

Recent Advances and Prospects in the Differentiation of Pancreatic Cells From Human Embryonic Stem Cells

Josué Kunjom Mfopou,¹ Bing Chen,^{1,2} Lina Sui,¹ Karen Sermon,² and Luc Bouwens¹

Recent studies with human embryonic stem (hES) cells have established new protocols for substantial generation of pancreatic progenitors from definitive endoderm. These findings add to the efficient derivation of definitive endoderm, which is controlled by Wnt and *Nodal* pathways, and delineate a step forward in the quest for alternative β -cell sources. It also indicates that critical refining of the available strategies might help define a universal protocol for pancreatic differentiation applicable to several cell lines, therefore offering the possibility for transplantation of immune-matched or patient-specific hES-derived β -cells. We appraise here the fundamental role that bone morphogenetic protein, fibroblast growth factor, and retinoid signaling play during pancreas development, and describe a fundamental emergence of their combination in recent studies that generated pancreatic cells from hES cells. We finally enumerate some prospects that might improve further differentiation of the progenitor cells into functional β -cells needed in diabetes cell therapy. *Diabetes* 59:2094–2101, 2010

Overview of embryonic pancreas development. The regulation of pancreas development from the gut endoderm is a complex process that involves a carefully balanced interplay between several common signaling pathways. Studies in several model organisms indicate conservation of the main mechanisms of pancreas development from lower to higher vertebrates (Table 1). Gastrulation in the developing embryo (E7.5 in mice) results in the formation of three germ layers, namely the ectoderm, the endoderm, and the mesoderm. It appears that these germ layers do not further develop in isolation from each other, but signals emanate from one to pattern the other and vice versa (1). Several structures temporally participate in these early tissue interactions including the notochord (2), the cardiac mesoderm, the septum transversum mesenchyme and the lateral plate mesoderm (3), the aortic endothelial cells and the vitelline veins (4), and the developing pancreatic mesenchyme (5).

As far as the pancreas is concerned, specific growth and differentiation factors released by adjacent tissues control a set of transcription factors expression, resulting in the patterning of the ventral and dorsal prepancreatic endoderm. The initial interactions usually confer competence to respond to additional inductive signals that establish organ determination and specification at partic-

ular time points referred to as competence windows. Although our present knowledge of extrinsic factors that control patterning, proliferation, and differentiation cues throughout the developing pancreas is not yet complete, there is strong evidence emanating from all vertebrate developmental models that complex spatio-temporal combinations of common signaling pathways are crucial for appropriate specification of pancreatic cell fates from the endoderm (6–9). These involve, for instance, the hedgehog, Wnt, retinoid, and Notch pathways, as well as Activin/bone morphogenetic protein (BMP), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) signal transductions.

An overview of the roles played in pancreas development by the most relevant growth and differentiation factors is summarized in Table 2. A detailed sequential presentation is difficult to address because many signaling events are not clearly dissociated from each other, while others function redundantly or intermittently at several developmental stages. It is also worth noting that the developmental effects elicited by these common signals can vary significantly according to the cell type being stimulated or according to the stage of development when the signal occurs. This level of complexity is further enhanced by the interplay between transcription factors that lie downstream of these extracellular stimuli and are responsible for observed developmental features.

During the past two decennia, considerable progress has been achieved in understanding pancreas embryogenesis thanks to the numerous genetic studies involved in transcription factors. Most of them belong to the homeobox domain, the paired-box, or the basic helix-loop-helix families. The sequential expression of different transcription factors that control pancreas development has been extensively reviewed elsewhere (6–8,10). Only a brief summary of their expression pattern is given in Fig. 1 with special focus on 1) the early pancreatic epithelial cells, 2) the pancreatic endocrine progenitors, and 3) the endocrine subtypes selection.

Efficient definitive endoderm differentiation from many human embryonic stem cell lines and protocols. It is presently well recognized that attempts to generate pancreatic cells from pluripotent cells should first establish a definitive endoderm (DE) population as occurs in vivo. Wnt and *Nodal* signals were identified as sufficient to induce DE from mouse and human embryonic stem (hES) cells, but also from induced pluripotent stem (iPS) cells. In this model, high concentrations of activin A (ActA) are used to mimic the function of the endogenous endoderm inducer *Nodal* (11). Beside the activation of this pathway, initial treatment with Wnt3a or BMP4 was found to improve the efficiency of DE induction by setting up a transient mesendoderm progenitor population, especially when cultures are performed in feeder cell-free conditions

From the ¹Cell Differentiation Unit, Diabetes Research Centre, Vrije Universiteit Brussel, Brussels, Belgium; and the ²Department of Embryology and Genetics, Vrije Universiteit Brussel, Brussels, Belgium.
Corresponding author: Luc Bouwens, lucbo@vub.ac.be.
Received 29 March 2010 and accepted 12 June 2010.
DOI: 10.2337/db10-0439

© 2010 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

TABLE 1
Time scale of pancreas development in mouse and human

Organogenesis and differentiation events	Mouse	Human
Fusion dorsal aortae	E8	—
Mesenchyme condensation over dorsal gut endoderm	<E9.5	<E26
Endoderm evaginates in condensed dorsal mesenchyme	E9.5	E26
Endoderm evaginates in condensed ventral mesenchyme	E10	E32
First endocrine cells (insulin, glucagon)	E9.5–10.5	E52
Gut rotation, contact, and fusion of ventral and dorsal buds	E12–13	E37–56
Branching morphogenesis, acinar differentiation, distinct cellular architecture, β -cells amplification	E13–14	E70–90
Endocrine cells accumulation beside ducts	E14–18	E75
Endocrine coalescence and islets formation	>E18	E91–105
Birth	E22	E260–280

The major sequences of developmental processes are similar in mouse and human embryonic pancreas. The striking difference resides in the duration of each stage, which can vary by a factor of 5 to 15.

(12,13). Because active PI3K signaling is inhibitory for DE differentiation from embryonic stem (ES) cells, antagonists of this pathway (LY294002, Wortmanin) were also included in some protocols, resulting in an increased efficiency (14,15). In addition, supplementation of sodium

butyrate was found to support endoderm differentiation when combined with ActA (16), though it remains unclear how this chemical participates in the signaling network required for the definitive endoderm.

