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Milestones of Pancreatic Beta Cell Differentiation from Embryonic Stem Cells

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ABSTRACT

Transplantation of pancreatic islets of Langerhans has proven effective in setting diabetic patients insulin-free for periods longer than a year, resulting in a net improvement in generic measures of quality of life. Although very promising, this treatment cannot yet be generally applied because of shortage in brain-dead heart-beating islet donors. Several non-endocrine pancreatic cells and non-pancreatic cells are presently investigated for their possible use as alternative sources of islets needed for diabetes cell therapy. Among them, embryonic stem (ES) cells are valued for their high proliferative capacity and their potential to generate progenies of all three developmental germ layers. Till date, several attempts to generate pancreatic epithelial cells, namely insulin-producing beta cells from rodent and primate ES cells have been initiated by different groups and resulted in diverse success rates. One of the most striking limiting factors has been the lack of a comprehensive efficient and reproducible protocol taking into account the main features of normal pancreas development *in vivo*. This review focuses on the successive achievements in this field, with special credits to the ideological milestones of the so far proposed protocols for generation of beta cells from ES cells.

Keywords: activin, definitive endoderm, diabetes, insulin, pancreas development

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INTRODUCTION

Diabetes mellitus is the leading metabolic disease, a growing public health burden that presently affects 200 million individuals worldwide and shows estimates up to 366 millions by the year 2030 (Wild *et al*. 2004). Two major types of diabetes are recognized and clinically predominant: Type I or insulin-dependent diabetes that follows immune attack to beta-cells with a resulting loss of endogenous insulin production, and Type II or non-insulin-dependent diabetes which develops in a context of overweight and insulin resistance. Despite the key improvements in diabetes management during the past 20 years, it remains not yet absolute that severe debilitating complications can be prevented by these treatments (The Diabetes Control and Complications Trial Research Group 1993). Indeed, exogenous insulin supply or other pharmacological agents do not perfectly mimic the glucose sensing and insulin release functions of beta cells within islets of Langerhans.

In order to restore the loss of endogenous insulin production in type I diabetes and in subsets of advanced type II diabetes, pancreas transplantation was considered, but has been mainly limited by technical issues. Cell replacement therapy, namely transplantation of purified islets of Langerhans into the portal vein of the liver, is currently considered as an interesting alternative to insulin therapy in the management of type I diabetic patients with poor metabolic control and frequent episodes of hypoglycemia. This procedure improves patients' quality of life and leads to insulin independence for periods as long as 5 years, though only 10% of transplanted patients still do not require insulin at these later stages (Shapiro *et al*. 2000; Ryan *et al*. 2005;

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Invited Review

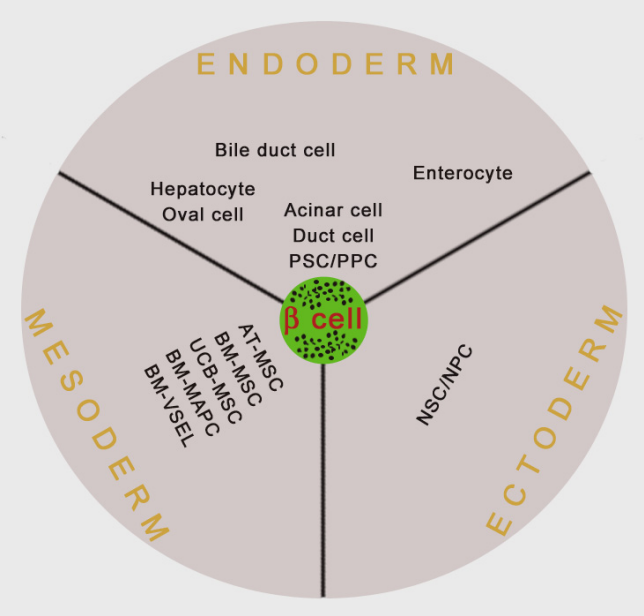

Fig. 1 Potential sources of beta cells. Several studies have documented the possible generation of beta-like cells from differentiated cells of the endoderm lineage (liver, bile duct, intestine, exocrine pancreas), or from stem cells that have their niche in the mesoderm- or ectoderm-derived organs. It is at present widely accepted that pluripotent embryonic stem cells can be directed towards a beta cell phenotype following an initial step of definitive endoderm differentiation. PSC/PPC: putative pancreatic stem or pancreatic progenitor cell; NSC/NPC: neural stem or neural progenitor cell; AT-MSC: adipose tissue-derived mesenchymal stem cell; BM: bone marrow-derived MSC; UCB-MSC: umbilical cord blood-derived MSC; BM-MAPC: BM-derived multipotent adult progenitor cell; BM-VSEL: BM-derived very small embryonic-like cell.

