. The two basic domains brought together through dimerization bind specific hexanucleotide motifs termed E boxes with consensus sequence CANNTG [9].

The first bHLH motif was identified in the murine transcription factors E12 and E47 [10]. Since then, more than 300 other bHLH proteins have been identified in organisms ranging from yeast to humans. Owing to the large number of identified bHLH factors, they have been classified into 6 major groups, A, B, C, D, E and F on the basis of their evolutionary relationships, residue conservation at certain positions in the bHLH domain, E box binding specificity and the presence of additional domains [11-13]. In this review, we will mainly discuss data regarding Group A proteins but it should be noted that, with the exception of group F, bHLH proteins belonging to all the bHLH phylogenetic groups have been described in the developing pancreas (Table 1).

Group A includes the ubiquitously-expressed E12-related proteins which are collectively known as E proteins (E12, E47, E2-2 and HEB) as well as tissue-specific bHLH proteins such as the Neurogenin/NeuroD factors, Ptf1a and Mist1, which will be described in detail later in this review. It should be noted that they are often referred to as Class I (E proteins) or Class II (tissue-specific) owing to their initial classification based upon tissue distribution patterns and dimerization capabilities [9]. Group A factors classically bind to E boxes with the sequence CAGCTG or CACCTG. In many instances, Class II proteins require binding with E proteins to form active heterodimers and binding of these heterodimers to DNA results in transcriptional activation of target genes. Remarkably, groups of highly related Class II bHLH factors often act sequentially to govern cell fate decisions and differentiation programs in multiple tissues [14-16].

The function of bHLH proteins is subject to different layers of regulation and thereby these factors can impose a tight control over gene expression profiles. On one hand, because bHLH

Table 1. bHLH transcription factors with reported expression in the embryonic pancreas and their known or suspected roles during pancreatic development.

Phylogenetic groups	Features	Members	Role in pancreatic development	Ref.
GROUP A	Class I (E proteins) ubiquitous and Class II, lineage-specific. Bind CAGCTG or CACCTG	NeuroD1	Endocrine cell differentiation and survival	[136]
		NeuroD2	Embryonic expression, unknown function	[100]
		Neurog3	Endocrine cell specification	[76]
		Math6	Embryonic expression, unknown function	[157]
		Mist1	Exocrine cell differentiation	[63]
		Ptf1a	Specification and expansion pancreatic progenitors/ exocrine cell differentiation	[23]
GROUP B	Contain leucine-zipper domain (bHLH-LZ) Mad/Myc/Max network Bind CACGTG or CATGTTG	Myc	Proliferation of pancreatic epithelial and acinar precursor cells	[158]
GROUP C	Contain PAS domain Bind ACGTG or GCGTG. Response to environmental toxins /O_2	HIF1a	β-cell differentiation	[159, 160]
GROUP D	Lack basic DNA-binding domain Dominant negative regulators of Group A proteins	Id1	Embryonic α-cells, unknown	[161]
		Id2	Pancreatic progenitor expansion, inhibition of NeuroD1	[162]
GROUP E	Contain "Orange" domain and WRPW or YRPW motifs Bind CACGCG or CACGAG (N boxes) Transcriptional repressors	Hes1	Notch effector/Inhibition of endocrine cell development/inhibition of exocrine differentiation	[126]
		Hey1/2	Inhibition of exocrine differentiation	[163]

proteins are designed for dimerization with other proteins, mostly within the same family, the choice and availability of specific partners allow for a combinatorial regulatory system that can control transcriptional outputs in a spatial and temporal-dependent manner. Hence, the diversity coming from different combinations of dimeric proteins together with the heterogeneity in E box sequence recognized by these dimers makes possible the regulation by bHLH proteins of a wide array of developmental programs. Changes in subcellular localization (cytoplasm/nucleus) and post-translational modifications may also affect bHLH function, thus adding additional layers of complexity to the regulatory circuits governed by these proteins. Moreover, genetic knockout studies have indicated intricate cross-regulation among different bHLH genes further emphasizing the complexity of bHLH gene regulatory hierarchies. Ultimately, these bHLH networks need to be integrated with signaling pathways, other transcription factors or cell cycle genes to instruct cell fate and differentiation programs during development.

3. bHLH players in exocrine development and differentiation

3.1. Ptf1a

Ptf1a/p48 was initially identified as a DNA binding protein of the tissue-specific heterotrimeric

transcription complex called PTF1, which regulates digestive enzyme gene expression in the pancreas [17-19]. Protein alignments showed a high degree of conservation among Ptf1a mammalian genes and with its orthologues in zebrafish and fungi [20]. Later on, it was found that Ptf1a is also crucial for pancreatic organogenesis, serving roles during the specification of pancreatic progenitor cells, during growth and pancreatic morphogenesis of the epithelium and during the specification and differentiation of acinar cells. These functions are largely dependent on the nature of the PTF1 complex, which switches from containing the Supressor of Hairless, Rbpjκ, at early initial stages to containing Rbpjl as development progresses (see below). In addition to the pancreas, Ptf1a plays a central task in the generation of specific neuronal subtypes [21, 22].

3.1.1. Early roles of Ptf1a in pancreatic specification and acinar cell fate

Expression of Ptf1a was first described at E9.5, slightly later than Pdx1, in cells of the foregut destined to become pancreas [23, 24]. Subsequently, using newly developed antibodies, another study showed that it was already present as early as E8.5-E8.75 in both dorsal and ventral pre-pancreatic domains [25]. This early expression pattern of Ptf1a was linked with a signature gene expression profile associated with the multipotent condition [26]. Later on, at E12, a multipotent progenitor population located specifically at the branching tips of the growing pancreatic tree and that gives rise to the three pancreatic cell lineages (acinar, endocrine and ductal cells) is marked by a combination of the Ptf1a+, Pdx1+, $cMyc^{high}$ transcription factors and Carboxypeptidase A1 (Cpa1)+ [27]. As development progresses, Ptf1a expression decreases in the endocrine and ductal progenitors and becomes restricted to acinar cells, the only cell type that maintains its expression in adult pancreas [23, 28, 29].

