

# Pancreatic Development

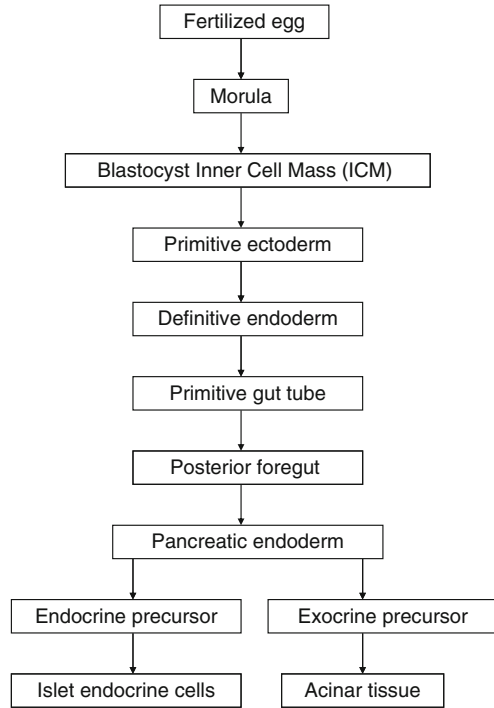
**Abstract** Pancreatic development is arguably the best-studied example of organogenesis. Both gain-of-function and loss-of-function studies conducted in mice over the last decade have contributed to our understanding of a basic “genetic roadmap” of pancreatic – and particularly endocrine – development. Here we review this knowledge from the onset of the pancreatic program in the foregut epithelium (with the expression of the critical regulators Pdx1 and Ptf1a) to the specification of ductal, exocrine, and endocrine cell types. A special emphasis is placed on the development of endocrine beta cells, which are destroyed in type I diabetes and therefore constitute the endpoint of many stem cell differentiation protocols.

**Keywords** Foregut epithelium • Pancreatic buds • Pdx1 • Ptf1a • Ngn3 • Secondary transition

## 1 Introduction

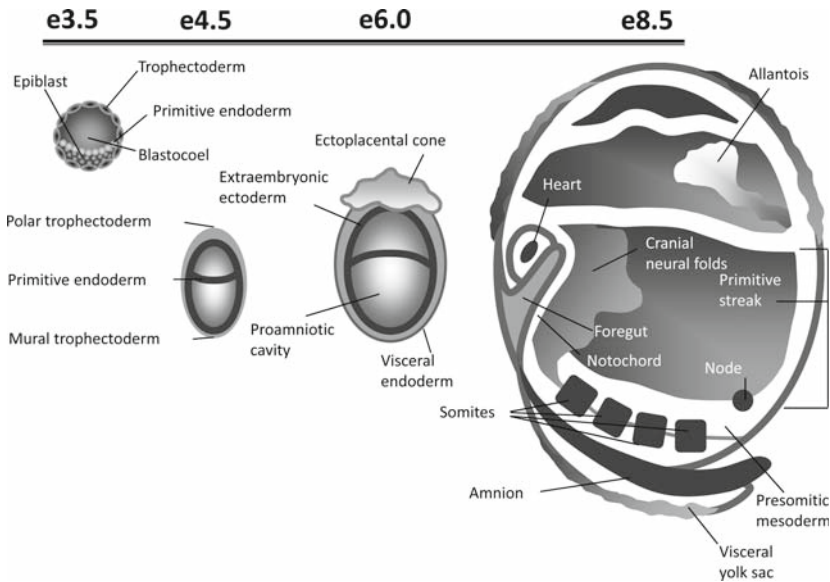
For obvious reasons, most of our knowledge on pancreatic development comes from the mouse model. Indeed, despite a few minor differences that will be pointed out throughout this chapter, the most important molecular players are highly conserved between mouse and human. Research conducted over the last decade has outlined a basic “roadmap” of the major molecular events that shape mouse beta cell development from the early blastocyst.<sup>47–49</sup> Critical developmental milestones are: (1) generation of definitive endoderm/gut epithelium; (2) pancreatic differentiation; (3) endocrine specification; and (4) beta cell differentiation. We will now describe what is known about this process (Fig. 13), emphasizing the role of the genes that act as master regulators of the transition between each stage and the next.

**Fig. 13** Intermediate developmental stages between fertilization and the formation of the pancreas (see main text for details)



## 2 Generation of Endoderm/Gut Epithelium

*Primitive endoderm* and *epiblast* are, respectively, the outer and inner layers of the inner cell mass (ICM) immediately before gastrulation (Fig. 14). The primitive endoderm will become part of the yolk sac, without contribution to the embryo proper. In contrast, the *definitive endoderm* is formed during gastrulation when epiblast cells leave the ICM through the primitive streak. There is an intermediate stage in definitive endoderm formation, called *mesendoderm*. Although visceral and definitive endoderm are similar, mesendoderm-specific genes such as *goosecoid* (*Gsc*) and *Brachyury* (*Bry*) do not appear during visceral endoderm differentiation,<sup>50–53</sup> and therefore can be used to identify true definitive endoderm.<sup>54</sup> The *anterior* part of the definitive endoderm will evolve into the foregut, from which pancreas, liver, and lungs will eventually bud out. The *posterior* definitive endoderm, on the other hand, becomes the midgut and hindgut, which will differentiate into large and small intestine. Nodal, a member of the transforming growth factor (TGF)- $\beta$  family, is the main signaling molecule responsible for the initial patterning of the primitive gut epithelium. The gradients of Nodal are finely tuned, as shown in experiments where significant reductions in its expression resulted in preferential formation of mesoderm at the expense of endoderm.<sup>55</sup> Additional studies imply that the regulation



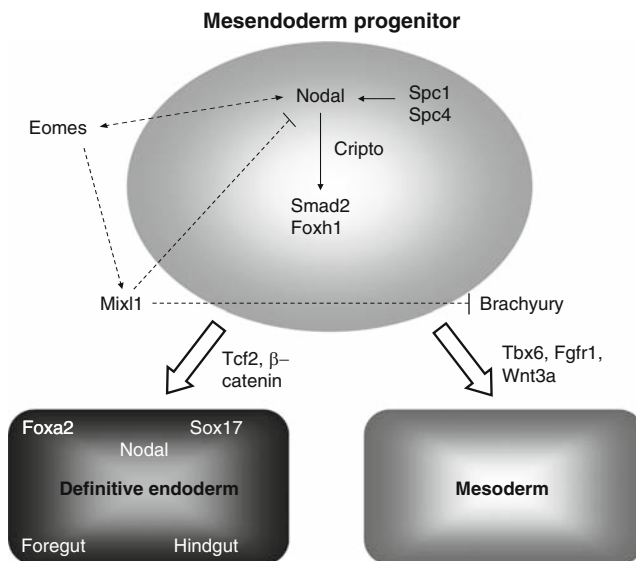
**Fig. 14** Initial stages of embryonic development after the formation of the blastocyst (e3.5). Adapted from Hogan et al. *Manipulating the Mouse Embryo-A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, 1994

of Nodal gradients is a dynamic process that involves not only the secretion of the protein, but also the activity of specific repressors such as *Drap1*.<sup>56–58</sup>

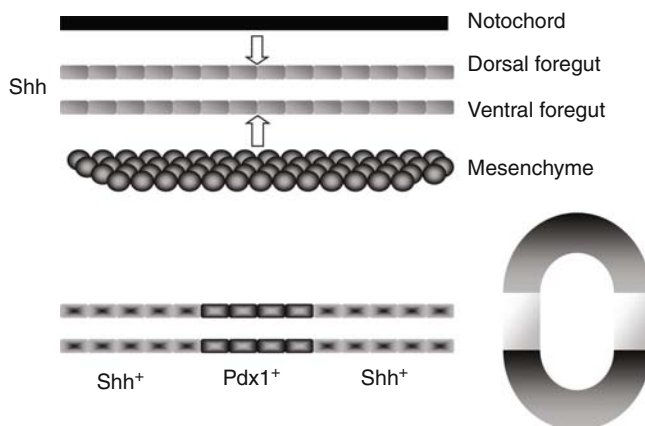
Many genes have been associated with the formation of true endoderm, including *Foxa1–2*, *Mixl1*, *Eomes*, *GATA4–6*, and several members of the *Sox* family (reviewed in<sup>53,59</sup>). Although there is a potential redundancy with other *Sox* genes, *Sox17* is essential for embryonic cells to become endoderm in mouse.<sup>60</sup> At least in *Xenopus*, *Sox17* also appears to be sufficient to induce endodermal fates.<sup>61</sup> A theoretical model for the cross-talk between these genes (adapted from<sup>53</sup>) is presented in Fig. 15.