Poggioli *et al*. 2006; Shapiro *et al*. 2006). Following a recent evaluation of glycemic control in type 1 diabetic recipients of islets grafts, it appears that the initial beta cell mass transplanted should be at least 2 x 10^6/kg body weight in order to achieve a functioning graft by post-transplant month 2 (C-peptide levels), avoid the need for a second graft and obtain a good metabolic control (HbA1c, coefficient of variation of glycemia) within the next 10 months (Keymeulen *et al*. 2006). The main challenge of a therapy with such critical requirements is that donor pancreata are not sufficiently available to allow for transplantation of the majority, not to mention all diabetic patients in need. This condition fostered interest of several research groups in the hunt for clinically relevant alternatives for cadaveric islet cells. These presently include the replication of beta cells, the (trans)differentiation of non-endocrine pancreatic cells (duct- and acinar cells, putative pancreatic stem/progenitor cells), the differentiation of non-pancreatic endoderm-derived cells (hepatocytes, bile duct cells, enterocytes), and the differentiation of adult (bone marrow-derived or adipose tissue-derived mesenchymal stem cells, neural stem/progenitor cells, liver oval cells) or embryonic stem cells (Kaczorowski *et al*. 2002; Bonner-Weir and Weir 2005; Fellous *et al*. 2006; Santana *et al*. 2006) (**Fig. 1**). Because of their high proliferative and self-renewal capacity, and their potential to generate progenies of all three developmental germ layers (ectoderm, mesoderm, endoderm) *in vitro* and in teratoma *in vivo*, ES cells are considered as one of the most promising source for generation of islet-like cells needed for diabetes cell therapy. More than six years have passed since the first study reported expression of insulin gene by progenies of mouse ES cells (Soria *et al*. 2000). Despite the large amount of work carried out in order to confirm and improve these initial findings in both rodents and primates ES cell lines, there are still important challenges remaining before the introduction of ES cells into clinics. This review will recapitulate the main strategic landmarks of the development of beta-cell therapy from ES cells.

ES CELL DISCOVERY AND POTENTIALS IN CELL THERAPY

EC cells as a model for embryological studies *in vitro*

Embryonal carcinoma (EC) cells are multipotent cell lines derived from spontaneous teratocarcinoma initially described in mouse testis, but which has been observed in human testis and ovary as well. They are thus considered as the stem cells of the germ cell-derived tumors (Kleinsmith and Pierce 1964; Friedrich *et al*. 1983). Several lines of EC cells have been derived from mouse and human teratocarcinoma and their multipotential property confirmed in single cell transplantation experiments (Martin and Evans 1975; Nicolas *et al*. 1976; Thompson *et al*. 1984; Pera *et al*. 1989). For decades, these cells have fuelled the basic *in vitro* studies of cell fate determination and differentiation at the molecular level; somewhat establishing the path to a better understanding of mammalian development. Indeed, it was very soon recognized that EC cell differentiation *in vitro* is an "orderly" process that mirrors the development of the early embryo, starting with the formation of an outer layer of endoderm cells as occurs at the surface of the inner cell mass. Therefore, these cells represented an important instrument in the investigation of processes otherwise inaccessible in the developing embryo (Martin and Evans 1975; Przyborski *et al*. 2004). The finding that EC cells share several properties with the inner cell mass (ICM) cells of blastocysts, as well as the knowledge gained in manipulating EC cells *in vitro* led to the development of techniques for derivation of their embryonic counterparts, namely embryonic stem (ES) cells from blastocysts of mouse (Evans and Kaufman 1981; Martin 1981) and later on human origin (Thomson *et al*. 1998).

Although both mice and human ES cells share some essential characteristics such as derivation from the preimplantation blastocyst (i), prolonged proliferation in an undifferentiated state (ii), and potential to form derivatives of all three germ layers (iii), there are several differences in their molecular features and in their respective manipulation in the lab. Whereas Oct4 and Nanog are well recognized endogenous transcription factors required for pluripotency in both lines, mES and hES cells differ in their requirements for feeder layer, leukemia inhibitory factor (LIF), bone morphogenetic protein 4 (BMP4), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ)/Activin/Nodal or Wnt signaling as exogenous factors sustaining undifferentiated state and inhibiting differentiation (Niwa *et al*. 1998; Mitsui *et al*. 2003; Chambers 2004; Humphrey *et al*. 2004; O'Shea 2004; Sato *et al*. 2004; Beattie *et al*. 2005; James *et al*. 2005; Vallier *et al*. 2005; Hao *et al*. 2006). These molecular dissimilarities fuelled investigations aiming at deciphering whether they relate to the species difference or to the time point in embryonic development at which the respective cell lines are derived (embryonic day (E) 3.5 in mouse, and E5 in human). It is now known that pluripotent stem cells can also be derived from pre-blastocyst mouse embryos (less than E3.5), as well as from mouse and rat post-implantation embryos. Interestingly, the post-implantation epiblast-derived mouse and rat pluripotent stem cells named EpiSCs, share similar self-renewal mechanisms (Activin/Nodal) with human ES cells and differentiate to all three germ layers *in vitro* and *in vivo* (Tesar 2005; Brons *et al*. 2007; Tesar *et al*. 2007).