The role of Ptf1a in early pancreatic development was first suspected though the analysis of several animal models. By lineage tracing analysis using the endogenous Ptf1a promoter to regulate the expression of the recombinase Cre, it was found that practically all acinar cells, >90% ductal and β-cells and >70% of α-cells are derived from Ptf1a-expressing progenitor cells [23]. In *Ptf1a* null animals, the dorsal rudiment develops into a rudimentary duct without acinar and islet cells while the ventral bud does not form, being the progenitors redirected to an intestinal fate and populating the duodenal epithelium [23]. Few immature endocrine cells are also found in the knockout mice and misallocated in the spleen [29]. Interestingly, the developmental phenotypes of *Pdx1* null and *Pdx1*; *Ptf1a* double null mice were found indistinguishable (with an early pancreatic bud formed [30]), suggesting that each factor may regulate the expression of the other at early stages. In agreement, Ptf1a was found to bind and activate region III of the *Pdx1* promoter, which confers Pdx1 expression in the early pancreas, although *in vivo* studies showed that it was not absolutely required for its induction but rather for its maintenance [30-32]. Conversely, reduced expression of Ptf1a was found at E10 in early dorsal pancreatic progenitors of *Pdx1* null mice [33].

In line with the studies in mice, knockdown studies in zebrafish and *Xenopus* determined the requirement for Ptf1a in the development of both endocrine and exocrine cell lineages [34, 35], favouring the notion of a global role of this transcription factor in pancreatic specification. Similarly, transgenic studies in *Xenopus* demonstrated a Ptf1a-mediated specification of the Pdx1-positive endodermal cells to a pancreatic fate, these cells converting both into exocrine and endocrine tissue [34, 36]. In addition, also in *Xenopus*, combined ectopic expression of Pdx1 and Ptf1a was shown to expand the pancreatic domain into the posterior endoderm [34, 36], suggesting the conversion of non-pancreatic precursors into pancreatic progenitor cells co-expressing Pdx1 and Ptf1a in this portion of the endoderm. Likewise, misexpression of Ptf1a in discrete regions of the stomach, duodenum and bile duct of *Hes1* null mice, leads to the reprogramming of $Ptf1a^{+}$ cells to multipotent pancreatic progenitor status and ectopic pancreas formation [43].

This critical role of Ptf1a in early pancreatic development was further highlighted with the finding that *PTF1A* mutations in humans associated to pancreatic and cerebellar agenesis [37, 38]. These mutations generate truncated

forms of PTF1A lacking the C-terminal 32 amino acids, suggesting a crucial function of the Ptf1a C-terminal region in mediating pancreatic and neuronal development.

In the pancreas, the early events mediated by Ptf1a are triggered by the embryonic PTF1 complex composed by three DNA-binding proteins: Ptf1a itself, an ubiquitous E protein and Rbpjκ, which is the best known mediator of the canonical Notch signaling pathway [39]. At the onset of the secondary transition, Rbpjκ is replaced by the Rbpjl form that is independent of Notch (see next section). The interaction of Ptf1a with the E protein allows the bHLH heterodimer to bind to DNA but, unlike other bHLH factors, this dimer requires Rbpjκ/l to bind specific sites and for transcriptional activity. The Ptf1a binding sites are bipartite with an E-box (CACCTG preferred) and a TC-box (TTTCCCACG) spaced one or two helical turns apart, center to center [40]. The Ptf1a/E protein heterodimer binds to the E-box and Rbpjκ/l binds to the TC-box [40, 41]. Binding of the PTF1 complex to DNA requires both boxes, and the spacing between these elements is critical for Ptf1a binding [42], which imposes a restrictive specificity and the exclusion of the PTF1 complex from simple E-boxes.

The binding of Rbpjκ is through two short conserved tryptophan-containing motifs near the C terminus of Ptf1a, which are similar to the motif of the Notch intracellular domain (NotchIC) that interacts with Rbpjκ. Indeed, binding of Ptf1a and the NotchIC to Rbpjκ is mutually exclusive. *In vivo*, a single amino acid change in one of these motifs that abrogates its ability to bind Rbpjκ but not to Rbpjl is sufficient to truncate pancreatic development at an immature stage without formation of acini and islets. These findings demonstrate that the Ptf1a-Rbpjκ interaction is required for early stages of pancreatic growth, morphogenesis and lineage fate decisions in a Notch signaling independent way [39]. Indeed, Rbpjκ mutants were phenotypically indistinguishable from the *Ptf1a*-null mice, suggesting that the main functions of Ptf1a at early stages are mediated by binding to Rbpjκ.

Not only the induction of Ptf1a expression is determinant for pancreas formation but also its expression levels are critical to correctly instruct exocrine development. *Ptf1a*-hypomorphic mutant mice showed similar defects as the null allele including pancreatic hypoplasia and misspecification of a substantial proportion of pancreatic progenitors to the common bile duct and duodenal cells [164]. However, in these mutants, cell growth, branching morphogenesis and subsequent exocrine cyto-differentiation were delayed. Remarkably, they also displayed an important decrease in the total number of β-cells, highlighting that Ptf1a dosage can affect endocrine differentiation. In this same line, in zebrafish, Ptf1a levels modulate the choice between endocrine and exocrine fates, with high Ptf1a amounts repressing the commitment to the endocrine lineage and concomitantly promoting the exocrine one [44]. Surprisingly, at a certain threshold of Ptf1a activity in milder hypomorphs endocrine cells develop to a greater extent [44], suggesting a higher production of early pancreatic progenitors leading to more endocrine cells, although this assumption needs to be proven. Yet, the alternative possibility that certain Ptf1a levels can induce endocrine specification is not supported by *in vivo* studies [30].