### 3 Pancreatic Differentiation

The interaction between the gut endoderm and the surrounding mesoderm is primarily mediated by *Sonic Hedgehog* (*Shh*) signaling.<sup>59,62,63</sup> *Shh* is highly expressed throughout the gut epithelium, but is down-regulated in a *Ptfla*(p48)/*Pdx1*<sup>+</sup> region that will later become the pancreas at e8. Both *Shh* repression and activation of *Ptfla* and *Pdx1* are defining events of pancreatic specification (Fig. 16). Chemical inhibition of *Shh* by the steroid alkaloid cyclopamine enhances pancreatic differentiation, as *Pdx1* expression is no longer restricted throughout the posterior foregut.<sup>64</sup> Conversely, ectopic expression of *Shh* under the control of the *Pdx1* promoter induces intestinal fates (including smooth muscle and interstitial cells of Cajal) instead of pancreatic fates (Fig. 17).<sup>65</sup>

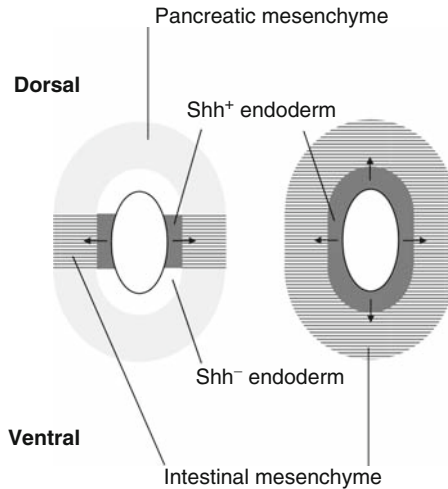


**Fig. 15** A theoretical model for the molecular interplay leading to the development of definitive endoderm from mesendoderm. Nodal signaling is essential for the specification of mesendodermal progenitors. Definitive endoderm formation requires the concerted activity of Mixl1,  $\beta$ -catenin, and Tcf2 (HNF-1 $\beta$ ). Mesoderm specification, in contrast, is influenced by Fgfr1, Tbx6, Brachyury, and Wnt3a. Different requirements for Foxa2, Sox17, and Nodal are found throughout the gut endoderm. Potential interactions between Eomes, Nodal, T, and Mixl1 are indicated with a *dotted line* (Adapted from Tam et al.<sup>53</sup>)



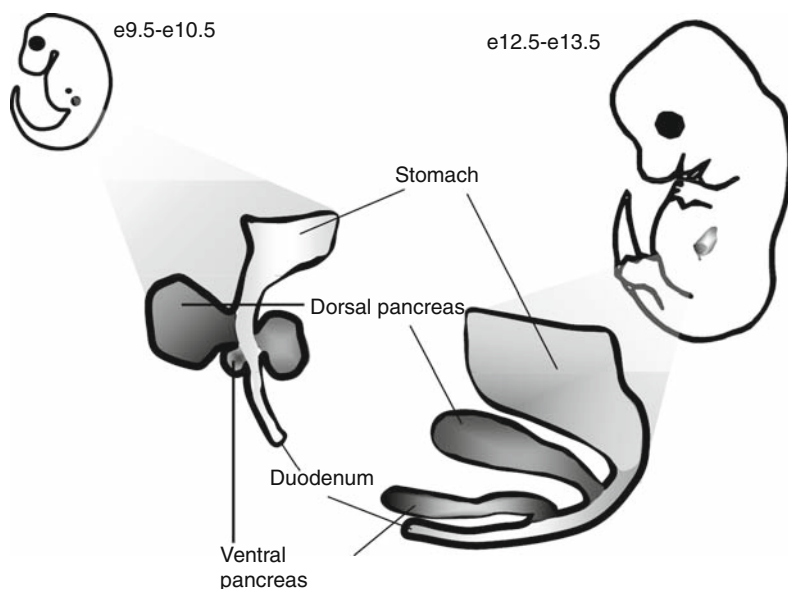
**Fig. 16** The pancreas is specified from a region of the embryonic foregut where Shh expression has been excluded due to active signaling from the notochord and surrounding mesenchyme. This region will express the pancreatic and duodenal homeobox 1 (Pdx1), as well as Ptf1 $\alpha$ (p48). A transversal cut of the foregut at this point would give a pattern similar to that depicted to the *right*, *bottom*: *top* and *bottom* Pdx1<sup>+</sup>/Shh<sup>-</sup> areas, which will form the dorsal and the ventral pancreatic buds upon evagination, and a *middle*, Pdx1<sup>-</sup>/Shh<sup>+</sup> region with pro-intestinal cells

**Fig. 17** During regular pancreatic development, an area is defined in the posterior foregut in which Pdx1 expression occurs at the expense of Shh. This patterns the early pancreas as two Shh-excluded regions that will bud out dorsally and ventrally (*left*). Shh<sup>+</sup> areas, in contrast, will adopt an intestinal fate. Ectopic expression of Shh under the expression of the Pdx1 promoter will extend the latter phenotype in every direction (*right*), preventing appropriate pancreatic specification (Adapted from Apelqvist et al.<sup>65</sup>)



### Pdx1

The pancreatic and duodenal homeobox 1 gene is also known as insulin promoter factor 1 (Ipfl) or islet/duodenum homeobox 1 (IDX1). In the adult mouse, it is selectively expressed in islet beta cells, where it binds to and regulates the insulin promoter.<sup>66</sup> Pdx1 is first expressed in the region of the foregut endoderm that will later become the pancreas and the duodenum (~e8.5 or 10-somite stage, see main text). Up to ~e10, it is uniformly expressed in the dorsal and pancreatic buds. Pdx1 is subsequently down-regulated in the entire organ, to reappear again in arising beta cells from e11 onward.<sup>67</sup> Lack of Pdx1 expression results in selective agenesis of the pancreas, both in knockout mice<sup>68</sup> and in humans with a single-nucleotide mutation.<sup>69</sup> However, it was also shown that the earlier events of pancreatic morphogenesis take place even in the absence of functional Pdx1, which suggests that Pdx1 acts in concert with other factors.<sup>70</sup> In addition to its well-studied role during pancreatic development, expression of Pdx1 is essential for the maintenance of the phenotype in adult beta cells, as evidenced by conditional knockout experiments.<sup>71,72</sup> Heterozygous Pdx1<sup>+/-</sup> mice exhibit an age-dependent worsening of glucose tolerance, reduced glucose-stimulated insulin release, and higher susceptibility to apoptosis.<sup>73</sup> The impaired glucose response of *Psammomys obesus*, a model of type 2 diabetes, was also associated to Pdx1 deficiency.<sup>74</sup> Because of its critical role in orchestrating the early events of pancreatic development, as well as in the acquisition of beta cell properties, Pdx1 has been extensively used as a tool for the differentiation of stem cells.