Lineage differentiation of ES cells: ectoderm-mesoderm-endoderm

Analysis of differentiated cells in teratocarcinomas from implanted EC cells obviously indicated that they belong to the three germ layers of mammalian development (ecto-

derm, mesoderm and endoderm), which is concordant with their definition as pluripotent cells. Also, their *in vitro* differentiation was shown to follow a similar pattern as *in vivo*, starting with the formation of spherical aggregates named embryoid bodies (EBs) because of their resemblance with mouse embryo at the egg-cylinder stage. In these aggregates, the initial sign of differentiation is the formation of an outer layer of endoderm cells showing enlarged endoplasmic reticulum and prominent microvilli. This cell layer is referred to as visceral endoderm, an equivalent of the extra-embryonic endoderm in vertebrate development (Martin and Evans 1975). The visceral endoderm of EBs surrounds a multilayered columnar epithelium, which represents the primitive ectoderm or the epiblast cells.

As embryonal carcinoma cells were at the root of ES cell discovery, the differentiation of the later was as well initially investigated via the formation of embryoid bodies (Martin 1981). EBs formation induced by hanging-drop or suspension culture in the absence of self-renewal factors has remained for a long time the primary step if not the unique setting of nearly all differentiation protocols. However EBs culture does not constitute an efficient method to obtain purified populations of differentiated cells amenable to cell therapy. Indeed, differentiation within EBs is not patterned as in the embryo and results into mixtures of several cell types even in conditions where extra-cellular molecules are added to favor one particular lineage (O'Shea 2004). It is nowadays widely recognized that ES cells can differentiate in two dimensions upon withdrawal of self-renewal factors, with or without concomitant supplementation of "fate-determining" molecules. In these settings, neuroectodermal differentiation represents the default differentiation pathway (Tropepe *et al*. 2001; Smukler *et al*. 2006).

Ectoderm and mesoderm are the germ layers whose differentiation from ES cells has been the most studied. There are currently established protocols for the differentiation of sensory and motor neurons, astrocytes, oligodendrocytes, as well as glial cells from mouse and human ES cells; and some studies further characterized the *in vivo* functional integration of differentiated cells (Brustle *et al*. 1999; Wichterle *et al*. 2002; Scheffler *et al*. 2003; Maye *et al*. 2004; Keirstead *et al*. 2005; Li *et al*. 2005). Similarly, cells originating from the mesoderm germ layer such as cardiomyocytes, skeletal myoblasts, osteoblasts, chondroblasts and endothelial cells can be efficiently differentiated from embryonic stem cells, and signals from molecules of the TGF-β/Activin/Nodal family play crucial roles in the initiation of mesodermal tissues (Johansson and Wiles 1995; Schuldiner *et al*. 2000; Pfendler *et al*. 2005; Tada *et al*. 2005; zur Nieden *et al*. 2005; Barberi *et al*. 2007). It is just recently that the derivation of cells representing definitive endoderm has been achieved via activation of SMAD (mothers against decapentaplegic homolog) pathway with high concentrations of activin-A, somewhat reproducing the findings from *Xenopus* animal cap assay in embryonic stem cells (Okabayashi and Asashima 2003; Kubo *et al*. 2004; D'Amour *et al*. 2005).

Theoretical basis for cell therapy applications

It is well recognized that several chronic and degenerative diseases are the result of a particular cell loss or functional failure. This includes, just to mention a few, the loss of dopaminergic neurons in Parkinson's disease, the loss of insulin-producing beta cell mass/function in diabetes, or the ischemic injury to contracting cardiomyocytes that results into myocardial infarction. Some of these diseases can be clinically controlled via supplementation of active molecules lost in the disease process. However such treatments only partially mimic the normal physiological function, therefore bring only temporary symptomatic relief and cannot halt the disease progression or cure the patient (**Table 1**). The ultimate treatment for such diseases would be the replacement of the initial functional cell population presently lost in the disease process, a procedure referred to as "cell replacement therapy" or "regenerative medicine". This theoretical assertion has been clinically implemented for decades with bone marrow transplants in hematopoeitic diseases. It was recently extended to selected diabetic patients by transplantation of pancreas or most usually purified islets of Langerhans, resulting in long-term independence from daily insulin injections and improvement of quality of life (Shapiro *et al*. 2000; Ryan *et al*. 2005; Keymeulen *et al*. 2006; Shapiro *et al*. 2006). The main challenge for its wide utilization in clinics is the limited availability of transplantation material, which currently is mainly procured from heart-beating brain-dead donors. The scientific community strives to find alternatives to cadaveric donors. To this point, embryonic stem cells, on the basis of their wide developmental capacities, represent an abundant source of functional cells, provided their differentiation to the desired cell type could be adequately controlled *in vitro*. As such, the

Table 1 Scope of regenerative medicine. Diseases that are related to loss of endogenous cell mass or function can be treated by replacement of the default cell population with cells from exogenous sources or by stimulating a regenerative process from endogenous progenitors when these latter are known. Exogenous cells could be obtained by *in vitro* differentiation of autologous cells, or via directed differentiation of pluripotent embryonic stem cells. Diseases that can benefit from such therapy options encompass several organs representing the three mammalian germ layers. A short list of the most common ones is given in the table.