The standard and modified protocols for DE induction from ES cells allow for the derivation of at least 60–80% cells that express the characteristic markers FOXA2, SOX17, GSC, and CXCR4 (Table 3) but not the visceral endoderm marker SOX7 (13,14,16–20). Whether the quest for a universal β -cell differentiation protocol is a desired myth or an achievable reality, it appears clear from the literature that combined Wnt3a and ActA treatments efficiently activate DE phenotype in a vast majority of hES cell lines. In other words, the molecular events that lead DE differentiation in vivo have been largely—if not completely—unrevealed and implemented in vitro. Despite the recently described differences in the response of hES cell lines to differentiation cues (21), the variability and the low success rate in the outcome of initial pancreas differentiation protocols might first of all reflect the lack of such a universal developmentally based strategy that thoroughly reproduces in vivo events in the culture dish after the DE stage.

Noggin and retinoids: key players in blocking in vitro liver induction and favoring pancreas specification from definitive endoderm. When maintained in culture without added growth factors or when transplanted under the kidney capsule of immunocompromised mice, hES-derived definitive endoderm cells could spontaneously generate hepatocyte-like cells characterized by the expression of AFP, albumin (ALB) (Fig. 2A), or hepatocyte-specific antigen (12,17,20). This tendency for hepatic

TABLE 2
Overview of growth and differentiation factors that participate in vertebrate pancreas development

Stages	GDFs	Sources	Functions
Specification (E8.5–9.5)	Wnt	Mesoderm	Inhibit foregut, pancreas, and liver
	Shh, Ihh, Dhh	Endoderm	Anti-pancreatic
	Activin B, FGF2	Notochord	Repress hedgehog, pro-pancreatic (Dend)
	FGF–BMP	Cmes, LPM	Pro-hepatic/anti-pancreatic (Vend)
Expansion (E9.5–12.5)	Noggin	Dmes	Repress BMP (Dend), pro-pancreatic
	RA	LPM, Dmes	Gut patterning, bud and Pdx1 induction
	VEGF-A	Dorsal aorta	Pdx1, insulin, Ptf1a induction
	FGF1–7-10	Pmes	Epithelium proliferation, branching
	GCG	Alpha cell	Induce early insulin cells
	EGF, betacellulin	Pepi	Epithelium proliferation, pro-endocrine?
Differentiation (E12.5–E15.5)	Delta, jagged	Pnep	Anti-endocrine, progenitor proliferation
	Activin A, B	Endocrine	Anti-exocrine/pro-endocrine (via NGN3)
	BMP4–5-7	—	β -cell differentiation?
	TGFB1–2-3	Pepi, acini	Pro-endocrine, islet architecture
	RA	Pmes	Pro-endocrine (NGN3 induction)
	HGF	Pmes, pepi	Pro- β -cell
	GDF11 (BMP11)	Pepi	Pro-endocrine (NGN3 regulation)
	Follistatin	Pmes	Pro-exocrine/anti-endocrine
	Wnt (7 members)	Pmes	Control proliferation?
	Maintenance and function (>E15.5)	VEGF-A	Islet
GDF11 (BMP11)		Acini	Pro-endocrine?
HB-EGF		Duct, islet	Islet architecture?
EGF, betacellulin		—	β -cell proliferation?
GCG, Glp1		α -cells	Pro- β -cell, insulin synthesis
IHH		Islet	Pdx1 and insulin expression
Wnt		—	Postnatal pancreatic growth

Cmes, cardiac mesoderm; Dmes, dorsal mesenchyme; GCG, glucagon; GDF, growth differentiation factor; HB-EGF, heparin-binding epidermal growth factor; IHH, Indian hedgehog; LPM, lateral plate mesoderm; Pepi, pancreatic epithelium; Pmes, pancreatic mesenchyme; Pnep, pancreatic NGN3⁺ endocrine progenitor. Dend, dorsal endoderm; Vend, ventral endoderm.