**Fig. 18** Spatial distribution of dorsal and ventral buds throughout the early stages of development. The two anlagen will eventually fuse in one single organ

In the mouse, the areas defined by expression of *Pdx1* and repression of *Shh* will start to branch out dorsally and ventrally. This initial separation between the *dorsal* and the *ventral pancreas*<sup>75</sup> will persist until later in development, when the two primordia will fuse (Fig. 18). The influence of blood vessels in the overall development of the pancreatic primordia is well established. Thus, while removal of the dorsal aorta in frog embryos abrogated insulin expression, transgenic mice where the posterior foregut was ectopically vascularized developed hyperplastic islets and elevated insulin expression.<sup>76</sup> It is in this context that endothelial cell signaling has been identified as a major morphogenetic agent in pancreatic specification.<sup>77</sup>

### Ptf1 $\alpha$ (p48)

Ptf1 $\alpha$  is the  $\alpha$ -subunit of the pancreas-specific transcription factor 1 (Ptf1), a basic helix-loop-helix (Bhlh) protein first described as a DNA-binding element regulating the expression of  $\alpha$ -amylase 2, elastase 2, and trypsin in the acinar pancreas.<sup>78</sup> p48 knockouts have a complete absence of exocrine pancreatic tissue, suggesting that the gene is a key regulator of acinar tissue development.<sup>79</sup> This role was confirmed by the finding that endocrine cells (relocated to the spleen) were not affected by the abrogation of p48 expression. Later studies, however, found an additional role for p48 in the initiation of

pancreatic development,<sup>49,80</sup> because its expression is observed in the Shh-excluded area of the foregut endoderm around e8.5. The expression patterning at this stage of p48, but not that of Pdx1, is thought to be partially mediated by aortal endothelial signaling.<sup>81</sup> In *Xenopus*, the combination of both Pdx1 and p48 expression was sufficient to induce ectopic pancreatic formation,<sup>82</sup> but the initiation of mouse pancreatic development might require additional genes, such as Hlxb9 (see below).

### **HNF-6 (OC-1)**

Hepatocyte nuclear factor (HNF-6), also termed Onecut (OC)-1, is a member of the OC family of transcription factors, generally characterized by a single cut domain and a homeodomain distinct from that of other homeoproteins, including those of the cut subfamily.<sup>83</sup> During embryonic development, it is highly expressed in the developing central nervous system (CNS) and from e9.5 in the foregut–midgut junction and liver primordium. Pancreatic expression is detectable throughout the epithelium from e10.5 onward, although it seems to be excluded from the islets at e18.<sup>84</sup> Pancreatic growth and endocrine cell differentiation were severely impaired in Hnf-6 knockout mice, with an almost total abrogation of Ngn3 expression.<sup>85</sup> The same authors demonstrated that Ngn3 is indeed a downstream target of Hnf-6. Interestingly, however, islets were able to “regrow” after birth. This is consistent with the view that adult islet regeneration occurs typically through Ngn3-independent processes,<sup>86,87</sup> with only one known experimental exception (in which reactivation of the embryonic developmental program was observed after partial duct ligation; see the chapter “Pancreatic Regeneration”).<sup>88</sup> Notwithstanding this, the newly generated beta cells were defective in Glut-2 and these animals remained diabetic.<sup>85</sup> Additional studies demonstrated not only that Hnf-6 expression precedes that of Pdx1 in the foregut endoderm, but also that (1) the expression of the latter is delayed in Hnf6<sup>-/-</sup> embryos; and (2) Hnf-6 binds to the Pdx1 promoter and stimulates its activity.<sup>89</sup>

### **TCF2 (HNF 1beta)**

Transcription factor 2 (Tcf2), also called hepatocyte nuclear factor (HNF) 1beta is a POU homeobox transcription factor that has been associated with a variant of maturity-onset diabetes of the young (MODY). Other mutations of the gene result in pancreatic atrophy and hypoplasia in humans.<sup>90</sup> The gene is highly expressed from e8.5 in the entire foregut–midgut region and in the

(continued)

### TCF2 (HNF 1beta) (continued)

pancreatic primordia by e9.5, where it colocalizes with Ptf1 $\alpha$  and Pdx1.<sup>91</sup> Although Tcf2<sup>-/-</sup> knockout mice display early embryonic lethality due to defective formation of the visceral endoderm, tetraploid rescue with Tcf2<sup>-/-</sup> embryonic stem (ES) cells results in embryos that can proceed throughout development. In these embryos, the formation of the dorsal, but not the ventral pancreatic bud could be observed. However, this bud was hypoplastic throughout development and disappeared around e13.5.<sup>92</sup> This phenotype is similar to that of Ptf1 $\alpha$  knockouts,<sup>79</sup> albeit more severe; indeed, a Tcf2-binding site was identified in the Ptf1 $\alpha$  promoter, which would be consistent with a role of the former in the regulation of the latter.<sup>92</sup> Pdx1 expression, however, was still detectable at e9.5 in Tcf2<sup>-/-</sup> embryos, suggesting that the latter is not absolutely essential for the initiation of the pancreatic program. Experimental evidence indicates that both Hnf6 and Tcf2 are indispensable for Ngn3 expression.<sup>85,92,93</sup>

While branching and the progression of differentiation are arrested in Pdx1-null embryos (lack of *Pdx1* results in pancreatic agenesis<sup>68,69</sup>), the initial evagination of the pancreatic buds, and even the appearance of scattered insulin- and glucagon-positive cells, does still occur in the absence of *Pdx1*.<sup>70</sup> Recent evidence suggests that the expression of Ptf1 $\alpha$ , previously thought to be exclusively a marker of exocrine progenitor cells, may actually precede that of *Pdx1*.<sup>49,80,81</sup> Additional experimental evidence (e.g., simultaneous ectopic expression of both *Pdx1* and *Ptf1a* induces stable conversion of posterior endoderm into pancreas<sup>82</sup>) seems to confirm that the concerted action of both is necessary for the initiation of the pancreatic program.

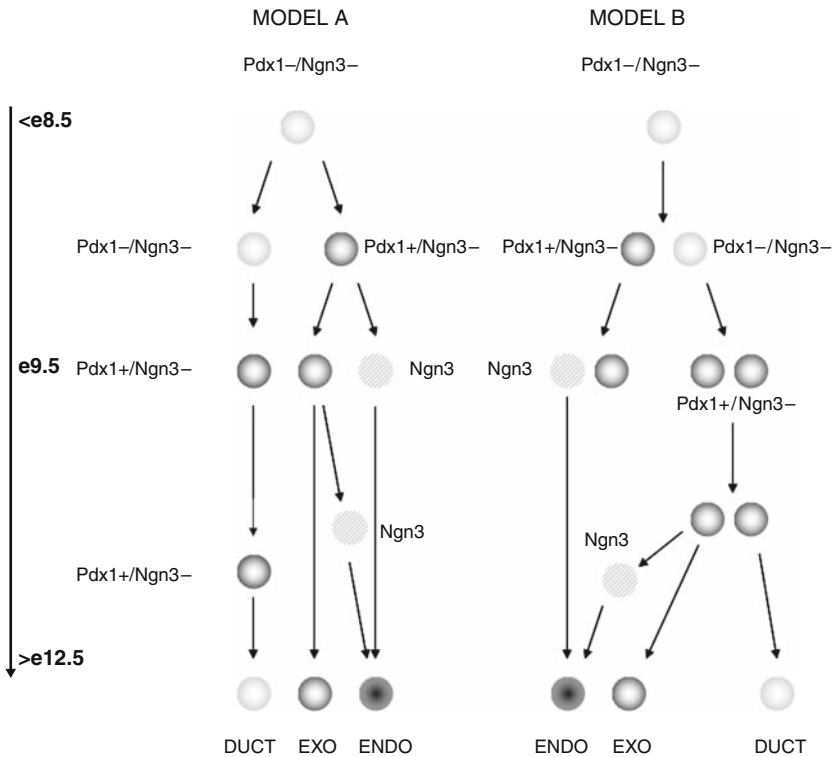
### Hlxb9

Human homeobox gene 9 (*Hlxb9*), also known as its encoded protein, HB9,<sup>94</sup> is expressed in fully differentiated beta cells and from very early on (eight somite stage, ~e8) in the notochord and the ventral and dorsal pancreatic endoderm.<sup>95</sup> *Pdx1*, in contrast, is expressed only in the ventral pancreatic endoderm at this stage of development. The observation that *Hlxb9* expression precedes that of *Pdx1* (at least in the dorsal anlagen) suggests an active role of this gene in shaping the early events of pancreatic specification. *Hlxb9* knockouts show a selective agenesis of the dorsal pancreas.<sup>95,96</sup> Although the ventral lobe still develops, its islets are smaller and beta cells within them less numerous, with evident reduction in beta cell-specific factors such as Nkx6.1 and Glut2.<sup>96</sup> Ectopic expression of *Hlxb9* beyond e8 in *Pdx1*-*Hlxb9* transgenic mice led to severe impairment in pancreatic development, with decreased endocrine and exocrine differentiation and a partial adoption of intestinal fates.<sup>97</sup>