On the other hand, the transcription factor Nkx6.1 previously recognized as an important player in α and β-cell development, has been recently reported to exert an effect opposite to Ptf1a by controlling the endocrine/acinar cell lineage choice in a critical time window that precedes the final lineage commitment of multipotent progenitors [45]. In this case, high levels of Nkx6.1 and Nkx6.2 antagonize Ptf1a action on acinar cell fate and instruct endocrine commitment from pancreatic progenitors. This effect is mediated in part by direct Nkx6.1 binding to and repression of the activity of the Ptf1a autoregulatory enhancer, which is required for the superinduction of Ptf1a at the onset of acinar cell development and for the maintenance of its expression in adulthood [46]. Consequently, sustained overexpression of Ptf1a throughout the epithelium in all pancreatic progenitors is sufficient to repress Nkx6.1 and to block endocrine differentiation [45].

The development of conditional knockout models for Ptf1a will be crucial to further define the distinct roles of Ptf1a at different developmental points. Another crucial aspect that needs further

investigation is the identity of the downstream mediators modulated by Ptf1a at these different stages. In fact, whereas some direct gene targets of Ptf1a have been recognized in neural development including surface molecules (Neph3, Nephrin) [47] and transcription factors (Ngn2) [48], very few have been identified in the pancreas except for digestive enzymes and other acinar-characteristic genes (see above and [49]). One of the identified pancreatic targets, Expdf, was found to be highly expressed in exocrine progenitors and differentiated cells in zebrafish [50]. *Expdf* knockdown studies demonstrated a loss and significant reduction of exocrine cells via lineage-specific cell cycle arrest, indicating that Expdf acts as a key exocrine cell determinant gene. Conversely, overexpression of *Expdf* led to exocrine pancreas overgrowth and a severe decrease in the endocrine mass, supporting the notion that this gene could mediate cell fate choice [50].

3.1.2. Ptf1a regulates acinar cell differentiation and maturation

Acinar cells are highly efficient factories designed for the production of digestive enzymes and other secretory proteins, their storage and their regulated exocytosis. To date, Ptf1a is thought to be the major determinant for terminal differentiation of these cells. As Ptf1a starts to accumulate in the nucleus of acinar cells after E13.5, the embryonic Rbpjκ form of the PTF1 complex is gradually swapped for the Rbpjl form, which is the exclusive complex that binds the promoters of acinar-specific genes in the adult [39, 40]. Rbpjl is itself a direct target of the embryonic PTF1-Jκ complex at early stages and the establishment of the new PTF1-L complex directs later on an auto-activation loop that allows the continued production of both Ptf1a and Rbpjl, thus stabilizing the levels of this complex and the maintenance of the acinar differentiated phenotype [39]. In line with this, pioneer experiments using an antisense RNA-mediated reduction of Ptf1a in an acinar cell line showed inhibition of the exocrine differentiation program [18]. Furthermore, functional binding sites for the PTF1 complex are present in the promoter regions of all of the acinar digestive enzyme genes examined [17, 51]. More recently, *in vivo* disruption of *Rbpjl* has demonstrated that this form in the PTF1 complex drives terminal acinar differentiation not only by maximizing secretory protein synthesis but also by enhancing mitochondrial metabolism and cytoplasmic creatine phosphate energy stores and by contributing to the packaging and secretory machinery, thus ensuring maintenance of acinar-cell homeostasis [49]. Interestingly, a comprehensive comparison of the expression profiles between these mutants and wild type mice defined a highly selective set of potential target genes (acinar secretory proteins, components of secretory apparatus etc) in which transcriptional regulators were largely excluded [49].Therefore, the main function of the PTF1 complex containing Rbpjl appears to be the increase in acinar cell function after the secondary transition.

Despite the crucial role of the PTF1 complex in orchestrating key steps of pancreatic development, little is known with regard to the molecular mechanisms that regulate its activity. Ptf1a shares a high homology with other members of the bHLH family in its DNA-binding domain, yet its amino- (N) and carboxy (C)-domains share no similarity with known proteins. The N-terminal region of Ptf1a contains two activation domains which recruit transcriptional co-activators such as the histone acetyltransferase p/CAF [52]. p/CAF acetylates Lys^{200} of Ptf1a and this step is crucial for the functional cooperation between Ptf1a and Rbpjl. Because this effect is selective for PTF1-L and is not observed for PTF1-Jκ [52], it is thought that p/CAF may be an important regulator of acinar differentiation at the onset of the secondary transition. Further, p/CAF promotes the nuclear accumulation of Ptf1a through a mechanism which is not fully dependent on its acetyltransferase activity but likely through its interaction with Ptf1a [52].

In addition to E proteins, Ptf1a can interact with bHLH factors from other phylogenetic groups. For instance, Ptf1a physically interacts with the bHLH repressor and Notch effector Hes1 (Group E), and this interaction inhibits PTF1-dependent transcriptional activity [53]. Interestingly, this would provide a potential mechanism by which Notch would prevent PTF1 from binding to target DNA without affecting Ptf1a expression. In this way, Notch would modulate PTF1 activity prior

to the secondary transition, coincidently with Hes1 and Ptf1a expression in dedicated exocrine progenitors [54]. After the onset of acinar differentiation around E14.5, Hes1 levels are silenced in acinar cells in parallel with a higher increase in PTF1 activity. The molecular mechanisms underlying the Hes1-dependent inhibition of PTF1 remain to be fully defined.

Ptf1a activity may also be regulated by the Inhibitor of Differentiation (ID) factors (Group D of bHLH proteins), which are characterized by the absence of the basic DNA-binding domain. ID factors are known to dimerize with group A bHLH factors and, because the resulting ID-bHLH hetorodimers are unable to bind DNA, they act as dominant negative regulators of class A proteins [55]. However, it has been proposed that ID factors impede Ptf1a transcriptional activity through retention of the Ptf1a protein in the cytoplasm [56]. The importance of the subcellular distribution as a mechanism to regulate acinar gene expression and exocrine function has been unveiled in various studies. For instance, cytoplasmic localization of Ptf1a has been reported in acinar tumours [57], in human preneoplasic lesions and in mice models overexpressing an oncogenic form of Ras in the pancreas or the CCK2 receptor in acinar cells [56, 58]. In many of these studies, this mislocalization is associated with defects in acinar cell differentiation. In fact, earlier studies showed that Ptf1a lacks a nuclear import signal and needs to interact with E47 to translocate into the cell nucleus [41, 59]. Thus, one possible mechanism for redistribution of the protein to the cytoplasm might be mediated by Id3 through the disruption of the Ptf1a-E47 complex. Indeed, Id3 can be immunoprecipitated from this complex in response to proliferative signals concurrently with cytoplasmic Ptf1a relocalization [56]. Nonetheless, a direct interaction of Ptf1a with ID proteins has not been demonstrated so far.