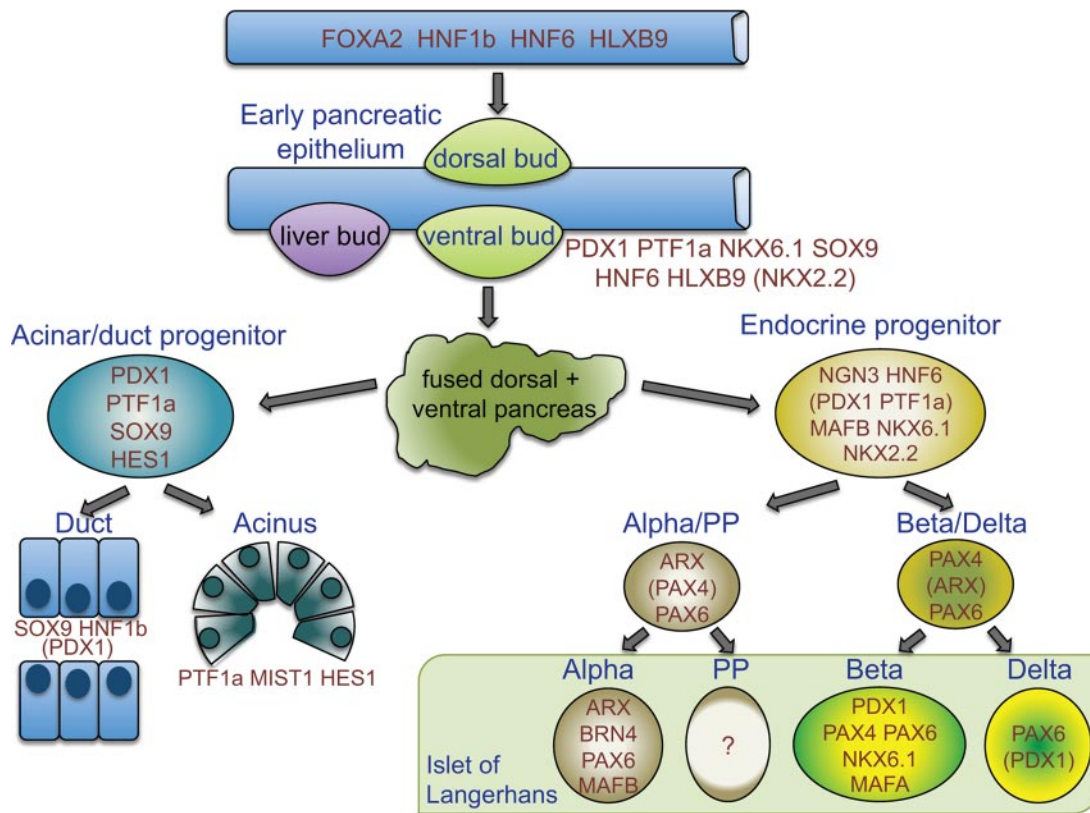


FIG. 1. Overview of transcription factors (TFs) expression during β -cell development. Expression of different combinations of TFs determines the sequential lineage segregation in the developing pancreas. Not only are the combinations of TFs important, but the expression level of a particular TF is known to guide differentiation as well. Low protein levels are indicated in parentheses. The most relevant TFs that are landmarks for the selection of each stage/lineage are shown.

lineage commitment was also reported in several studies wherein hES-derived DE cells were exposed to BMP and/or FGF, two growth factors implicated in embryonic liver induction and specification (13,20,22,23). The contribution of these pathways was further demonstrated by the treatment with BMP antagonist Noggin and FGF receptor antagonist SU5402, leading to the complete abrogation of hepatic gene expression from DE progenies that otherwise was mainly composed of hepatocytes. As suggested from animal studies, these experiments also indicated that FGF did not function in the induction but essentially contributed to the amplification of liver progenitors initially induced by BMP signaling (20). Therefore, the molecular signals elicited by BMP and FGF are the main factors driving hepatocyte differentiation from hES-derived DE cells.

In vivo, BMP activity generated by the adjacent mesoderm-derived structures and operating in the ventral endoderm is associated with hepatic induction during early embryonic development. On the contrary, ventral pancreas progenitors are located away from the midline endoderm with active BMP signaling, whereas BMP inhibition in the dorsal endoderm plays a crucial function in the acquisition of a pancreatic fate (3,24). The requirement for BMP antagonism is reverted after pancreas induction since this organ requires BMP signaling later on for the maintenance of Pdx1 expression and further differentiation. This underscores the existence of a tight competence window and the versatile functions of growth factors during development, which complicates their in vitro implementation for directed differentiation of ES cells (24).

Retinoic acid (RA) plays a crucial role shortly after gastrulation in endoderm patterning and in the induction of PDX1 expression by pancreas progenitors in several organisms from *Xenopus* to humans, which indicates the conservation of its activity during evolution (25–27). This role for RA has already been evaluated during mouse and human ES cell differentiation in vitro and has proven to be relevant for the induction of pancreatic gene expression, notably detectable levels of PDX1 (17,23,28–32).

In line with the in vivo recognized functions of these two signaling pathways on pancreas induction, recent studies have combined BMP inhibition (Noggin supplementation) with retinoid signaling immediately after the DE induction stage and succeeded in producing large amounts (up to 80%) of PDX1⁺ pancreas progenitor cells (Table 3), (13,14,20). Concomitantly, the proportion of hepatocytes that could be detected in these conditions was significantly reduced (Fig. 2A and B), indicating an efficient blockade of their induction upon BMP antagonism (20,33). This combination was efficient not only in different hES cell lines, but also in iPS cells (14), suggesting that the basic molecular events that should be monitored in order to design a universal pancreatic differentiation protocol are being progressively clarified. These minimally include the signals for the blockade of hepatic induction on the one hand and those required for pancreatic induction (namely PDX1 expression) on the other (Fig. 3). The necessity for such a combination was recently highlighted by treatment of hES-derived DE cells with Noggin in the absence of RA; this blocked the expression of liver markers, but did not generate PDX1⁺ cells (20,23). This requirement for combining RA treatment with BMP antagonism

TABLE 3
Derivation of PDX1⁺ progenitors from hES cells

References	DE induction	Pancreas induction	Differentiation	Key features
D'Amour (2006)	ActA + Wnt3a; ActA + FBS	FGF10 + Cyclo (4d); FGF10 + Cyclo + RA (4d)	DAPT + Ex4; IGF1 + HGF + Ex4	7% INS ⁺ cells, double + endocrine cells; no glucose response
Johannesson (2009)	ActA + Wnt3a; ActA + FBS	FGF4 + RA (+/- Cyclo)	—	32% PDX1 ⁺ cells, very low <i>INS</i> expression
Cai (2009)	ActA; ActA + ITS	(DE replating on 3T3) FGF7 + RA (6d)	HGF + Ex4 + NA (6d)	>90% PDX1 ⁺ ; co-expression FOXA2, HNF1b, HNF4a, HNF6, NKX6.1; some INS ⁺ cells
Jiang (2007)	ActA + NaBut (7d)	EGF + bFGF + NG (14d); EGF + NG (7d)	NA + IGF2 (5d); NA (2d)	EBs after DE induction; budding PDX1 ⁺ INS ⁺ clusters; 3D is better
Kroon (2008)	ActA + Wnt3a; ActA + FBS	FGF7 (4d); NG + RA + FGF7 (4d)	Transplantation in immunodeficient mice	Endocrine cells in grafts, glucose response in vivo at 3 months, protection from STZ effect
Vallier (2009)	ActA + BMP4 + bFGF (3d)	NG + RA + FGF10 + SB431542 (6d)	—	PDX1 ⁺ clusters, culture in feeder-free settings
Zhang (2009)	ActA + Wortmanin (4d)	NG + RA + FGF7 (4d); EGF (5d)	bFGF + Ex4 + BMP4 + NA + ITS (7d)	20% PDX1 ⁺ , 25% INS ⁺ , low glucose response, ITS issue not addressed
Mfopou (2010)	ActA + Wnt3a; ActA + FBS	NG + RA + Cyclo (8d); FGF10 + Ex4 + Compound E (4d)	NA + Ex4 + IGF1 + BMP4	50–80% PDX1 ⁺ in 4 hES lines, co-expression FOXA2, SOX9, HNF1b, HNF6, NKX6.1, low <i>PTF1a</i> . Some INS ⁺ cells