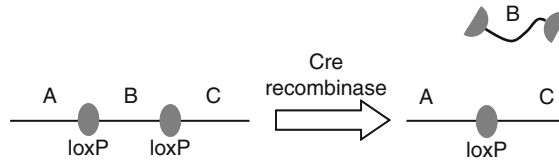


## 4 Ductal and Exocrine Specification

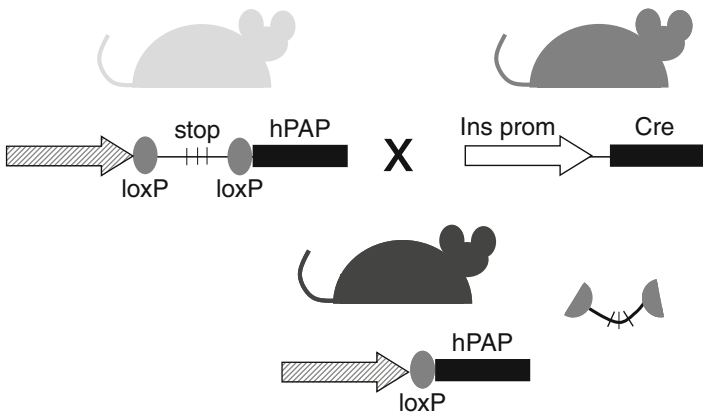
CRE-ER<sup>TM</sup> technology (see box below and Figs. 19 and 20)<sup>98,99</sup> has allowed the development of invaluable lineage-tracing experiments to ascertain the origin of each cell type within the pancreas. Using this technology, Gu et al.<sup>100,101</sup> noticed that, when marking Pdx1<sup>+</sup> cells at any point during the initial stages of pancreatic development (e9.5–e11.5), the label could be detected in cells of every pancreatic tissue (endocrine, exocrine, and ductal). This served as additional confirmation of earlier knockout experiments showing that the entire pancreas arises from Pdx1<sup>+</sup> progenitor cells.<sup>68</sup> However, when tamoxifen-induced labeling of Pdx1<sup>+</sup> cells was performed before e9.5 or after e11.5, the marker could only be seen in acinar and endocrine cells. Thus, it appears that ductal progenitors are specified from Pdx1<sup>+</sup>



**Fig. 19** Two hypothetical models to explain the acquisition of each main cell fate within the developing pancreas. (Adapted from Gu et al.<sup>100,101</sup>) In model A, the divergence between ductal and acinar/endocrine tissue occurs before e9.5. Ductal-committed cells acquire Pdx1 expression between e9.5 and e11.5. Thus, during this time window there are two distinct Pdx1 populations. In model B, the divergence occurs between e9.5 and e11.5. According to this hypothesis, suppressive or inductive signals received during this time will determine whether Pdx1<sup>+</sup> cells acquire duct or endocrine/exocrine fates. In both cases, Ngn3 expression is responsible for the specification of endocrine cell types



**Fig. 20** Cre/loxP excision. The Cre recombinase will cut anything comprised between two loxP sites, leaving a single one. The original loxP-flanked fragment is lost



**Fig. 21** When a transgenic mouse containing a loxP-flanked stop codon between a constitutive promoter (*striped arrow*) and a cellular marker such as human placental alkaline phosphatase (hPAP) is mated with another strain containing a Cre cassette driven by a tissue-specific promoter (in this case insulin, *open arrow*), the resulting offspring will display the hPAP label only in the insulin-producing cells. This system can be further refined by using Cre<sup>ER</sup>, which will not penetrate the nucleus and excise the loxP-flanked DNA unless tamoxifen is given to the animal. This alternative strategy allows for the generation of a “pulse” in which specific cell types can be labeled only at one point. Their progeny will inherit the label, but cells that start expressing the chosen promoter (here, insulin) after the administration of tamoxifen will not be labeled

cells during this time window, and then set aside. According to this observation, endocrine cells are specified from Pdx1<sup>+</sup> cells upon disruption of the Notch pathway, transient Ngn3 expression and down-regulation of Ptf1 $\alpha$ ; exocrine cells arise from Pdx1<sup>+</sup> cells where both Notch signaling and Ptf1 $\alpha$  expression persist; and, finally, ductal cells originate from a specific subset of Pdx1<sup>+</sup>/Ngn3<sup>-</sup> cells that appear between e9.5 and e11.5. Figure 21 presents two specification models based on different divergence points.

### Cre/loxP and CRE-ER<sup>TM</sup> Systems for Lineage Tracing

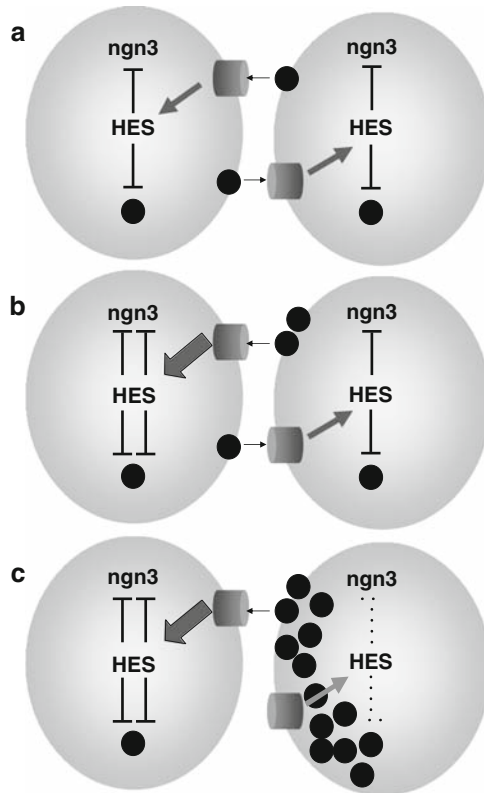
The CRE-ER<sup>TM</sup> transgenic system<sup>98,99</sup> is based on the fusion of the Cre recombinase (which excises any fragment of DNA comprised between any two loxP sites; Fig. 20) to the ligand-binding domain of a modified estrogen receptor.<sup>102</sup> In short, whenever Cre is produced in any specific tissue as a result of a conventional Cre/loxP strategy (Fig. 21), the protein remains cytoplasmic unless the animal is injected with tamoxifen. In that case, the Cre protein is internalized into the nucleus and it will cut between loxP sites. If the result of this cut is the expression of a marker such as alkaline phosphatase, that cell and its progeny will be permanently labeled.

## 5 Endocrine Specification

Endocrine differentiation occurs through a *lateral inhibition* process<sup>103</sup> mediated by *Notch* signaling. Cells in which the *Notch* receptor is activated by the ligands *delta* or *serrate* express high levels of *HES-1*, which in turn represses the pro-endocrine gene *Ngn3*. Lower levels of *Notch* signaling may randomly occur in individual cells, where *HES-1* expression will not be up-regulated. In the absence of its repressor, *Ngn3* will be expressed robustly, and the cell will adopt a pro-endocrine fate<sup>104–107</sup> (Fig. 22).