Germ layer	Organ	Disease	Cellular or molecular basis	Actual treatment
ENDODERM	Pancreas	Diabetes mellitus	Beta-cell loss (mass and/or function), insulinopenia	Daily insulin injections, pancreas or islets TPT
	Liver	Metabolic liver diseases	Mutation of genes encoding metabolic enzymes	Metabolic control, palliative support, liver TPT
		Cirrhosis	Primary or cryptogenic, fibrosis	Symptomatic relief, liver TPT
	Lungs	Cystic fibrosis	Mutation CFTR gene, multiple organs affected	Organ dependent symptomatic relief, lung TPT
MESODERM	Skeletal muscle	Duchenne muscular dystrophy	Genetic lack of dystrophin	Palliative support
	Myocardium	Myocardial infarction	Ischemic cell death => scar formation and arrythmias	Emergency management for reperfusion, stem cell trials
		Dilated cardiomyopathy	Presenilin mutation	Heart function improvement, Pacemakers, Heart TPT
	Kidney	Alport Syndrome	Mutation Collagen-IV: structural damage of glomerular BM	Long-term dialysis, Kidney TPT
ECTODERM	DOPA neurons	Parkinson's disease	Loss of midbrain DOPA neurons	Dopaminergic + antidyskinesis drugs, brain stimulation
	Motor neurons	Amyotrophic lateral sclerosis	Mutation SOD1 gene => motor neuron destruction	Palliative support
	Brain	Alzheimer's disease	Presenilin mutation	Palliative support

Abbreviations: BM, basement membrane; CFTR, cystic fibrosis transmembrane conductance regulator; DOPA, dopaminergic; TPT, transplantation; SOD1, superoxide dismutase

scope of regenerative medicine possibly will be opened to many other chronic or degenerative disturbances than diabetes, as depicted in **Table 1**.

CHRONICLE OF PANCREATIC DIFFERENTIATION FROM ES CELLS

The pioneer study: antibiotic selection of ES cell clones with active insulin promoter

The first study to demonstrate the expression and synthesis of insulin by ES-derived cells was published by Soria et al. in 2000. Mouse ES-cells were transfected with an antibiotic resistance gene driven by insulin promoter sequence, ensuring the survival of only insulin-expressing cells upon supplementation of the antibiotic geneticin to the culture medium. After several rounds of culture and final maturation in the presence of nicotinamide and low glucose concentrations, 8 clones of geneticin-resistant cells were identified out of 784. The insulin content of the cells generated by this procedure amounted to 90% of mouse islet insulin content and they were able to restore normoglycemia when transplanted in animals rendered diabetic by streptozotocin injection (Soria *et al*. 2000). This study constituted the proof of principle that ES cells could synthesize insulin. However the antibiotic selection pressure and the low success rate (1 out of 100 clones), and the need of DNA transfection represent limitations to potential expansion and clinical application to many candidate islets transplant recipients.

Spontaneous differentiation of ES cells to pancreatic cells: an orderly yet stochastic world!

Although the study by Soria *et al.* (2000) selectively generated cells expressing insulin *in vitro*, a second type of proof that ES cells could follow the pancreatic pathway was the demonstration that rhesus monkey ES cells injected in severe combined immuno-deficient (SCID) mice give rise to teratomas containing fully differentiated gut like structures with branching ducts, as well as cells transcribing pancreatic-islet specific genes such as *glucagon*, *somatostatin*, *β-glucokinase* and glucose transporter-2 (*Glut2*) (Jacobson *et al*. 2001). A parallel study used human ES cells (H9 cell line) cultured as embryoid bodies (EBs) in suspension up to 30 days and showed for the first time that between 60 to 70% EBs contain insulin-positive cells (1-3% total cells). These EBs initially expressed mRNAs of transcription factors necessary for pancreas development (*pancreas duodenum homeobox-1* (*Pdx1*), *neurogenin-3* (*Ngn3*)), followed by those of beta cell markers *insulin*, *glucokinase* and *Glut2* (Assady *et al*. 2001). Mouse ES cells were allowed to form EBs and differentiated for 2 weeks on gelatin-coated dishes in the presence of 10-15% serum and nicotinamide. These cultures generated 0.3% insulin-positive cells, however detection of *insulin1* expression required the use of nested-PCR. Differentiated cells could be maintained and subcultured up to 9 times in 15% serum and nicotinamide. This maintenance was achieved at least in part via proliferation of insulin-positive cells or their progenitors as indicated by bromodeoxyuridine (BrdU) incorporation (Ibii *et al*. 2007).