Finally, another important aspect for understanding the role of Ptf1a in the pancreas is brought up by the discovery of Ptf1a functions that are independent of its transcriptional activity. In this regard, Ptf1a displays an antiproliferative activity by inducing a delay in G1-S progression through the up-regulation of $p21^{Cip}$ and $p27^{Kip}$ [60]. Remarkably, this effect is independent of the bHLH domain and the PTF1 activity but requires instead the C-terminal region of Ptf1a, pointing again to the relevance of this protein domain to modulate some Ptf1a-dependent functions. These results provide new insights of a potential role of Ptf1a in the coupling of differentiation and cell cycle exit during exocrine differentiation, a feature that is also shared by the exocrine bHLH factor Mist1.

3.2. Mist1 is involved in terminal differentiation of pancreatic acinar cells

Mist1 is another Class II bHLH factor that is essential for the normal development of serous acinar cells in various secretory glands, including the pancreas and salivary glands [61, 62]. During pancreatic development, Mist expression starts as early as E10.5 and becomes restricted to the acinar cells. In *Mist1* null mice, endocrine cells develop normally and the exocrine pancreas lineage is specified properly but the development of individual cells and the establishment of normal acinar cell polarity is disrupted, leading to defective zymogen granule trafficking, Ca^{2+} signaling and altered regulated exocytosis [63, 64]. In addition, the expression of connexin 32, a component of the gap junction connexon, is compromised which results in the breakdown of intercellular communication and defective secretion [65]. The impairment of exocytosis may be also explained in part by the altered expression of other candidate target genes whose products are involved in secretory vesicle formation and transport such as the Ras-like Rab3d and Rab26 proteins or in maintaining appropriate levels of cytosolic Ca^{2+} such as the secretory pathway Ca^{2+}-ATPase 2 (SPCA2) [62, 66, 67].

Moreover, both deletion of *Mist1* and over-expression of a dominant negative form of Mist1 in acinar cells, lead to intracellular enzyme activation and clear signs of stress such as cytoplasmic vacuolization and nuclear dysplasia [63, 68]. With aging, these alterations are accompanied with the development of lesions reminiscent of chronic pancreatitis and appearance of cells undergoing acinar to ductal metaplasia. Altogether, these results indicate that, in addition to regulating terminal differentiation, Mist1 is also required for the maintenance of acinar cell identity. As a way to regulate these processes, Mist1 also controls

cell growth decisions. For instance, *Mist1*$^{-/-}$ acinar cells exhibited a higher proliferative index as compared to wild type cells, a phenotype that could be rescued by Mist1 overexpression *in vivo* [69]. The antiproliferative activity of Mist1 was mediated through a direct induction of *p21*^{Cip} at the promoter level which, surprisingly, was independent of E boxes but required Sp1 binding sites [69]. This E-box independent transcriptional regulation mediated by Mist1 is not unique to the *p21*^{Cip} gene and is possibly extended to others, including Connexin 32 [70].

Mist1 is also particular in that, unlike most class II bHLH proteins, it preferentially forms homodimer complexes that can bind to a unique DNA target site known as the TA-E-box in the promoter-enhancers of target genes [70]. For transcriptional activation, the N- and C-terminal domains are dispensable but an intact bHLH domain is necessary. This domain was shown to recruit coactivators belonging to the p300/CBP family [70]. Thus, CBP, but not p/CAF, was found to positively influence Mist1 activity in an *in vitro* system, whereas p/CAF selectively modulates Ptf1a activity [52]. These observations reveal the specificity of bHLH factors in recruiting selected co-activators in exocrine cells. Further work is needed to identify common and unique molecular pathways used by distinct bHLH factors to direct exocrine development. In addition, as both Ptf1a and Mist1 are expressed as early as E10 in pancreatic progenitors a black box remains to be investigated regarding a possible cooperation/ network between these two bHLH factors at early stages of exocrine development.

4. Endocrine bHLH factors

4.1. Neurogenin3 is a master switch for endocrine differentiation

Neurogenin3/Atonal homolog 5 (hereafter Neurog3) is a Class II bHLH factor highly related to *Drosophila*'s proneural protein Atonal. Neurog3 exhibits a restricted expression pattern being found in scattered cells in the embryonic pancreas and gastrointestinal tract and in discrete regions of the developing nervous system [71-73]. Neurog3 is a master switch for the endocrine lineage in the pancreas. All pancreatic endocrine cells derive from pancreatic progenitors that transiently express high levels of Neurog3 [74, 75]. Genetic ablation of *Neurog3* in the mouse results in nearly complete loss of pancreatic endocrine cells as well as islet cell precursors, thus demonstrating a non-redundant and essential role for Neurog3 in endocrine cell specification in the pancreas [76, 77]. Conversely, gain-of-function studies demonstrate that Neurog3 is sufficient to drive endocrine differentiation in permissive cellular contexts *in vivo* [78-83] and *in vitro* [84-88]. Therefore, in an analogous way to proneural bHLH factors, Neurog3 can function as a proendocrine gene. In addition to the pancreas, Neurog3 is also required for the formation of endocrine cells in the intestine and stomach [73].

Mutations in the *NEUROG3* gene have been described in patients with congenital malabsorptive diarrhea that associates with a reduced number of enteroendocrine cells [89]. Surprisingly, these patients are not diabetic at birth and, therefore, it is inferred that they are born with enough insulin-producing cells to maintain euglycemia. While this may indicate the existence of a NEUROG3-independent pathway for islet cell genesis in humans, it should be noted that the mutations are hypomorphic and the resulting proteins retain most of their capacity to induce endocrine differentiation when ectopically-expressed in chick embryos [90].