### Ngn3

Neurogenin 3 (Ngn3) encodes a class B Bhlh factor. Knockout studies have shown its requirement for the development of all the endocrine cell lineages of the pancreas.<sup>105</sup> Thus, *Ngn3*<sup>−/−</sup> mice are born without islets. The pro-endocrine role of *ngn3* has also been demonstrated in gain-of-function studies. Both ectopic *ngn3* expression<sup>104,106</sup> and lineage tracing experiments<sup>100,101</sup> indicate that *ngn3* is a cell-autonomous determinant and true marker of endocrine progenitor cells. The pattern of adoption of endocrine cell fates seems to follow a specific timeline, suggesting that *ngn3*-positive cells adapt their responses to a changing milieu of signals present in the bud microenvironment. Early *ngn3* expression in pancreatic progenitor cells (e8.5–e9) results in their differentiation into glucagon-producing cells; adenoviral expression of *ngn3* in adult human ductal cells leads to neuroendocrine differentiation<sup>65</sup>; and ectopic expression of *ngn3* in the chick gut results in endodermal cell differentiation into endocrine cells that cluster in the mesenchyme.<sup>108</sup> Furthermore, when the Ngn3 protein was engineered with a TAT protein transduction domain, it was shown to promote endocrine cell differentiation in vitro.<sup>109</sup>



**Fig. 22** Lateral specification mechanisms leading to endocrine cell differentiation. Pancreatic progenitors at an early stage of development can be considered functionally equivalent. **(a)** Any cell will both secrete the Notch ligand (delta or serrate, *black circles*) and receive it from the neighboring cell. Activation of the Notch receptor leads to the upregulation of Hairy Enhancer of Split-1 (HES-1), which is a potent repressor of the pro-endocrine gene *Ngn3*. HES-1 will also block the production of more ligand, but this blockade is leaky. A basal level of ligand is still produced, which maintains the system in an unstable equilibrium. In the presence of active Notch signaling, further differentiation does not occur. The activation of this pathway is normally associated with proliferation. **(b)** If one of the two cells in the model expresses more ligand than the other, the effect on HES-1 in the receiving cell will be proportional. While *Ngn3* remains repressed, cells where HES-1 is over-activated as a result of overstimulation exert a stronger blockade on the production of ligand. **(c)** Repression of ligand secretion in one cell results in down-regulation of HES-1 in the other. If HES-1 is de-repressed, so is (a) its production of ligand (which will be secreted in large amounts to keep the other cell undifferentiated); and (b) the expression of *Ngn3*, which will induce a pro-endocrine fate in the cell where Notch has been down-regulated

### Isl1

Isl1 is a LIM homeodomain protein - a family of proteins featuring a DNA binding homeodomain and two LIM domains, zinc-binding motifs that mediate protein-protein interactions.<sup>110</sup> It was originally identified as a regulator of the rat insulin I gene enhancer,<sup>111</sup> and is ubiquitously expressed in

all mature islet endocrine cell types. During development, Isl1 expression is detected immediately upon maturation of these cells ( $\sim$ e9 for glucagon-positive cells,  $\sim$ e10.5–11 for insulin-positive cells), as well as in the mesenchyme of the dorsal bud.<sup>112</sup> As expected, Isl1 knockout mice fail to develop pancreatic endocrine cells. The dorsal pancreatic mesenchyme is also absent, which in turn impairs exocrine cell differentiation in the dorsal pancreas. The latter effect, however, could be rescued in vitro by coculture with pancreatic mesenchymal tissue extracted from wild-type animals.<sup>112</sup>

### **Brn-4**

Brain-4 (Brn-4/Pou3f4) is a class III POU homeodomain-containing protein. It is expressed both in neural tissue during CNS development and in pancreatic glucagon-producing cells as early as e10,<sup>113</sup> where it seems to bind to the G1 element of the proglucagon gene proximal promoter. Although Brn-4 is the very first marker of alpha cells (preceding the expression of Pax6 and even Isl1), endocrine cell formation is perfectly normal in Brn4<sup>-/-</sup> mutants.<sup>114</sup> This is a surprising observation, and more so in view of the fact that ectopic expression of Brn4 from the insulin promoter results in the coexpression of insulin and glucagon in beta cells.<sup>115</sup> Taken together, these data suggest that Brn4 acts at an early developmental stage, but is not essential for the alpha cell fate determination.

The differentiation into each endocrine cell type within the islet is preferentially observed at specific time points during embryogenesis (alpha cells since e9.5; beta cells since e10.5;  $\delta$  cells since e14.5; and PP cells since e18.5), suggesting that *Ngn3*<sup>+</sup> cells adapt their responses to an evolving milieu of signals.

### **BETA2/NeuroD**

The beta-cell E-box transactivator 2 (BETA2, also known as NeuroD) is a cell-restricted Bhlh first isolated from insulinoma cells, where it was shown to be a component of the native insulin E-box-binding complex with an associative preference to the ubiquitous Bhlh factor E47.<sup>116</sup> Mice where BETA2/NeuroD has been knocked out display a dramatic reduction in the number of beta cells, impaired islet morphogenesis and some additional abnormalities in the exocrine pancreas, such as defects in the apical-basal polarity that result in the inability of acinar cells to secrete zymogen granules.<sup>117</sup> The main effects of this knockout were observed after e15.5, suggesting that this transcription factor is needed at a relatively late stage of endocrine cell development. *Ngn3* was found to be a critical upstream regulator of BETA2/NeuroD expression during islet specification.<sup>118</sup>

## 6 Beta Cell Differentiation

Little is known about the extracellular signals that drive beta cell specification from *Ngn3*<sup>+</sup> progenitors. Animals lacking *Nkx6.1*<sup>119</sup> and *Nkx2.2*<sup>120</sup> have defects in beta cell formation. *MafA* has also been implicated in the terminal differentiation of beta cells, particularly in the beta cell-specific reactivation of Pdx1.<sup>121</sup> However, several observations point to *Pax4* as the main hallmark of beta cell differentiation: The knockout of this gene results in the total absence of beta cells,<sup>122</sup> but not alpha cells; its expression peaks between e13.5 and e15.5, which coincides with the period of maximal differentiation of beta cell precursors<sup>122,123</sup>; and shortly after endocrine specification, *Ngn3* colocalizes with *Pax4*,<sup>124</sup> which suggests that the latter may be one of the targets of the former.<sup>125,126</sup>

Also, ES cells transfected with *Pax4* were induced to express insulin at much higher levels than untransfected controls.<sup>127</sup> Recent evidence indicates that *Pax4* and *Arx* are mutually repressed, and that the balance between the two determines  $\alpha$  (*Arx*) or beta (*Pax4*) specification from *Ngn3*<sup>+</sup> progenitors.<sup>128–130</sup>

### Nkx2.2

The NK2 homeobox 2 (*Nkx2.2*) gene belongs to a family of genes involved in the differentiation of many tissues.<sup>131</sup> *Nkx2.2* expression is observed both in the ventral CNS and in mature alpha, beta, and PP cells. The gene is activated from very early on during pancreatic specification (~e8), but – in a pattern similar to that of Pdx1 – becomes restricted to specific islet cell types later in development.<sup>120</sup> *Nkx2.2* mutant mice lack beta cells and have lower amounts of other islet endocrine cell types. Further analyses of these islets, however, show a sizeable population of hormone-negative undifferentiated cells. While Pdx1 expression remains largely unaffected in *Nkx2.2* knockouts at the onset of pancreatic specification (~e8.5), the relative strength of its signal is significantly reduced during the secondary transition (~e13.5 onward). This led to the hypothesis that *Nkx2.2* might be required for the terminal differentiation of beta cells.<sup>120</sup>