Recent models of pancreas differentiation from hES cells integrate BMP antagonism and retinoid signaling early after definitive endoderm induction. This allows for concomitant hepatic blockade and pancreas induction from definitive endoderm cells. Cyclo, cyclopamine; DAPT, *N*-[*N*-(3,5-Difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (gamma secretase inhibitor); EBs, embryoid bodies; Ex4, exendin-4; FBS, fetal bovine serum; ITS, insulin selenium transferring supplement; NA, nicotinamide; NaBut, sodium butyrate; NG, Noggin; STZ, streptozotocin; 3D, three dimensional. The text in bold marks protocol with combination of NG and RA.

could also be seen from another angle. Indeed, retinoid signaling was shown to activate BMP expression in several *in vivo* and *in vitro* systems including embryonic carcinoma cells and differentiating mouse embryoid bodies (34–36). It is not yet clear whether this inductive effect on BMP also occurs during hES cell differentiation. Nevertheless, considering the *in vivo* function of BMP signaling in the midgut endoderm, it appears reasonable that both retinoid signaling and BMP antagonism were required in these studies for strong induction of a pancreatic fate from the DE cells while preventing hepatic differentiation. However, it is likely that the effect of RA is context-dependent because when applied on hES-derived DE cells cultured at low density, it resulted in the inhibition of Smad 1/5/8 phosphorylation (23). It remains to be elucidated whether this later effect represents an artifact of *in vitro* culture or a specific retinoid effect at the single cell level.

Other studies made use of RA combined with FGF ligands (FGF4, FGF7, FGF10) or of Noggin combined with FGF2 and EGF to induce pancreatic cells albeit at a lower efficiency than for combined Noggin and RA (Table 3) (13,14,16,17,19,23). Our unpublished observations suggest that FGF signals favor hepatic gene expression in the absence of BMP antagonism, but the addition of FGF ligands together with BMP antagonism (Noggin) and RA enhances pancreatic gene expression. Furthermore, the induction of PDX1⁺ cells by combined Noggin and RA requires at least a basal level of FGF activity (via the extracellular signal-regulated kinase/mitogen-activated protein kinase [ERK/MAPK] pathway [Fig. 3]) given that the addition of the MAPK-inhibitor U1026 prevented pancreatic cell differentiation (20). Because RA is known to

act synergistically with FGF signaling during endoderm patterning, supplemented FGF can be seen as an enhancing factor in protocols that already integrate Noggin and RA (13,14,19). This view might resolve the nature of factors X suggested as involved in the indirect effects of RA in *Xenopus* dorsal endoderm (37). It was recently suggested that in the presence of FGF7, RA can efficiently induce PDX1⁺ progenitors from hES-derived DE cells seeded at low density (5–50,000 cells/cm²) and that this effect is paralleled by inhibition of Smad1/5/8 phosphorylation, therefore recapitulating BMP antagonism described above (23).

Recent analysis of the signaling network during early liver and pancreas development in 3–6 somites-stage mouse embryos further indicated that inhibition of the activin signaling significantly increases the proportion of cells fated toward PDX1 expression. These findings therefore suggest that the addition of activin inhibitors to the above-mentioned cocktails might still boost the occurrence of PDX1⁺ cells in hES cell cultures (13,24).

Despite the fact that combining BMP antagonism with retinoid signaling efficiently generates PDX1⁺ pancreatic progenitors from hES-derived definitive endoderm, the requirement for hedgehog antagonism with cyclopamine during pancreas induction from these cells has not been clarified by these studies. The initial report by D'Amour et al. (18) indicated the necessity to antagonize hedgehog signaling that is indeed activated during ES cell differentiation, and it was shown to also play a negative role *in vivo* by limiting endocrine and exocrine genes expression in embryonic (E12.5) mouse pancreatic explants (38,39). However, additional studies using Noggin, FGF ligands,