In the mouse, pancreatic expression of Neurog3 is initially detected at around E10.5 concomitant with the appearance of the first glucagon-positive cells. Neurog3 levels decline thereafter until around E12.5-13 when they increase again coinciding with the major differentiation wave of the secondary pancreatic transition to decline yet again after birth [91]. During the secondary transition, Neurog3+ cells appear as scattered cells at or near embryonic ducts, are mainly non-proliferative and express early endocrine differentiation genes but lack expression of mature endocrine markers such as islet hormones [71, 78, 82, 92, 93]. Therefore, Neurog3 expression marks endocrine-lineage committed progenitors in the developing pancreas. Neurog3 dimerizes with E47 and stimulates transcription of target genes [94], some of which are endocrine differentiation transcription factors themselves,

i.e. *NeuroD1*, *Pax4*, *Nkx2.2* or *Insm1* [95-99]. Subsequently, these transcription factors cooperatively carry on the endocrine differentiation program initiated by Neurog3. Nonetheless, genome-wide studies in gain and loss-of-function studies have revealed that Neurog3 may regulate expression of a much broader range of genes involved not only in transcription but also in other cellular functions such as signaling or cellular organization [85, 86, 100, 101]. Future work should assess whether these genes are genuine targets of Neurog3 and establish their relevance in the pro-endocrine actions of this transcription factor.

A recent study has tracked the fate of single Neurog3+ cells using a *Neurog3-Cre*-based genetic system and shown that Neurog3+ are unipotent, that is, each Neurog3+ is the precursor of a single endocrine cell [92]. Importantly, these observations imply that endocrine cell numbers at birth will be grossly similar to the total number of Neurog3+ cells formed during pancreatogenesis and thus reinforce the relevance of properly controlling the Neurog3+ cell population during embryonic development. To this aim, several signaling pathways contribute to regulate the allocation of pancreatic progenitors into the endocrine and exocrine lineages. The first of these pathways to be recognized was Notch signaling (discussed in a separate section below). Besides Notch, transforming growth factor β (TGFβ) signaling has also been shown to modulate the endocrine/exocrine ratio during pancreatic development. Follistatin produced by the surrounding mesenchyme antagonizes the TGF-β family and has a repressive effect on the development of the endocrine pancreas [102]. One of the candidate molecules antagonized by follistatin is activin. Reductions in activin signaling in mice result in hypoplastic islets [103, 104]. Furthermore, the proximal Neurog3 promoter contains an activin responsive element, indicating that activin signaling has a direct impact on the *Neurog3* gene [105]. In contrast to the positive effect of activin, another TGFβ ligand, GDF11 (or BMP11), which is expressed throughout the pancreatic epithelium when Neurog3+ cells first appear, negatively regulates the production of Neurog+ cells [106].

Just as Ptf1a levels regulate exocrine cell fate determination as discussed earlier, the level of Neurog3 expression is also a limiting step for ensuring proper endocrine commitment from individual pancreatic progenitors. Low Neurog3-expressing precursors fail to become endocrine cells and, remarkably, they retain certain degree of plasticity to revert to alternate (exocrine or ductal) cell fates [107]. Since Neurog3 is transiently expressed in a narrow time window in pancreatic progenitors [74, 108], this means that Neurog3 expression must be tightly regulated to guarantee, firstly, its rapid induction to certain threshold levels to initiate differentiation and, secondly, its subsequent repression as the endocrine differentiation program is switched on. Several transcription factors that are expressed in pancreatic progenitors can trans-activate the *Neurog3* gene including Hnf6 [109], Hnf1 [110], Sox9 [111] and Pdx1 [112] and hence may be involved in initial induction of Neurog3 expression. Another transcription factor, Myt1, is both a target and an activator of Neurog3 and thus it forms with Neurog3 a feed-forward expression loop that can enhance the levels of both proteins in endocrine progenitors [77]. On the contrary, virtually nothing is known with regard to the mechanisms that mediate Neurog3 repression in differentiating endocrine cells. Neurog3 can inhibit its own promoter in luciferase reporter assays [110], which would nicely fit in with the transient nature of Neurog3 expression. However, this mechanism would also prevent the high levels of Neurog3 needed for endocrine commitment. Further confounding this notion, Neurog3 has been shown to induce its own expression in cultured duct cells [85]. While these positive regulatory loops have been reported for many other lineage-specific bHLH factors [113-115], it will be important to establish whether this mechanism may regulate Neurog3 expression *in vivo*.

Neurog3 mRNA is expressed in more cells than the Neurog3 protein [91], suggesting that Neurog3 expression may also be regulated post-transcriptionally. In this regard, four microRNAs targeting Neurog3 transcripts have been described in the embryonic and adult pancreas, which may inhibit Neurog3 translation at specific times and

cells [116]. Alternative splicing of the *Neurog3* mRNA has also been proposed as another potential mode of regulation [117]. In the pancreas, Neurog3 would be alternatively spliced such that the majority of the isoforms would not code for protein [117], thus ensuring that only a small subset of cells will make enough Neurog3 to initiate endocrine differentiation. Finally, Neurogenins are quite unstable proteins that can be polyubiquitinated for degradation by the proteasome. Binding with partner E-proteins would promote their stabilization and accumulation when their activity is needed [118]. In sum, several superimposed layers of regulation, transcriptional and post-transcriptional, but also at the epigenetic and activity levels, are likely to control Neurog3 expression during pancreatic development to guarantee adequate endocrine mass and function in the adult.