### Nkx6.1

*Nkx6.1* is another member of the NK homeodomain family, which can be found both in the pancreas and the CNS.<sup>132</sup> *Nkx6.1* expression is first detected from e10.5 in the pancreatic epithelium. At e15.5, it colocalizes either with Pdx1<sup>+</sup> cells (postmitotic beta cells, mostly) or with *Ngn3* (immature progenitors). At later developmental stages, as well as in the adult organ, *Nkx6.1* is exclusively restricted to beta cells.<sup>119</sup> Knockouts display a marked deficiency of beta cells, which could be traced back to the initiation of the secondary

transition, during which beta cell neogenesis was severely impaired. *Nkx2.2* expression was not affected, suggesting that this gene is upstream of *Nkx6.1*. This was further confirmed by double *Nkx2.2/Nkx6.1* knockout experiments, whose phenotype was identical to that of *Nkx2.2*<sup>-/-</sup>.<sup>119</sup>

### **Pax4**

Paired box-containing gene 4 (*Pax4*) belongs to a multigene family that shares a conserved motif, termed “paired box.”<sup>133</sup> Both *Pax4* and *Pax6* have, in addition, a homeodomain.<sup>134</sup> *Pax4* expression is first detected in the pancreas at around e9.5, and is later restricted to beta cells. Knockout mice lack both beta and delta cells, but alpha cells appear to compensate for their absence with a much higher than normal representation in the islet, which is suggestive of a “default” alpha cell differentiation pathway. Several lines of evidence point at *Pax4* as a direct downstream target of *Ngn3*.<sup>123–126</sup> As discussed in the main text, the balance between *Pax4* and *Arx* might be critical for the adoption of alpha or beta cell fates.<sup>128–130</sup>

### **Pax6**

Perhaps best known for its role in eye development,<sup>135–138</sup> *Pax6* is also expressed both in the developing pancreas (scattered throughout the fore/midgut cells at e9.5; colocalizing with arising alpha cells at e9.5; with alpha or beta cells at e15.5) and with alpha, beta, or delta cells in the adult pancreas – but not in acinar tissue.<sup>139–141</sup> Knockout mice show both a very significant reduction in all hormone-producing cells (with alpha cells largely absent) and an abnormal distribution of the remaining ones within the islet.<sup>134,140</sup> A model for alpha and beta cell specification based on relative levels of *Pax4* and *Pax6* is presented in Fig. 23.

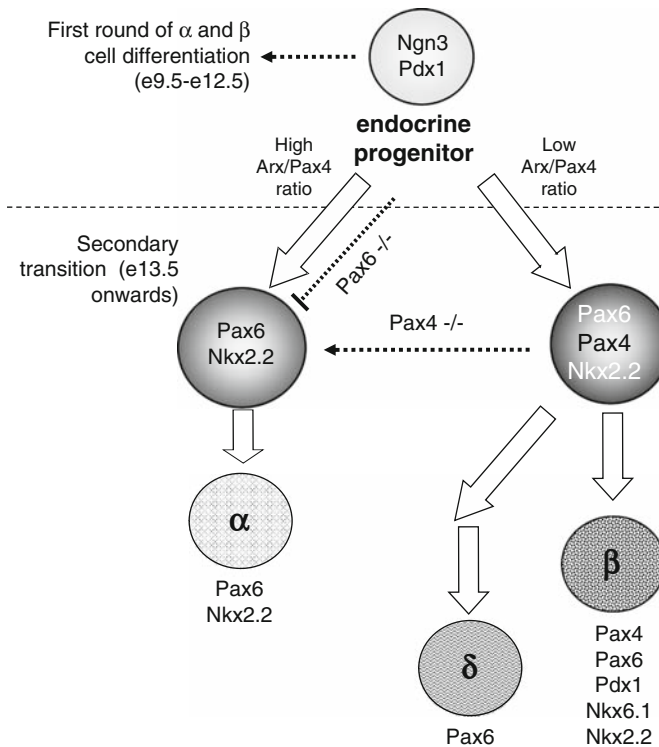
### **Arx**

The aristaless-related homeobox gene (*Arx*) contains both a C-terminal stretch of amino acids known as the OAR/aristaless domain and a prd-class homeobox domain. Like many other genes involved in pancreatic development, *Arx* was first identified in the mouse CNS.<sup>142</sup> *Arx* expression is observed in the proliferating epithelium of the pancreatic anlage at e9.5, and then is progressively restricted to endocrine cell types.<sup>129</sup> *Ngn3* knockouts do not express *Arx*, indicating that the former is upstream of the latter.<sup>129</sup> *Arx*<sup>-/-</sup> mutants lack alpha cells and display a concomitant increase in delta cells (and, to a lesser extent, beta cells).

(continued)

**Arx** (continued)

This effect is exacerbated in double Pax4/Arx mutants, where both alpha and beta cell subsets are largely replaced by delta cells.<sup>128</sup> Arx, however, does not seem to be required for early alpha cell differentiation, because glucagon-positive cells can be readily detected until e12.5 in mutant embryos, which suggests that the main role of Arx is during the secondary transition. Taken together, these observations support a model where Arx promotes alpha cell differentiation and antagonizes that of beta cells (from e12.5 onward) and delta cells (from e14.5 onward). Because Pax4 has an opposing activity, the relative levels of each protein will likely dictate cell fate after e12.5 (see Fig. 23)



**Fig. 23** A model for alpha, beta, and delta cell specification. Experimental evidence suggests that the first insulin-positive and glucagon-positive cells that appear prior to the secondary transition are a developmental dead end. Relative levels of the mutually repressing proteins Arx and Pax4 will define two populations of endocrine progenitors during the secondary transition. Both are characterized by the expression of Nkx2.2 and Pax6. However, Pax4 expression is only detected in those cells that will give rise to both beta and delta cells. The effect of knocking out either Pax6 or Pax4 is indicated in dotted lines. Figure 24 shows an alternative model where two distinct pancreatic progenitor cells exist before and after the secondary transition (Adapted from St-Onge et al.<sup>140</sup> and Collombat et al.<sup>128,129</sup>)



### **MafA/MafB**

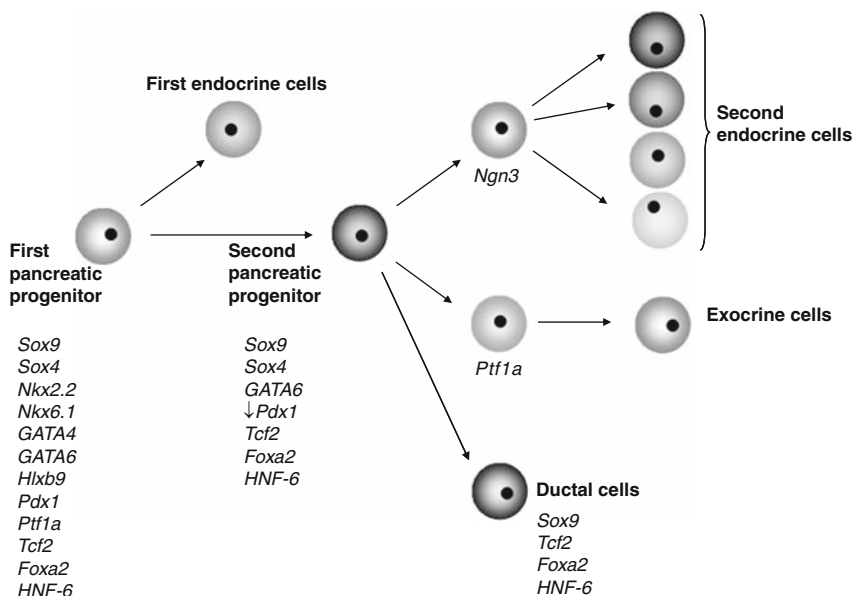
Maf transcription factors (containing a basic leucine zipper) have been associated with the regulation of multiple differentiation processes.<sup>143–146</sup> The best-characterized Maf factors expressed in the pancreas are MafA and MafB, which are preferentially expressed in beta and alpha cells, respectively.<sup>121</sup> The role of these factors has been difficult to ascertain. MafA knockout mice, for instance, display glucose intolerance and develop diabetes; however, there are no developmental effects associated with the mutation.<sup>147</sup> Recent evidence suggests that a switch from MafB to MafA might be critical for the embryonic maturation and prolonged survival/function of beta cells.<sup>121</sup> However, MafB has also been recently shown to be essential for the appropriate regulation of Pdx1, Nkx6.1, and GLUT-2 in the final stages of maturation of beta cells.<sup>148</sup>