These first generation studies therefore indicated that in the absence of any selection pressure, a limited proportion of ES cells spontaneously differentiate to pancreatic phenotype both *in vivo* and *in vitro*. Sequential analysis of pancreas transcription factors and markers over a 5-week differentiation course indicated a stepwise transition through characteristic stages of pancreas development, suggesting recapitulation of the normal islet cyto-differentiation from ES cells (Kahan *et al*. 2003). However, in these settings, the proportion of cells committing to the pancreatic lineage remains very low and unpredictable and they have a very low insulin content (**Table 2**), raising several issues:

(1) Detection: the limited number of ES-derived beta-like cells and their low cellular insulin content represent a technical challenge for *in vitro* analysis, especially when the detection system relies on activation of insulin promotor. Similarly, differentiated EBs cells express insulin mRNA at levels at least 100.000 times lower than whole pancreas level (Kahan *et al*. 2003; Mfopou *et al*. 2005; Lavon *et al*. 2006). In order to ease *in-situ* identification of the rare insu-

Table 2 Insulin expression in ES-derived beta-like cells. Several protocols have been used to generate beta-like cells from embryonic stem cells. Assuming no major discrepancy in technical assessment of beta cell function among different groups, these cells vary considerably in their intracellular insulin content (ICIC) and in their glucose-stimulated insulin secretion (GSIS). The levels achieved so far usually represent only limited fraction of that recorded from genuine pancreatic beta cells. In contrast to other insulin secretagogues, glucose response of these cells is poor.

ES cells	Major protocol features	ICIC (a)	GSIS (a)	GSIS (b)	References
Mouse	Insulin promoter cell trapping, NA	16500	318.40	6.8	Soria *et al.* 2000
	Nkx6.1 cell trapping, NA	160	4	5.0	Léon-Quinto *et al.* 2004
	Nestin+ selection, ITS-FGF-NA	145	2.87	-	Lumelsky *et al.* 2001
	Nestin+ selection, PI3K inhibitor	11300	390	-	Hori *et al.* 2003
	Nestin+ seclection, feeder free	-	4.73	2.5	Moritoh *et al.* 2003
	Nestin+ selection	-	§	1.0	Sipione *et al.* 2004
	Nestin+ selection, GLP1-NA	§	§	4.4	Bai *et al.* 2005
	Nestin+ selection	36	8	1.0	McKiernan *et al.* 2007
	Ectoderm commitment	0.53	-	-	Roche *et al.* 2005
	o-ex Pax4, nestin+ seclection	98.70	19.7	2.0	Blyszczuk *et al.* 2003
	o-ex Pax4, no nestin+ seclection	168	27	1.6	Blyszczuk *et al.* 2004
	o-ex Pax4, PI3K inhibitor	170	§	-	Lin *et al.* 2007
	o-ex Pdx1, nestin+ selection	54	10.9	1.3	Miyazaki *et al.* 2004
	o-ex Nkx2.2	-	§	1.3	Shiroi *et al.* 2006
	FP factors, Insulin+ cell trapping	2700	600	4	Vaca *et al.* 2005
	DE induction, RA-B27-FGF2-NA	-	1.3	6.0	Shi *et al.* 2006
Human	Nestin+ selection	-	7.3	0.6	Hansson *et al.* 2004
	Nestin+ selection, suspension	-	§	1.5	Segev *et al.* 2004
	Nestin+ selection	-	-	4.0	Baharvand *et al.* 2006
	DE induction > PGT > PFG > PE	§	§	1.0	D'Amour *et al.* 2006
	DE induction, RA-FGF2-NA	-	0.2	2.0	Jiang *et al.* 2007a
	DE induction-Noggin-EGF-FGF2	§	§	3.7	Jiang *et al.* 2007b
Mouse islets		20310	-	-	León-Quinto *et al.* 2004
MIN6 cell line		≈ 4000	≈ 150	4.0	McKiernan *et al.* 2007

Abbreviations: -, not determined; §, expressed in other units; (a), ng/mg protein; (b), fold increase over basal condition; ActA, activin-A; DE, definitive endoderm; FP, fetal pancreas; GLP1, glucagon-like peptide-1; NA, nicotinamide; o-ex, over-expression upon transfection or nucleofection; PE, pancreatic endoderm; PFG, posterior foregut; PGT, primitive gut tube; RA, retinoic acid;

lin-positive cells differentiated with this protocol from mouse ES cells, the zinc-chelating agent dithizon was used based on the known requirement of zinc for insulin packaging in pancreatic beta cell granules. In contrast to negative cells, selected dithizon-positive clusters showed in classical RT-PCR a comparable expression profile (*forkhead box A2* (*Foxa2*), *Pdx1*, *pro-insulin1*, *pro-insulin2*, *glucagon*, *Glut2*) with isolated pancreatic islets. However these cells were not further evaluated in a glucose-stimulated insulin secretion (GSIS) assay nor transplanted into diabetic animals (Shiroi *et al*. 2002).