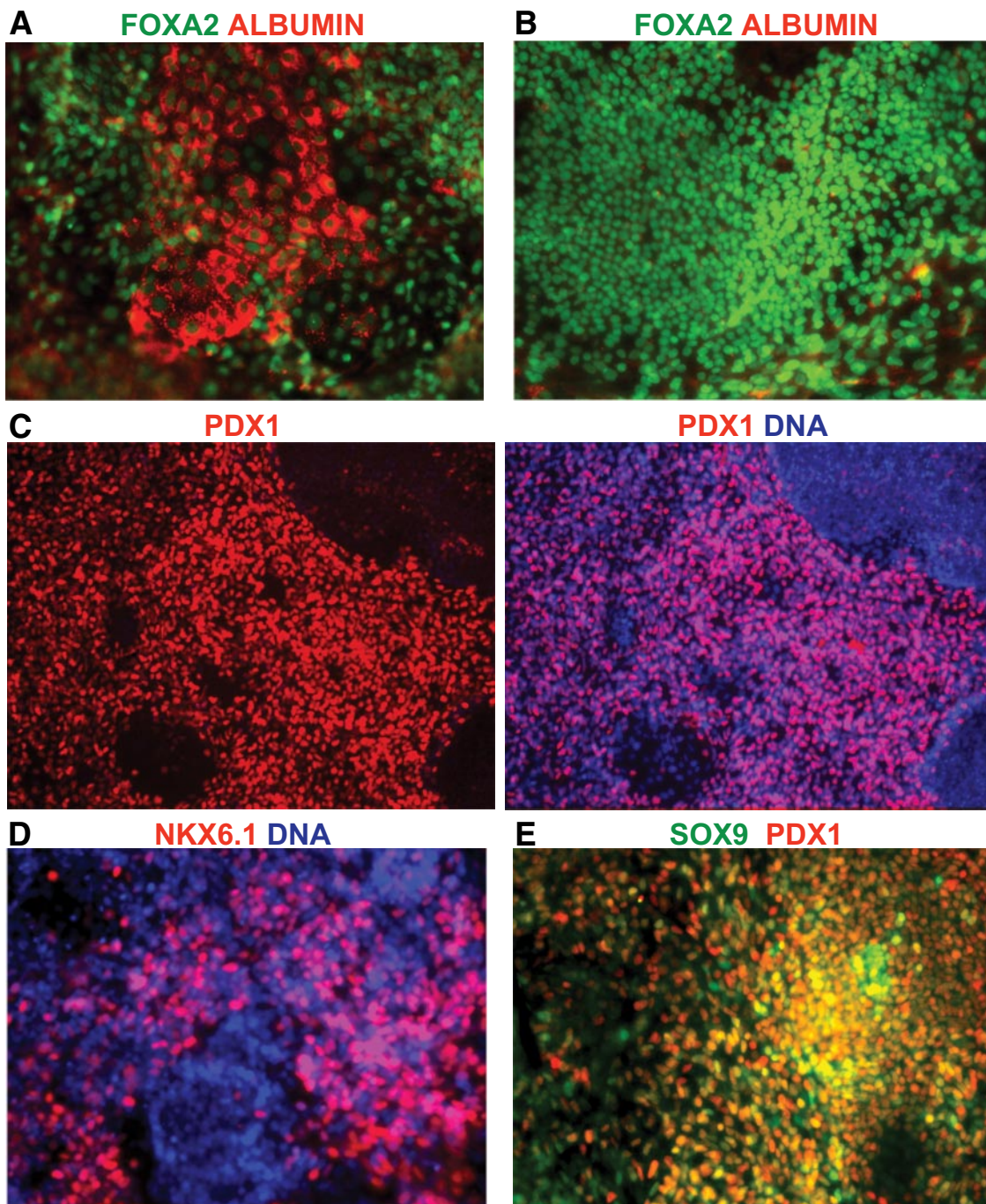


FIG. 2. Expression of pancreas-related transcription factors in hES-derived DE progenies treated with Noggin, RA, and cyclopamine. FOXA2 and albumin expression in control cultures (A) and following treatment with Noggin, RA, and cyclopamine (B). Note the major increase in FOXA2 and loss of ALB-expressing cells. C: Large clusters of PDX1⁺ progenitors are detected, representing 50–80% cells. D: Several NKX6.1⁺ cells are also detectable, the majority of which express lower levels of PDX1 (not shown). E: Co-expression of PDX1 and SOX9 transcription factors in differentiated cells. (A high-quality digital representation of this figure is available in the online issue.)

and/or RA after DE induction succeeded in generating PDX1⁺ cells without the use of hedgehog inhibitors (14,17,19,23). Owing to the previously described increase in the expression of hedgehog ligands and effectors upon DE induction, we added cyclopamine to the combination of Noggin and RA in our experimental setup, but it remains unclear whether this was mandatory for the efficient differentiation of PDX1⁺ cells that we observed (20,38,39). While waiting for conclusive data on this issue, we speculate that supplementation of hedgehog antagonists would become obsolete in settings where combined Noggin and RA

treatment of DE cells essentially induces a “true pancreatic endoderm” cell type, which is normally devoid of hedgehog activity. Gli1 transcripts profiling in cells treated with Noggin and RA combination would help to clarify this issue.

Amplification of early pancreas progenitors by FGF signaling. Pancreatic progenitor cells are characterized by their expression of a subset of transcription factors. For instance, the combined expression of PDX1, FOXA2, SOX9, HNF6, NKX6.1, and PTF1a (Fig. 3) define the multipotent pancreas progenitor (9,33). In the endoderm, the last two transcription factors are more specific to the

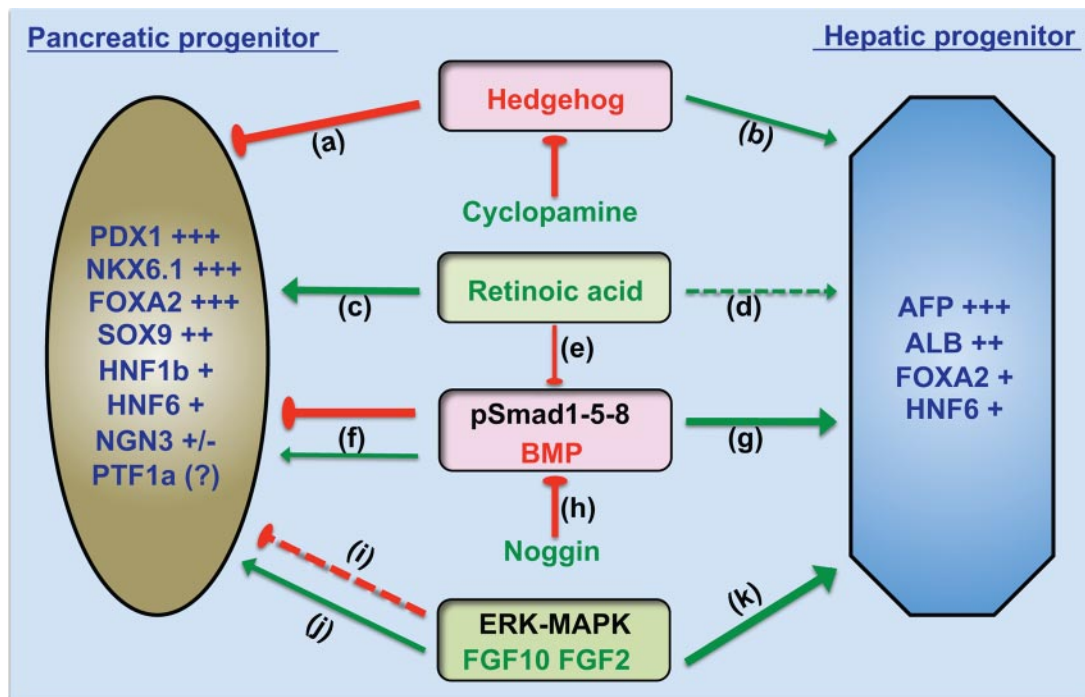


FIG. 3. Molecular pathways involved in pancreas and liver differentiation from hES cells. Four main pathways regulate the early stages of pancreas differentiation from definitive endoderm and control the acquisition of hepatic vs. pancreatic fate. Hedgehog signaling is well characterized as a potent inhibitor of pancreatic initiation (a) that is expressed in the hepatic domain (b) under the influence of FGF signaling. It functions in the establishment of organ domains. This pathway can be blocked by treatment with the alkaloid cyclopamine. Retinoic acid is expressed in the developing pancreas and participates in the induction of PDX1 expression (c). It is also suggested to contribute to hepatic gene expression to a certain degree (d). An inhibitory effect of retinoic acid on Smad1-5-8 phosphorylation (e) was demonstrated on hES-derived definitive endoderm seeded at low density, which contrasts with the early studies indicating activation of BMP signaling by retinoic acid. BMP is well described as an inhibitor of early pancreas development (f) in contrast to its requirement for hepatic initiation (g). This potent inhibitory effect at early stages gets reverted afterward and pancreatic progenitors require BMP signaling (f). The inhibition of BMP signaling in the pancreatic domain is under the control of Noggin (h). As for BMP pathway, FGF signaling via ERK/MAPK also controls early pancreas induction with high concentration being inhibitory (i) whereas low concentrations are required (j). In late stages, FGF plays in the proliferation of pancreas progenitors (j). On the contrary, this pathway represents an amplification signal (h) for the ALB⁺AFP⁺ hepatoblast. The markers displayed in the progenitors recall the current status of protein detection from differentiated hES cells.