### **Foxa2 (HNF3beta)**

The winged-helix transcription factor Foxa2 (also known as hepatocyte nuclear factor 3beta [HNF3beta]) is one of the first true markers of definitive endoderm.<sup>149,150</sup> Despite its expression throughout the development of the pancreas, not many studies have looked into its role in this process. These studies are chiefly based on conditional loss of function. Thus, selective abrogation of Foxa2 expression in beta cells results in insulin hypersecretion, leading to profound hypoglycemia.<sup>151</sup> In fact, Foxa2 has been shown to control Pdx1 expression in mature beta cells.<sup>152</sup> However, the onset of Pdx1 expression and pancreatic specification is not affected in mice where the Foxa2 has been selectively inactivated in the developing endoderm.<sup>153</sup> These animals, however, are severely hypoglycemic and die within days of birth, due to a dramatic reduction (>90%) in the number of alpha cells. They show impaired maturation, but not initial specification of alpha cells.<sup>153</sup>

## **7 The Secondary Transition**

The secondary transition is a phenomenon first observed in the pioneering studies on the developing pancreas conducted by Pictet and Rutter in the early 1970s.<sup>154,155</sup> In short, it can be described as a secondary wave of synchronized endocrine and exocrine differentiation. Although there is no clear-cut initiation, it is generally acknowledged that the secondary transition starts around e13.5 in the mouse. Recent studies suggest that changes in the TGF- $\beta$  signaling pattern might be responsible for this developmental phase.<sup>156</sup> Throughout its course, the epithelium branches extensively: the termini of the ducts give rise to exocrine cell types, and the cells lining the ducts become a niche for pancreatic progenitor cell proliferation and differentiation.



**Fig. 24** A differentiation model contemplating the existence of two distinct pancreatic progenitor cells before and after the secondary transition (Adapted from Lynn et al. 2007<sup>157</sup>)

It has been reported that the progenitor cells detected during the secondary transition are different from those that give rise to the first differentiated cells before e13.5. Thus, the early progenitors generally express markers such as *Ptf1α*, *Hlxb9*, *Pdx1*, *Sox4*, *Nkx2.2*, and *Nkx6.1*, which are later restricted to differentiating cells during the secondary transition<sup>157</sup> (Fig. 24).

### Prox1

Expression of Prospero-related homeobox 1 (*Prox1*) was first observed in the CNS, developing eye lens, liver, and pancreas. In the latter, *Prox1* can be detected from e9.5 in the dorsal bud,<sup>158</sup> but other authors pin down the initiation of *Prox1* expression to an even earlier time point, before the divergence between liver and pancreas.<sup>159</sup> At any rate, *Prox1* expression is detected throughout pancreatic development in endocrine, but not exocrine tissue. In adult mice, *Prox1* signal was stronger in ductal cells than in the beta cell component of the islets.<sup>160</sup> *Prox1* mutations lead to embryonic lethality at around e15.5, due to severe developmental aberrations. The phenotypic manifestation of *Prox1* knockout is an arrest in pancreatic growth from e11.5, with an increase of exocrine cell differentiation at the expense of the progenitor cells responsible for the secondary transition. This has led to the hypothesis that *Prox1* might have a role in repressing exocrine differentiation from bipotential endocrine/exocrine progenitors.<sup>160</sup>

### Sox4/Sox9

The SRY/HMG box (Sox) family of genes is involved in many developmental processes. At least 11 of these genes are expressed in the pancreas during embryonic development.<sup>161</sup> Among these, the best characterized are *Sox4* and *Sox9*. *Sox4* is broadly expressed in the epithelium throughout development, but later becomes restricted to hormone-producing cells. The pancreas of *Sox4* knockout mice appears to be normal until the initiation of the secondary transition. However, experiments where pancreatic buds were explanted at e13.5 and grown in vitro (the mutation is lethal at around e14<sup>162</sup>) showed a dramatic reduction in the differentiation of beta and other endocrine cell types.<sup>161</sup> This observation suggests that *Sox4* might be involved in facilitating the secondary transition. *Sox9* is expressed in the pancreas as early as e10.5, where it can be found together with *Pdx1*<sup>+</sup>, but not differentiated cells.<sup>157</sup> The same pattern applies at later stages of development: at e14.5 it is detected coexpressed with *Pdx1*<sup>+</sup> undifferentiated cells and *Ngn3*<sup>+</sup> progenitors. Furthermore, reporter experiments demonstrated that *Sox9* is involved in the regulation of the *Ngn3* promoter.<sup>157</sup> Taken together, these observations suggest a role of *Sox9* as a critical component of the molecular network responsible for the maintenance of pancreatic progenitor cells.

## 8 Do Physical Factors Play a Role in Pancreatic Development?

Progress in our understanding of the influence of nonchemical agents in the progression of embryonic development is consolidating old disciplines such as biophysics and shaping new ones, such as mechanobiology. It is known, for instance, that mechanical forces generated by the division of cells in a confluent setting (such as that found in living tissues) are able to regulate the cellular pathways of proliferation and differentiation.<sup>163</sup> This is an example in which the form of the tissue (a physical parameter) would be not just the outcome, but also the effector of certain developmental programs. This knowledge is currently being applied for the design of better in vitro differentiation protocols that take into account not only the adequate biochemical milieu, but also physical determinants such as tensile strength and the nature of the substrate.<sup>164–170</sup>

Another example is that of bioelectrical fields, whose clear-cut influence on cell behavior and tissue patterning, morphogenesis, and regeneration remains largely ignored by mainstream developmental biologists. Thus, it is known that applied fields can cause cell differentiation and even dedifferentiation,<sup>171–175</sup> and that regeneration depends on the bioelectrical properties of the tissue.<sup>175,176</sup> Indeed, it has been hypothesized that biomagnetic fields provide the coordinates for cell migration and

tissue branching.<sup>177,178</sup> For instance, ion channel-generated fields precede and predict the appearance of limbs in several species, whereas the suppression of electrical activity results in arrest of growth and differentiation.<sup>161,179–181</sup>

While bioelectric fields constitute the essence of pancreatic beta cell function, nothing is known yet about the potential influence of this factor in their development. The same applies to virtually every other physical agent studied so far in this context, including oxygenation. The latter is especially surprising, because the role of molecular oxygen in shaping development has already been well established in many tissues and organs, including mammalian placentation, adipogenesis, cardiovascular/pulmonary development, bone morphogenesis, and general stem cell behavior (reviewed in<sup>182,183</sup>). This void has been explored in a tentative manner only recently,<sup>184</sup> but there is still a clear need to experimentally validate working models in which the oxygen-sensing machinery would directly affect critical pancreatic developmental pathways such as Notch and Wnt/beta-catenin.<sup>185</sup> Cells detect low oxygen concentrations by means of the hypoxia-inducible factor 1 (HIF-1) protein. HIF-1 is a heterodimer consisting of two subunits: the beta domain, which is expressed constitutively, and the oxygen-dependent alpha protein.<sup>186</sup> The latter is degraded at high pO<sub>2</sub>s but remains intact at lower tensions.<sup>186,187</sup> When activated, the HIF-1 dimer is known to participate in the regulation of a variety of cellular processes,<sup>186,188–191</sup> including progenitor cell self-renewal and proliferation.<sup>188,192–194</sup>

Since mammalian embryogenesis occurs at a very low oxygen tension before the initiation of blood circulation,<sup>182,195–198</sup> the early stages of pancreatic development are also expected to take place under hypoxic conditions. However, at e13.5 (coinciding with the onset of the secondary transition in the mouse), blood starts to flow in the pancreatic rudiments.<sup>199</sup> In fact, this might be considered a consequence of HIF-1-mediated stimulation of blood vessel formation in a hypoxic environment.<sup>186,188</sup>

As mentioned earlier in this chapter, the Notch pathway is typically associated with proliferation and the maintenance of an undifferentiated state.<sup>200</sup> Indeed, down-regulation of Notch signaling is critical for the activation of Ngn3 and subsequent endocrine differentiation (Fig. 22).<sup>48,104,106,201–203</sup> Because Notch is activated by HIF-1alpha under hypoxic conditions,<sup>188,192,204–206</sup> it is plausible that higher oxygenation (such as that expected from e13.5 onward) might partially disable this pathway.