(2) Maturation: The low insulin content of differentiated cells might suggest they are still immature. Supplementation of nicotinamide, a soluble group B vitamin, in the last days of differentiation appeared to increase the number of insulin-positive cells as well as insulin content. It is not yet known whether this effect occurs via improved differentiation and maturation, or by protection of differentiated insulin-producing cells from noxious apoptotic stimuli (Gale 1996; Vaca *et al*. 2003).

(3) Cellular origin and identity: genuine pancreatic beta cells differentiate from precursors that belong to the definitive endoderm germ layer. It was unclear whether ES-derived insulin-producing cells were ontologically similar to their pancreatic counterpart. Indeed, a *hepatocyte nuclear factor-6* (*Hnf6*)-null mutant ES cell line in which the pro-endocrine transcription factor *Ngn3* is not induced still gives rise to insulin-positive cells in the outer layer of EBs which is known as visceral endoderm (Martin 1981). Furthermore, gene expression analysis showed increased expression of *octamer binding transcription factor-4* (*Oct4*) and nuclear receptor subfamily 2, group F, member 1 (*Nr2f1*, also known as *COUP-TF1*) in differentiated cells, suggesting that they belong to the extra-embryonic visceral endoderm. This tissue is known to share several TFs (Foxa2, Pdx1) and proteins (alpha-fetoprotein (AFP), insulin) with embryonic endoderm (Houard *et al*. 2003; Milne *et al*. 2005).

(4) Molecular regulation: the cascade of transcription factors that control pancreas development is initiated and fine-tuned by extracellular signals binding to specific receptors and activating downstream targets. Extended expression profiling of signaling pathways involved in embryonic pancreas regulation (Hedgehog, Notch, TGFβ, FGF, Wnt) suggested that the microenvironment of EBs is inappropriate for normal pancreas development, therefore explaining the limited amount of insulin-producing cells that spontaneously differentiate from ES cultures (Mfopou and Bouwens 2005; Mfopou *et al*. 2005).

Selection of nestin-positive progenitor cells

The second-generation studies tried to improve the efficiency and yield of insulin-producing cells by use of exogenous signaling molecules to direct ES cell differentiation *in vitro*. Owing to the similar developmental features recognized for the pancreas and the central nervous system, a protocol initially designed to generate neural precursors from mouse ES cells was adapted to derive insulin-positive cells. In the course, nestin-positive cells were selected by plating EBs in serum-free medium with insulin–selenium–transferin–fibronectin (ITSFn) supplement, expanded in medium containing FGF2, N2 and B27 supplements, and further differentiated by FGF2 withdrawal and addition of nicotinamide. The generated insulin-positive cells were intimately associated with β-III tubulin-positive neuronal cells, and appeared to increase their insulin secretion 3- to 4-fold in response to glucose, tolbutamide, carbachol and 3-Isobutyl-1-methylxanthine (IBMX). However, these cells failed to normalize blood glucose levels after subcutaneous transplantation into diabetic animals (Lumelsky *et al*. 2001). Similar findings were recorded by other groups using mouse or human ES cell lines with slight modifications in growth factors supplementation, and insulin content was found to significantly increase (30-fold) when differentiated cells were finally cultured as aggregates in suspension (Kania *et al*. 2003; Rajagopal *et al*. 2003; Segev *et al*. 2004; Baharvand *et al*. 2006; Ibii *et al*. 2007). Fujikawa *et al*. applied the same strategy to two mouse ES cell lines and showed that insulin-producing cells also stained for C-peptide (a by-product of pro-insulin processing) but only transiently rescued hyperglycaemia in streptozotocin-induced diabetic mice. The major reason for this secondary failure was teratoma formation from few undifferentiated Oct4-positive cells present in the original transplant (Fujikawa *et al*. 2005).

Several other modifications were applied to the original protocol of Lumelsky et al. Replacing B27 supplement by a phosphoinositide 3-kinase (PI3K) inhibitor in the last stage of differentiation reduced cell growth and neurite outgrowth, and resulted in 30-fold increased insulin content. However, these insulin-enriched cell clusters prolonged survival but failed to completely normalize glycaemia in transplanted animals (Hori *et al*. 2002). On the contrary, supplementation of exendin-4, its analogue glucagon-like peptide 1 (GLP1) or glucose-dependent insulinotropic polypeptide (GIP) to cultures of purified nestin-positive cells significantly increased *Pdx1* expression, insulin content (45-50%) and insulin secretion (35-400%), resulting in reversal of hyperglycaemia in transplanted SCID mice (Lester *et al*. 2004; Bai *et al*. 2005; Marenah *et al*. 2006; Yue *et al*. 2006). Upon nestin selection and differentiation, transgenic ES cells constitutively expressing paired box gene-4 (Pax4) showed a three and five-fold increase in insulin-positive cell percentage and insulin content respectively, when compared to wild type ES cells. This function of Pax4 appeared not to affect the early events of pancreas development (*Pdx1*, *sonic hedgehog* (*Shh*), *islet-1* (*Isl1*)) but only genes known to regulate endocrine differentiation (*Ngn3*, *Pax6*). The cells derived by this procedure also normalized blood glucose in transplanted diabetic animals (Blyszczuk *et al*. 2003). With the use of transgenic technology, Moritoh et al. demonstrated the expression of *insulin2* promoter-driven LacZ in mouse ES cells that underwent nestin selection in ITSFn medium, the positive cells being found both in the primitive ectoderm inner cell layer and in the outer visceral endoderm layer of EBs. However as already noticed by others, RT-PCR analysis did not show expression of the orthologous pancreas specific *insulin1* gene, suggesting that non pancreatic insulin-producing cells are generated by these techniques (Moritoh *et al*. 2003).