One important and yet poorly-understood aspect of Neurog3 function is how the different endocrine sublineages (α, β, δ and PP) are specified in Neurog3+ cells. On one hand, the existence of combinatorial and counter-regulatory loops between specific sets of transcription factors downstream of Neurog3 is known to direct differentiation towards particular endocrine fates. For instance, α/PP versus β/δ cell fates are promoted through an inhibitory cross-regulatory circuit between the transcription factors Arx and Pax4 [119-121]. On the other hand, there is also evidence that Neurog3+ cells may be pre-biased to particular cell fates prior to Neurog3 expression. For example, Nkx6.1 is needed before Neurog3 expression for acquisition of the β cell fate [122]. In this same line, the finding that Neurog3+ cells are most likely unipotent [92] argues strongly, yet does not prove, that islet cell fate allocation occurs before Neurog3 expression. However, where, when and which factors (autonomous and non-autonomous) instruct these cell fate decisions are at present unknown.

Finally, despite that robust Neurog3 expression is lost as pancreatic endocrine precursors undergo differentiation, low to moderate levels of Neurog3 expression can be found in some differentiated endocrine cells after birth indicating that Neurog3 may serve additional roles postnatally [74, 123, 124]. Accordingly, loss of Neurog3 in islet Pdx1+ cells results in abnormal endocrine function, thus demonstrating the involvement of this factor in the maturation and maintenance of islet cell function [123]. Intriguingly, an important amount of the Neurog3 protein expressed in adult β-cells appears to be located in the cytoplasm as opposed to the mostly-nuclear localization of Neurog3 in embryonic cells [124]. The functional implications of this observation remain to be defined.

4.2. Interplay between Neurog3 and Ptf1a and Notch signaling in the regulation of endocrine versus exocrine cell fate decisions

Several studies in mice indicate that the Notch signaling pathway regulates pancreatic cell fate decisions through control of Neurog3 cell numbers. For instance, null mutants for the Notch ligand delta-like 1 or the intracellular mediator Rbpjκ exhibit accelerated and massive commitment of pancreatic progenitors to the endocrine lineage [82]. Conversely, activation of Notch signaling through ectopic expression of an active NotchIC leads to a dramatic decrease in both mature endocrine and exocrine cells indicating that active Notch signaling may select for a progenitor epithelial population [125]. The *Neurog3* promoter contains binding sites for the bHLH repressor Hes1, which is activated and is a downstream effector of Notch [110]. Therefore, in pancreatic progenitors, high Notch activity is thought to repress *Neurog3* gene expression via Hes1 [82, 110, 126]. In agreement, *Hes1* mutants present severe pancreatic hypoplasia due to early commitment of pancreatic progenitors to become endocrine cells [126].

Stochastic variation in Notch signaling may result in low Hes1 expression in a subset of pancreatic progenitors thus permitting Neurog3 expression. The number of Neurog3+ cells has been proposed to be further restricted though “lateral inhibition” of Notch that prevents neighbouring cells from activating Neurog3 and thus becoming endocrine cells. While such a mechanism is experimentally supported by data from mutations in the Notch pathway [126], “lateral inhibition” has not been conclusively demonstrated in the pancreas as it is described in *Drosophila*. For example, it is not clear if, as predicted by this model, Neurog3+ cells activate Notch ligands and whether adjacent cells express Notch receptors and up-regulate

Hes1 *in vivo*. Quite the reverse, Hes1 or Notch receptors appear to be widely expressed in epithelial cells prior to the secondary transition pancreas. This Notch-mediated *Hes*1 activation throughout the precursor cell population is referred to as "suppressive maintenance" [127] and is defined by the suppression of cell differentiation (Hes1 concomitantly represses Neurog3 gene expression [110] and inhibits Ptf1a activity [54] and the maintenance of the progenitor state (Figure 1).

Further complicating this model is the fact that the key Notch downstream transcription factor Rbpjκ, which mediates signaling through all Notch receptors, is at the same time part of the exocrine-differentiation complex PTF1. Remarkably, it has been postulated that this non-canonical aspect of Notch is key in regulating endocrine/exocrine cell fate decisions. Once Neurog3 is induced, these cells appear to require high Notch activity again to actively inhibit exocrine differentiation through changes in the availability of Rbpjκ [128]. The receptor Notch2 is thought to act by sequestering Rbpjκ from Ptf1a, thus ensuring selection of the endocrine fate by Neurog3+ progenitors. Indeed, *Ptf1a* mRNA and the corresponding protein have been detected in these progenitors [26, 128]. On the contrary, for exocrine differentiation to occur, Notch activity needs to be repressed as Hes1 can bind and inhibit the transcriptional activity of