pancreas, whereas PDX1, FOXA2, SOX9, and HNF6 are also detected in the adjacent duodenum, stomach, and liver. Following the induction of pancreas progenitor cells from hES-derived DE cells, PDX1 co-expression with FOXA2, SOX9, and HNF6 was demonstrated in a context where hepatic (AFP, ALB) and intestinal (CDX1, CDX2) markers were not induced or only minimally induced (Fig. 2) (14,17,20). However, none of the above-mentioned studies have assessed the expression of all the six transcription factors by the pancreatic progenitors obtained from hES cells. More specifically, demonstration of PTF1a expression has remained elusive until now, possibly owing to the lack of an optimal antibody against this protein. The same holds true for NGN3, which has been detected at the protein level in only few studies. In our experimental setup, *PTF1a* transcripts were detected only at low levels, whereas a significant emergence of NKX6.1-positive cells was denoted. Nevertheless, when combined with the demonstration of INS and PDX1 co-expression at later stages, these findings point toward the pancreatic nature of the differentiated PDX1⁺ cells. Additional efforts are required to obtain their efficient differentiation toward pancreatic endocrine cells in vitro (Table 3) (14,20).

During pancreas development, the PDX1⁺ progenitor cells are amplified thanks to signals emanating from the pancreatic mesenchyme. FGF10 controls this mesenchymal-to-epithelial interaction, and its over-expression beyond the competence window (E11.5–E13.5 in the mouse) significantly induces progenitor cell proliferation and arrests their terminal differentiation (40,41). This signaling

operates via activation of the Notch pathway, which temporally prevents progenitor differentiation toward the endocrine or exocrine fates (42). In agreement with these, FGF10 or EGF supplementation induces the proliferation of hES-derived PDX1⁺ cells, suggesting the contribution of ERK/MAPK and AKT activation in this effect (14,16,20). Furthermore, before the proliferation step, establishment of the PDX1⁺ progenitors from hES-derived definitive endoderm was also shown to be highly dependent on ERK/MAPK signaling and possibly mediated by FGF2 (20,43).

Prospects for further endocrine differentiation of hES cell-derived pancreatic progenitors. The initiation of endocrine differentiation from the PDX1⁺ progenitors relies on the activation of NGN3 expression, which should be paralleled or preceded by downregulation of Notch activity and further sustained by additional induction of PAX6 (Fig. 1) (44,45). Although the transcription factors cascade regulating pancreatic endocrine differentiation has been largely explored, to date the instructive extracellular signals that trigger their coordinated expression remain elusive. To this end, there is a growing list of proposed growth factors and chemicals whose effects will need to be extensively assessed in the endocrine commitment and maturation of PDX1⁺ progenitors obtained in vitro from hES-derived DE cells. These include but are not limited to Wortmannin and LY294002 that are PI3K inhibitors (46), VEGF (4,47), RA (26), Conophylline (48), Wnt, and Hedgehog ligands (49,50), and antagonists of the Notch pathway such as gamma secretase inhibitors, Notch

ligands, and soluble Notch. Not only is the nature of these factors important, but their timely combination will also be of consideration given the demonstration of a competence window for endocrine patterning beyond which the endocrine differentiation arrest becomes irreversible (41). For instance in our experimental setup (Table 3), FGF10 was supplemented for controlling PDX1⁺ cell proliferation together with a gamma secretase inhibitor (Compound E) for Notch antagonism in view of endocrine induction (20). Considering the above-mentioned crosstalk between FGF10 and Notch activity in the proliferation of the PDX1⁺ progenitors, such a protocol therefore included two phenomena that were actually antagonistic. This issue might explain the very limited progression toward endocrine cells that we obtained, a hypothesis that will need further evaluation by dissociating FGF10 treatment from Notch inhibition in this protocol.

The next step after endocrine commitment will be the further endocrine differentiation and maturation by use of old and new players such as betacellulin, activin, exendin-4, insulin-like growth factor, HGF, nicotinamide, bone morphogenetic protein, FGF, and RA (14,16,18,20,26,28). Similarly, three-dimensional cultures will merit further investigation in order to mimic the actual environment of the β -cells in islets as was recently described in differentiating hES cells (16). Some of these factors have been used singly or in combination on PDX1⁺ progenitors generated from hES cells. Because of the complex nature of pancreatic endocrine differentiation, which is presently not yet elucidated, the generation of mature and functional β -cells has not been efficiently achieved *in vitro*. The demonstration of human-specific β -cell functions in animal models following transplantation of pancreatic progenitors derived from hES cells (19,32,51) underscores this gap in our current knowledge. Based on these data, it has been proposed that diabetic patients could be grafted with pancreatic progenitors derived from hES cells, eliminating the need for finding the efficient strategy for terminal differentiation *in vitro*. However, this approach would be hampered by teratoma formation from cells that were not fully differentiated at the time of transplantation (see below).