Rebuttal of nestin selection as a prerequisite to differentiate pancreatic cells

Although several studies could find insulin-positive cells from ES cell cultures following nestin-positive cell selection and differentiation, the expression of *insulin2* transcripts as detected by RT-PCR appeared to be very low and inconsistent, and that of *insulin1* was almost not unambiguously demonstrated. In addition, transgenic cell lines with enhanced-green fluorescent protein (eGFP) or β-D-galactosidase (LacZ) driven by *insulin* or *Pdx1* promoter indicated a wide discrepancy between reporter positive cells (absent and 1 out of 100.000 cells) and cells staining positive with anti-insulin antibodies (10-30%). The finding that many insulin-positive cells were also positive in terminal transferase dUTP nick end labeling (TUNEL) assay suggested insulin uptake by apoptotic cells from culture medium in which this hormone is supplemented at very high concentrations (up to 25 μg/ml; contributed by ITSFn, N2 or B27 supplements). Furthermore, fluorescein isothiocyanate (FITC)-labelled insulin added to culture medium was shown to concentrate in cells staining positive with antibodies to insulin (Rajagopal *et al*. 2003; Kitano *et al*. 2006; Lavon *et al*. 2006; Ibii *et al*. 2007; McKiernan *et al*. 2007). Additional examination of insulin-positive cells differentiated from mouse and human ES cells via nestin selection indicated a lack of cytoplasmic insulin storage granules, very rare co-staining with C-peptide and a lack of typical glucose responsiveness recognized for genuine pancreatic

beta cells. These cells were actually found to be of neuronal phenotype: an initial selection for ectodermal neuro-epithelial progenitors still resulted in similar or even higher percentages of insulin-positive cells in the absence of endoderm markers (Hansson *et al*. 2004; Sipione *et al*. 2004; Roche *et al*. 2005). In order to confirm the uptake hypothesis, assays specifically detecting insulin of the ES cell line species, or those detecting radioactive-labeled newly synthesized insulin were developed and showed that upon selection and differentiation of nestin-positive cells, the majority of cellular insulin content is medium-derived and only a very tiny fraction could be attributed to *de novo* synthesis (Paek *et al*. 2005a, 2005b; McKiernan *et al*. 2007).

The options of TFs shortcuts: forcing cells to follow the pancreatic pathway!

During pancreatic development, several transcription factors act in a well-scheduled and complex cascade to instruct the differentiation of all pancreatic cell types being exocrine, endocrine or ductal. Some of these factors are crucial for the differentiation of a particular cell type. For instance, Pdx1 and pancreas specific transcription factor-1a (Ptf1a) are required in the progenitors of all pancreatic cells, whereas the lack of Ngn3 results in complete absence of all endocrine cells, and that of Pax4 improves alpha cell differentiation at the expense of beta cells (reviewed in Jensen 2004). The expression levels of many of these genes achieved during *in vitro* ES cell differentiation are usually far bellow the situation in pancreas (Mfopou *et al*. 2005). In order to mimic their functions in ES cell differentiation to pancreatic derivatives, several groups introduced transgenes over-expressing these factors in pluripotent cells and followed their differentiation. The proendocrine gene *Ngn3* introduced in embryonal carcinoma cells or in embryonic stem cells induced higher levels of downstream TFs and endocrine hormones expression. Addition of γ-secretase inhibitor to block the Notch pathway that gets upregulated in Ngn3-expressing cells also potentiated transgene effects in terms of insulin-, glucagon- and somatostatin-positive cell differentiation (Vetere *et al*. 2003; Treff *et al*. 2006). Downstream of Ngn3, another transcription factor Pax4 is responsible for beta cell fate. Cells constitutively expressing Pax4 and differentiated with the nestin selection protocol were enriched in insulin. More recently, nucleofection of Pax4, which ensures only transient expression as occurs *in vivo* led to higher expression levels of pancreatic endocrine markers and a 3-fold increase in insulin-positive cells compared to mock-nucleofected controls (Blyszczuk *et al*. 2003; Lin *et al*. 2007). Pdx1 plays a central role in early stages of pancreas initiation and in beta cell function later on. Its overexpression in transgenic mouse ES cells before nestin selection increased the proportion of insulin-positive C-peptide-positive cells, but no transcripts of the pancreas specific *insulin1* could be detected and the resulting cells showed only marginal response to glucose stimulation (**Table 2**). In another study, *insulin1* transcripts were detected but were lost during subsequent passaging of differentiated cells and could be rescued by additional transfection with neurogenic differentiation-1 (NeuroD1) adenovirus construct (Miyazaki *et al*. 2004; Saitoh *et al*. 2007). In human ES cells, increased Pdx1 activity was achieved by exposing EBs to TAT-Pdx1 fusion protein. Protein-transduced EBs showed a 20- and 30-fold upregulation of *Pdx1* and *insulin* expression respectively. In contrast to this study, Lavon et al. found no induction of *insulin* expression in Pdx1 over-expressing human ES cells differentiated as EBs *in vitro*, but only after teratoma formation *in vivo* suggesting the requirement of additional factors (Kwon *et al*. 2005; Lavon *et al*. 2006). On the other hand, transgenic mouse ES cells over-expressing NK2 homeobox transcription factor-2 (Nkx2.2) were shown to generate as from 2 weeks culture a higher proportion of insulin-positive and dithizon-positive cell clusters that expressed both *insulin1* and *insulin2* genes (Shiroi *et al*. 2005). Although transcription factors required for pancreas development are usually expressed at low levels during ES cell differentiation, their single or combined over-expression after DNA transfection might result in extremely high activities that do not anymore match with physiological conditions. Furthermore, this procedure does not delicately recapitulate the complex cascade of TFs known to regulate pancreas development. To this end, activation of insulin transcripts after over-expression of Pdx1 for instance might merely result from DNA binding and not factual differentiation to beta cells.