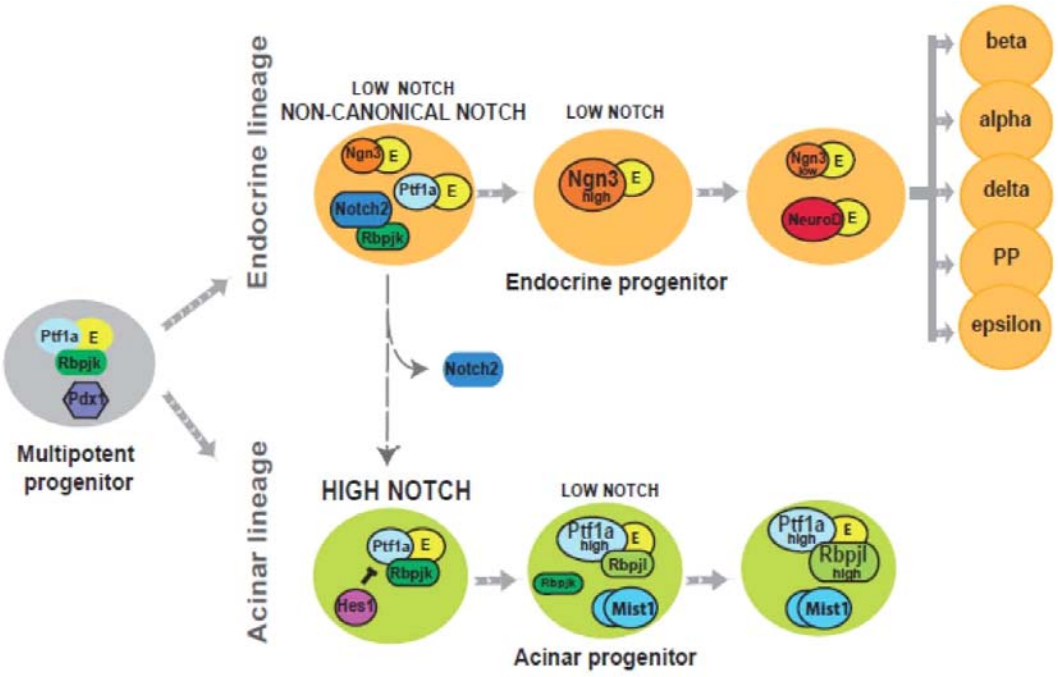

Figure 1. Key bHLH proteins at the crossroads between endocrine and acinar lineage decisions during pancreatic development. The embryonic trimeric complex PTF1-Jk which contains Ptf1a, an ubiquitous E protein and Rbpjk, is expressed in multipotent pancreatic progenitors with other non-bHLH transcription factors such as Pdx1. Low Notch signaling permits expression of the proendocrine bHLH factor Neurog3 in a subset of pancreatic progenitors. A non-canonical Notch pathway mediated by the Notch receptor Notch2 further ensures endocrine cell commitment from the Neurog3+ precursor: Notch2 activity sequestrates Rbpjk from PTF-Jk, thus repressing activity of this complex which, at this stage, would promote acinar cell fate commitment. Neurog3 expression transiently rises in endocrine progenitors switching on expression of NeuroD, which then carries on differentiation of endocrine cells. On the other hand, high Notch activity in pancreatic progenitors translates into expression of the bHLH repressor Hes1 in dedicated acinar progenitors. Hes1 inhibits the activity of the PTF1 complex thus preventing premature acinar differentiation. The nature of the PTF1 complex changes at the secondary transition and Rbpjk is gradually substituted for Rbpjl. This new PTF1-L complex regulates acinar cell function. Mist1 contributes to further differentiation of these cells.

Ptf1a. Overall, these findings indicate that Neurog3+ cells are bipotential (endocrine/exocrine) and that non-canonical Notch can resolve the dominance between the bHLH proteins Neurog3 and Ptf1a in determining cell fate commitment from pancreatic progenitors.

4.3. NeuroD1 is important for survival and functional competency of endocrine cells

NeuroD1 is another Class II bHLH factor that, like Neurog3, is highly related to *Drosophila*'s proneural protein Atonal. NeuroD1 is expressed in the nervous system, the pancreas and the gastro-intestinal tract. Within the pancreas and contrarily to Neurog3, NeuroD1 is highly expressed in differentiated β-cells where it functions as a key regulator of genes encoding mature β-cell markers such as the *insulin, sulfonylurea receptor (SUR1)* or *glucokinase* genes among others [129-131]. Indeed, NeuroD1 is important for the activation but also for regulation of these genes in response to physiological stimuli such as glucose [129, 132]. Deletion of *NeuroD1* in β-cells results in metabolically immature cells zand severe glucose intolerance [133]. In humans, heterozygous mutations in the *NEUROD1* gene have been linked to an autosomal dominant form of early-onset type 2 diabetes called maturity-onset-diabetes of the young or MODY [134]. Moreover, homozygous loss-of-function mutations in the *NEUROD1* have been recently linked to permanent neonatal diabetes [135]. The reported associations between the *NEUROD1* locus and the etiology of human diabetes highlight the importance of this transcription factor both in the function and in the development of islet cells.

In addition to maintaining normal β-cell function, NeuroD1 plays important roles in terminal differentiation and survival of endocrine cells during embryogenesis. Homozygous deletion of the *NeuroD1* gene in mice results in premature death within 5 days after birth due to severe hyperglycemia [136]. Close examination of the pancreases from *NeuroD1* knockout animals revealed that islets failed to form and that β-cells were severely decreased (74%) while the other endocrine subtypes were less affected (39% for α-cells and 18% for δ-cells). These defects became apparent after E14.5 but prior to E17.5, thus coinciding with the secondary transition. Loss of endocrine cells was accompanied by a substantial increase in apoptosis, thus suggesting that absence of *NeuroD1* affects survival of endocrine cells rather than endocrine specification [136]. Interestingly, the dramatic pancreatic phenotype of *NeuroD1* null animals was partially rescued through genetic backcrossing [137]. In the rescued animals, β-cell but not α-cell mass was increased and this increase most likely involved augmented β-cell replication from the existing islets. Despite that newly generated β-cells remained poorly differentiated these findings underscore the non-essential role of NeuroD1 for β-cell differentiation in this model.

Neurog3 induces endogenous *NeuroD1* gene expression in pancreatic cells [84, 95, 100] through direct binding to the *NeuroD1* promoter [95]. In contrast, in the converse experiment, NeuroD1 cannot activate Neurog3 expression [85]. Epistatic analysis *in vivo* shows loss of NeuroD1 expression in *Neurog3* null animals, further confirming that NeuroD1 lies downstream of Neurog3 during pancreatic differentiation [76]. Expression of NeuroD precedes that of endocrine post-mitotic markers such as Pax6 or Isl1, pointing to a role of this factor in cell cycle withdrawal [93]. In this model, NeuroD1 would cause endocrine precursors to withdraw from the cell cycle through activation of cyclin-dependent kinase inhibitors such as $p21^{Cip1}$ [138, 139] to later carry on, in post-mitotic cells, the differentiation program initiated by Neurog3. Since Neurog3 is down-regulated in differentiated islet cells, high NeuroD1 expression would then be maintained through alternative mechanisms including auto-regulation [85, 140] or the coordinate action of other transcription factors present in adult β-cells [141]. However, the less severe phenotype of *NeuroD1* knockout animals as compared to *Neurog3* knockouts suggests that this linear transcriptional cascade (Neurog3→NeuroD1) must contain additional branches that can partly compensate for the absence of NeuroD1 in the *NeuroD1* null animals. It is conceivable that other *NeuroD* paralogs that are also activated by Neurog3 exert redundant roles during endocrine differentiation [100].