Molecular beacons for cell selection. Although the differentiation of specific cell types such as pancreatic cells can be established through their characterization with several markers, it remains obvious that no protocol currently offers a factual conversion of all cultured stem cells to the desired phenotype. Therefore, the possibility exists that other cell types or even multi- or pluripotent cells might be transplanted to the diabetic recipients, which raises safety issues. Indeed, despite the fact that functional β -cells developed *in vivo* after transplantation of hES-derived pancreatic cell preparations, teratoma formation was also observed in at least 15% of grafts (19,51,52). Several tools have been investigated in order to select out the undifferentiated cells that express surface markers (SSEA4, SSEA3, TRA-1-60, TRA-1-81) or to specifically isolate the desired cell type (cell-trapping) that have been modified to express an antibiotic resistance gene, a fluorescent or a bioluminescent reporter under CK19, PDX1, or INS promoter (53–55). Whereas these techniques would allow for the selection of differentiated β -cells up to purity, transplantation of such cells is presently not approved given the genotoxic, the mutagenic, and the oncogenic risks associated with transgenic approaches. New transgenic designs are currently under development to specifically address these issues.

Concluding remarks. The *in vitro* differentiation of functional β -cells from human pluripotent cells (hES and iPS) represents a major challenge for diabetes cell therapy in the future. Progress toward this goal already encompasses the efficient generation of definitive endoderm and PDX1-expressing pancreas progenitors. The recent successful studies indicate that the differentiation of PDX1⁺ progenitors concomitantly requires at least two events after DE establishment in order to mimic the *in vivo* situation: blockade of hepatic induction by BMP antagonism and induction of pancreas progenitors by retinoid signaling (Fig. 3). Optimization of this step might still prove beneficial in order to unravel the role of FGF and hedgehog modulation and in order to definitely establish cells that undoubtedly show combined expression of the major transcription factors recognized for the multipotent pancreas progenitor, namely PDX1, PTF1a, and NKX6.1. In addition, the recently described temporal shift in the requirement for BMP antagonism to a need for BMP signaling during mouse pancreas development will merit implementation in hES cell differentiation. Despite the latest data indicating differentiation of the PDX1⁺ progenitors into insulin/C-peptide⁺ cells, we believe that additional work is needed for an optimal *in vitro* control of the progression toward NGN3-expressing endocrine progenitor cells and for the maturation of glucose-sensing and insulin-secretion functions in the β -like cells. A step forward toward these goals will certainly shorten the time frame for achieving our objective of using hES cells as alternative sources of functioning β -cells in diabetes cell therapy.

ACKNOWLEDGMENTS

This work was supported by a Vrije Universiteit Brussel (VUB) research grant under the code GOA41-Vrije Universiteit Brussel. J.K.M. is a postdoctoral research fellow with the VUB.

No potential conflicts of interest relevant to this article were reported.

J.K.M. researched the data, contributed to the discussion, and wrote, reviewed, and edited the manuscript. B.C. contributed to the discussion. L.S. contributed to the discussion. K.S. contributed to the discussion and reviewed/edited the manuscript. L.B. contributed to the discussion and reviewed/edited the manuscript.

REFERENCES

1. Wells JM, Melton DA. Vertebrate endoderm development. *Annu Rev Cell Dev Biol* 1999;15:393–410
2. Cleaver O, Krieg PA. Notochord patterning of the endoderm. *Dev Biol* 2001;234:1–12
3. Zaret KS. Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 2002;3:499–512
4. Lammert E, Cleaver O, Melton D. Induction of pancreatic differentiation by signals from blood vessels. *Science* 2001;294:564–567
5. Gittes GK, Galante PE, Hanahan D, Rutter WJ, Debase HT. Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. *Development* 1996;122:439–447
6. Bonal C, Herrera PL. Genes controlling pancreas ontogeny. *Int J Dev Biol* 2008;52:823–835
7. Gittes GK. Developmental biology of the pancreas: a comprehensive review. *Dev Biol* 2009;326:4–35
8. Kim SK, Hebrok M. Intercellular signals regulating pancreas development and function. *Genes Dev* 2001;15:111–127
9. Oliver-Krasinski JM, Stoffers DA. On the origin of the beta cell. *Genes Dev* 2008;22:1998–2021
10. Jensen J. Gene regulatory factors in pancreatic development. *Dev Dyn* 2004;229:176–200