$\downarrow O_2 \uparrow HIF-1 \propto \uparrow Notch \uparrow Endocrine\ progenitor\ cell\ proliferation$   
 $\uparrow O_2 \downarrow HIF-1 \propto \downarrow Notch \uparrow Endocrine\ progenitor\ cell\ differentiation$

Higher oxygenation could have effects on the developing pancreas other than Notch down-regulation and a wave of endocrine differentiation. For instance, Wnt/beta-catenin signaling (which is key for the maintenance of undifferentiated

proliferating exocrine progenitor cells<sup>207, 208</sup>) is inhibited in hypoxia.<sup>209, 210</sup> Therefore, high oxygen conditions present after e13.5 may also promote exocrine progenitor cell proliferation.

$\Downarrow O_2 \Downarrow Wnt/\beta\text{-catenin} \Downarrow \text{Exocrine progenitor cell proliferation}$   
 $\Uparrow O_2 \Uparrow Wnt/\beta\text{-catenin} \Uparrow \text{Exocrine progenitor cell proliferation}$

Finally, oxygen tension has been linked to chromatin reorganization through acetylation. In general, acetylated loci have a less compact DNA coiling, which leads to high transcription levels. However, if there is strong histone deacetylase (HDAC) activity, gene silencing ensues. HDAC-mediated repression of gene expression is associated with hypoxia in a HIF-1 $\alpha$ -mediated manner.<sup>182</sup> At least three important genes involved in the progression of pancreatic development (namely Pdx1, NeuroD/Beta2, and Ngn3) have already been shown to be repressed in hypoxic conditions through the action of HIF-1 $\alpha$ -dependent HDACs.<sup>211–213</sup> In fact, such knowledge has already been applied to enhance the rate of beta cell differentiation by means of forcing HDAC down-regulation in normoxia.<sup>214, 215</sup>

## 9 Correspondence Between Mouse and Human Pancreatic Development

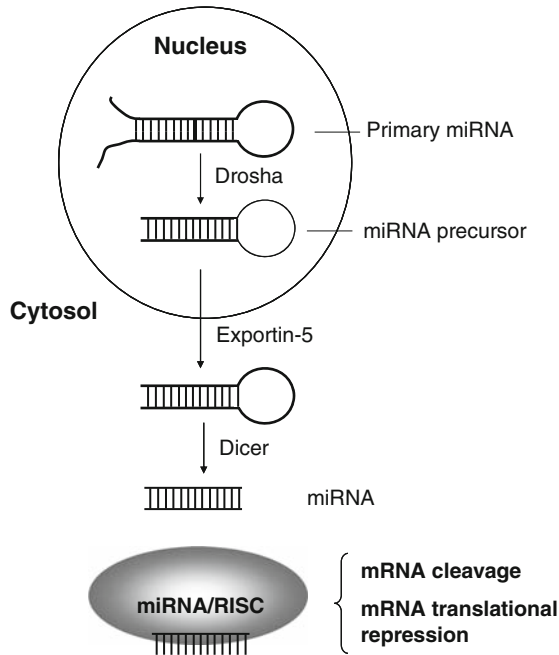
Despite the seemingly perfect conservation across species of most transcription factors already discussed, even a superficial analysis of human pancreatic development reveals striking differences with that of the mouse. The following are just a few examples: (1) the relative speed of the transition between the initiation of pancreatic development and the emergence of the first hormone-positive cells in the epithelium is remarkably faster in the mouse. According to the relative timeframe observed in the latter, the detection of the first endocrine cells in humans would take place no later than 4.5/5 weeks postconception (w.p.c.). However, extensive immunohistochemical analyses of the developing human pancreas show that the first endocrine cells are not detectable prior to 7 w.p.c., more than 3 weeks after pancreatic specification.<sup>216</sup> The biological significance of this relative delay is not fully understood yet. (2) The first endocrine cells to appear during human pancreatic development are beta cells (7 w.p.c.). In contrast with the mouse,  $\alpha$  cells do not appear until later, at around 8.5 w.p.c.<sup>216</sup> (3) Islets assemble just prior to term in mouse, whereas human islets are fully formed from 12 to 13 w.p.c.<sup>217, 218</sup> Table 2 compares chronologically the main events of fetal pancreatic development in both mouse and human.

**Table 2** Correspondence between human and mouse pancreatic development

Mouse developmental stage	Event	Human developmental stage
e8.5–e9.5	Initiation of pancreatic development; evagination from the primitive foregut; Pdx1 expression	3–4 w.p.c.
e9.5–e10.5	Immunodetection of glucagon expression	8.5 w.p.c.
e10.5–e11.5	Immunodetection of insulin expression	7 w.p.c.
e12.5	The two pancreatic buds fuse at the base	8 w.p.c.
e13.5–e14.5 onward	Formation of acini from ducts Initiation of secondary transition Immunodetection of somatostatin expression	8.5 w.p.c. onward
e16.5–e18.5	Immunodetection of PP expression Islet formation	10 w.p.c. 12–13 w.p.c.

### MicroRNAs and Pancreatic Development

MicroRNAs (miRNAs) are noncoding small RNAs (~19–22 nt) that regulate gene expression by posttranscriptional interference with specific messenger RNAs (mRNA)<sup>219,220</sup> (Fig. 25). Since each miRNA may have multiple target mRNAs, they are potentially capable of controlling very complex gene expression regulatory networks.<sup>221,222</sup> The function of most of them remains unknown, but some of their targets have been experimentally confirmed.<sup>223</sup> In beta cells, mir-375 has been shown to negatively control insulin secretion in by targeting myotrophin.<sup>224</sup> Several studies show that miRNAs regulate embryonic development and have tissue/cell-specific patterns.<sup>225,226</sup> miRNAs are necessary for murine islet differentiation,<sup>227</sup> and mir-375 inhibition has a deleterious effect on pancreatic development.<sup>228</sup> We have recently established that mir-7 is the most differentially expressed miRNA in islets,<sup>229</sup> and follow-up studies demonstrate that this miRNA was expressed in the human developing pancreas from week 9. The peak of expression was observed between weeks 14 and 18, coincident with an exponential phase of differentiation of hormone-producing cells.<sup>230</sup> Based on these intriguing findings, further research on the subject might unravel a potential implication of mir-7 in beta cell development. In conclusion, the study of miRNAs as macro-regulators of gene expression is an emerging field whose rapid advancement may completely redefine the way we understand the progression of development and the acquisition and maintenance of cell identity.



**Fig. 25** Primary miRNAs are cleaved in the nucleus by the RNase III endonuclease Drosha. This releases a ~60–70-nt stem loop pre-miRNA precursor, which in turn is actively transported to the cytoplasm by export receptor exportin-5. Once there, it is further processed by Dicer, another member of the ribonuclease III protein family. The ~21-nt double-stranded RNA cleavage product is subsequently separated into a single-stranded RNA by the action of helicases, and forms a ribonucleoprotein complex known as RNA-induced silencing complex (RISC). The RISC will guide the particular miRNA to its mRNA target. Upon binding to target RNA, RISC-miRNA would either cut it or repress its translation, depending on whether the homogeneity between the miRNA and the target mRNA is exact or incomplete, respectively



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