NeuroD1 shares with Neurog3 the ability to function as a pro-endocrine gene when ectopically

expressed in receptive cellular contexts [78, 85, 142]. Genome-wide expression analysis in cultured cells has revealed that both factors can regulate largely overlapping sets of genes, thus providing the molecular basis for their functional equivalence in these gain-of-functions approaches [100]. Nonetheless, this same study demonstrated that each factor could specifically activate a small subset of genes as well as differ in the timing and strength of the activation of a different group of common target loci. It is thus possible that a combination of intrinsic differences and cellular context-dependent effects dictates how these bHLH factors regulate endocrine differentiation. These are indeed important points to take into consideration when devising gene therapy approaches using these factors to create surrogate β-cells.

The transcriptional activity of NeuroD1 can be controlled by phosphorylation at different sites by the action of different kinases including GSK3β, ERK or CaMKII [143]. In some instances, phosphorylation of the same residue by two different kinases in two cellular contexts may lead to opposite effects [132, 144]. Apart from E proteins, NeuroD1 has been shown to interact with the coactivator p300, Cyclin D or ID factors [143]. Most of these protein-protein interactions have been identified in neuronal cell models or in mature endocrine cells, thus it remains to be determined whether these proteins may also cooperate in modulating NeuroD1 activity during differentiation of endocrine precursor cells in the embryonic pancreas.

5. bHLHs as regulators of pancreatic differentiation in ES differentiation models and *in vivo* : Potential switchers of cellular plasticity

Embryonic stem cells (ESC) are derived from the inner mass of the early embryo and can be expanded indefinitely *in vitro* while conserving their pluripotency. Furthermore, upon appropriate stimuli and culture conditions, they can give rise to all the cells of the body. Nearly a decade ago, it was first demonstrated that insulin-expressing cells could be generated from mouse [145, 146] and human [147] ES cells. However, the state of differentiation achieved in these initial approaches was limited, which led several groups to search for more effective approaches. The current most successful differentiation protocols have been based on the recapitulation of the sequence of signaling events that participate in pancreatic development during embryogenesis. This strategy has opened an efficient avenue to conduct definitive endoderm formation, pancreatic specification and differentiation, reminiscent of what occurs *in vivo* [148-152].

In addition to the manipulation of signalling pathways through the addition of specific activating or inhibitory molecules to ES cell cultures, the generation of engineered vectors that can efficiently deliver specific key gene products into ES cells has provided other means to try to increase the efficiency of the existing differentiation protocols. Some of the obvious candidates for this type of gene therapy schemes have been the lineage-specific bHLH regulators. For instance, the development of doxycycline-inducible Neurog3 expressing mouse ES cell lines allowed the generation of insulin expressing cells that were responsive to glucose although at a low frequency [86, 88]. In these cells, Neurog3 expression resulted in the up-regulation of *NeuroD1*, *Pax4*, *Nkx2.2* and *Insm1*, which are normally activated downstream of Neurog3 during β-cell development. However, using a knock-in ES cell line that has the EGFP coding sequence inserted into one allele of the Neurog3 locus, it was demonstrated that only 0.1%-0.6% of Ngn3-EGFP$^+$ cells gave rise to insulin-expressing colonies [153]. One possible explanation for these modest results is that the downstream effectors needed for hormone gene expression are not sufficiently induced in Neurog3-expressing ES cells. To solve this problem, it has been postulated that combinatorial expression of different developmental factors may lead to more efficient insulin gene expression induction. In line with this notion, stable expression of Pdx1 combined with the forced expression of NeuroD1 by adenoviral transduction has been shown to help to sustain insulin expression in differentiating ES cells [154]. Similarly, ectopic lentiviral-mediated expression of NeuroD1 favoured endocrine differentiation and insulin production in comparison to other ectopically expressed transcription factors, including Neurog3 [155].

On the other hand, transient expression of exocrine bHLH factors has been assayed to coax ES cells

to activate an acinar differentiation program. Thus, cells co-expressing Ptf1a and Mist1 by adenoviral gene transduction were shown to acquire features of acinar progenitor cells and exhibited a functional activity in response to specific secretagogues [156]. Altogether, these studies provide the proof of concept that key bHLH regulators may be used to generate functional pancreatic cells from ES cells *in vitro*.

Furthermore, the ability of bHLH factors to function at the crossroads of cell fate decisions has also been exploited to reprogram differentiated cells from different lineages into insulin-producing cells. For example, *in vivo* transduction of the combination of Neurog3, Pdx1, and MafA into adult acinar cells results in the generation of insulin-expressing cells that are functionally competent. Importantly, these newly-generated cells improve streptozotocin-induced diabetes without cell replication or transitioning through a dedifferentiated state [81]. Also, expression of Neurog3 in adult liver results in the trans-determination of hepatic progenitors, most likely oval cells, into islet cells that can induce long-term diabetes reversal in mice [79]. Similarly, expression of NeuroD1 in combination with the growth factor betacellulin results in the generation of insulin-positive cells in the liver [79, 142].

6. FUTURE DIRECTIONS

Although exciting new progress has been achieved in recent years with regard to the function of bHLH factors in pancreatic development, there is still a rather patchy understanding on the molecular mechanisms involved in the control of their expression and functional specificity. Future studies are expected to unveil the mechanisms by which these factors cooperate with non-bHLH proteins to modulate gene expression profiles during pancreatic development (a paradigm of such type of regulation is represented by the PTF1 complex), and how these transcriptional networks integrate with intercellular signaling pathways to promote proper pancreatic cell lineage allocation and function. In addition, here we have discussed a reduced set of bHLH proteins which are known to play crucial roles in cell fate decisions (Ptf1a Neurog3, Hes1) and cellular differentiation (Mist1 and NeuroD1). Nonetheless, additional bHLH factors are expressed in the developing and mature pancreas (Table 1) whose function ought to be explored in detail yet. In the future, this information will be fundamental in orchestrating the genesis of pancreatic cells *in vitro* and *in vivo* for therapeutic cell replacement strategies in regenerative medicine.

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