11. Tian T, Meng AM. Nodal signals pattern vertebrate embryos. *Cell Mol Life Sci* 2006;63:672–685
12. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* 2005;23:1534–1541
13. Vallier L. Differentiation of human pluripotent stem cells into pancreatic and hepatic progenitors using chemically defined culture conditions. Paper presented at *The European Molecular Biology Organization Workshop: Beta Cell Differentiation and Regeneration*, 26 February–1 March 2009, Peebles, Scotland, UK
14. Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y, Deng H. Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Res* 2009;19:429–438
15. McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE, Dalton S. Activin A efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells* 2007;25:29–38
16. Jiang J, Au M, Lu K, Eshpeter A, Korbitt G, Fisk G, Majumdar AS. Generation of insulin-producing islet-like clusters from human embryonic stem cells. *Stem Cells* 2007;25:1940–1953
17. Johannesson M, Stahlberg A, Ameri J, Sand FW, Norman K, Semb H. FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner. *PLoS One* 2009;4:e4794
18. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 2006;24:1392–1401
19. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* 2008;26:443–452
20. Mfopou JK, Chen B, Mateizel I, Sermon K, Bouwens L. Noggin, retinoids, and fibroblast growth factor regulate hepatic or pancreatic fate of human embryonic stem cells. *Gastroenterology* 2010;138:2233–2245
21. Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, Sato Y, Cowan CA, Chien KR, Melton DA. Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat Biotechnol* 2008;26:313–315
22. Hay DC, Zhao D, Ross A, Mandalam R, Lebkowski J, Cui W. Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities. *Cloning Stem Cells* 2007;9:51–62
23. Cai J, Yu C, Liu Y, Chen S, Guo Y, Yong J, Lu W, Ding M, Deng H. Generation of homogeneous PDX1⁺ pancreatic progenitors from human ES cell-derived endoderm cells. *J Mol Cell Biol* 2010;2:50–60
24. Wandzioch E, Zaret KS. Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science* 2009;324:1707–1710
25. Stafford D, Hornbruch A, Mueller PR, Prince VE. A conserved role for retinoid signaling in vertebrate pancreas development. *Dev Genes Evol* 2004;214:432–441
26. Ostrom M, Loffler KA, Edfalk S, Selander L, Dahl U, Ricordi C, Jeon J, Correa-Medina M, Diez J, Edlund H. Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into beta-cells. *PLoS One* 2008;3:e2841
27. Martín M, Gallego-Llamas J, Ribes V, Keding M, Niederreither K, Chambon P, Dollé P, Gradwohl G. Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. *Dev Biol* 2005;284:399–411
28. Jiang W, Shi Y, Zhao D, Chen S, Yong J, Zhang J, Qing T, Sun X, Zhang P, Ding M, Li D, Deng H. In vitro derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Res* 2007;17:333–344
29. Micallef SJ, Janes ME, Knezevic K, Davis RP, Elefanty AG, Stanley EG. Retinoic acid induces Pdx1-positive endoderm in differentiating mouse embryonic stem cells. *Diabetes* 2005;54:301–305
30. Nakanishi M, Hamazaki TS, Komazaki S, Okochi H, Asashima M. Pancreatic tissue formation from murine embryonic stem cells in vitro. *Differentiation* 2007;75:1–11
31. Shi Y, Hou L, Tang F, Jiang W, Wang P, Ding M, Deng H. Inducing embryonic stem cells to differentiate into pancreatic beta cells by a novel three-step approach with activin A and all-trans retinoic acid. *Stem Cells* 2005;23:656–662
32. Shim JH, Kim SE, Woo DH, Kim SK, Oh CH, McKay R, Kim JH. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. *Diabetologia* 2007;50:1228–1238
33. Van Hoof D, D'Amour KA, German MS. Derivation of insulin-producing cells from human embryonic stem cells. *Stem Cell Res* 2009;3:73–87
34. Rogers MB, Rosen V, Wozney JM, Gudas LJ. Bone morphogenetic proteins-2 and -4 are involved in the retinoic acid-induced differentiation of embryonal carcinoma cells. *Mol Biol Cell* 1992;3:189–196
35. Shen H, Luo Y, Kuo CC, Deng X, Chang CF, Harvey BK, Hoffer BJ, Wang Y. 9-Cis-retinoic acid reduces ischemic brain injury in rodents via bone morphogenetic protein. *J Neurosci Res* 2009;87:545–555
36. Rodriguez-Leon J, Merino R, Macias D, Gañan Y, Santesteban E, Hurlé JM. Retinoic acid regulates programmed cell death through BMP signalling. *Nat Cell Biol* 1999;1:125–126
37. Pan FC, Chen Y, Bayha E, Pieler T. Retinoic acid-mediated patterning of the pre-pancreatic endoderm in *Xenopus* operates via direct and indirect mechanisms. *Mech Dev* 2007;124:518–531
38. Mfopou JK, Bouwens L. Hedgehog signals in pancreatic differentiation from embryonic stem cells: revisiting the neglected. *Differentiation* 2008;76:107–117
39. Mfopou JK, De Groote V, Xu X, Heimberg H, Bouwens L. Sonic hedgehog and other soluble factors from differentiating embryoid bodies inhibit pancreas development. *Stem Cells* 2007;25:1156–1165
40. Bhushan A, Itoh N, Kato S, Thiery JP, Czernichow P, Bellusci S, Scharfmann R. Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development* 2001;128:5109–5117
41. Kobberup S, Schmeerr M, Dang ML, Nyeng P, Jensen JN, MacDonald RJ, Jensen J. Conditional control of the differentiation competence of pancreatic endocrine and ductal cells by Fgf10. *Mech Dev* 2010;127:220–234
42. Hart A, Papadopoulou S, Edlund H. Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. *Dev Dyn* 2003;228:185–193
43. Ameri J, Stahlberg A, Pedersen J, Johannesson JK, Johannesson MM, Artner I, Semb H. FGF2 specifies hESC-derived definitive endoderm into foregut/midgut cell lineages in a concentration-dependent manner. *Stem Cells* 2010;28:45–56
44. Cras-Méneur C, Li L, Kopan R, Permutt MA. Presenilins, Notch dose control the fate of pancreatic endocrine progenitors during a narrow developmental window. *Genes Dev* 2009;23:2088–2101
45. Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* 2000;97:1607–1611
46. Elghazi L, Rachdi L, Weiss AJ, Cras-Méneur C, Bernal-Mizrachi E. Regulation of beta-cell mass and function by the Akt/protein kinase B signalling pathway. *Diabetes Obes Metab* 2007;9(Suppl. 2):147–157
47. Lammert E, Gu G, McLaughlin M, Brown D, Brekken R, Murtaugh LC, Gerber HP, Ferrara N, Melton DA. Role of VEGF-A in vascularization of pancreatic islets. *Curr Biol* 2003;13:1070–1074
48. Kojima I, Umezawa K. Conophylline: a novel differentiation inducer for pancreatic beta cells. *Int J Biochem Cell Biol* 2006;38:923–930
49. Heiser PW, Lau J, Taketo MM, Herrera PL, Hebrok M. Stabilization of beta-catenin impacts pancreas growth. *Development* 2006;133:2023–2032
50. Nakayama S, Arakawa M, Uchida T, Ogihara T, Kanno R, Ikeda F, Azuma K, Hirose T, Kawamori R, Fujitani Y, Watada H. Dose-dependent requirement of patched homologue 1 in mouse pancreatic beta cell mass. *Diabetologia* 2008;51:1883–1892
51. Eshpeter A, Jiang J, Au M, Rajotte RV, Lu K, Lebkowski JS, Majumdar AS, Korbitt GS. In vivo characterization of transplanted human embryonic stem cell-derived pancreatic endocrine islet cells. *Cell Prolif* 2008;41:843–858
52. Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 2005;166:1781–1791
53. Shiraki N, Yoshida T, Araki K, Umezawa A, Higuchi Y, Goto H, Kume K, Kume S. Guided differentiation of embryonic stem cells into Pdx1-expressing regional-specific definitive endoderm. *Stem Cells* 2008;26:874–885
54. Ben-Yehudah A, White C, Navara CS, Castro CA, Ize-Ludlow D, Shaffer B, Sukhwani M, Mathews CE, Chaillet JR, Witchel SF. Evaluating protocols for embryonic stem cell differentiation into insulin-secreting beta-cells using insulin II-GFP as a specific and noninvasive reporter. *Cloning Stem Cells* 2009;11:245–257
55. Soria B, Roche E, Berná G, León-Quinto T, Reig JA, Martín F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 2000;49:157–162