The early stages of developmental mimicking *in vitro*

Considering the limitations of spontaneous differentiation and of ectodermal nestin-positive cell selection in generating "true" beta cells, it was increasingly recognized that one should follow the normal endodermal pathway to generate beta-like cells from ES cells. The third-generation studies manipulated the culture conditions in order to mimic effects of molecular signals known to initiate and/or regulate pancreas development from the gut endoderm *in vivo*. Indeed, the ventral and dorsal pancreatic buds evaginate from a particular region in posterior foregut endoderm devoid of *hedgehog* expression but highly transcribing *Pdx1*. They are under the influence of numerous signals emanating from adjacent tissues including notochord (activin-B and FGF2), lateral plate mesoderm (FGF4, bone morphogenetic protein-4 (BMP4), HGF, epidermal growth factor (EGF), activin), pancreatic mesenchyme (FGF10, retinoic acid), dorsal aorta and vitelline veins (Hebrok *et al*. 1998; Wells and Melton 2000; Bhushan *et al*. 2001; Lammert *et al*. 2001; Kumar *et al*. 2003; Martin *et al*. 2005). In addition, *in vitro* cultures of embryonic pancreas rudiments indicate that several factors including nicotinamide, betacellullin, GLP1 and activin-A improve proliferation or differentiation of fetal pancreatic epithelium and endocrine cells (Otonkoski *et al*. 1993; Demeterco *et al*. 2000). Considering all these data, several growth factors were tested on embryonic stem cells with the intention of directing their differentiation towards a pancreatic fate.

Embryoid bodies treated with serum followed by serum-free medium showed higher levels of endoderm genes, and when further exposed to a combination of activin-B, nicotinamide and exendin-4, the percentage of insulin-positive cells increased from less than 1% to 2.73%. In several other studies, addition of nicotinamide with other factors (N2 supplement, laminin, insulin) was proven efficient in increasing pancreatic gene expression in differentiated cells without any need for selection of nestin-positive progenitors (Blyszczuk *et al*. 2004; Ku *et al*. 2004; Rolletschek *et al*. 2004; Blyszczuk and Wobus 2006; Schroeder *et al*. 2006). Study of transgenic cells with GFP expression under Ngn3 promotor indicated that GFP-positive "pancreatic endocrine progenitors" are generated and represent up to 30% of total cells after secondary differentiation of EBs in the presence of activin-B, exendin-4 and nicotinamide (Ku *et al*. 2007). Retinoic acid, which was recently shown to be required during pancreas development in zebrafish, Xenopus and mouse embryos was also used in certain protocols. Alone or in combination with other molecules such as sodium butyrate, activin-A, FGF2 and nicotinamide, retinoid acid significantly induced *Pdx1* expression by ES cell progenies and the resulting cells could normalize blood glucose in transplanted diabetic animals (Micallef *et al*. 2005; Shi *et al*. 2005; McKiernan *et al*. 2007). Similarly, addition of hedgehog pathway inhibitors (anti-sonic hedgehog antibody or cyclopamine) to culture medium led to increased expression of both endocrine and exocrine pancreas markers (Leon-Quinto *et al*. 2004; Skoudy *et al*. 2004). As blood vessels also contribute to the molecular network of early pancreas development, overexpression of SHB (Src homology 2 domain containing adaptor protein B), an adaptor protein that functions downstream of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) recep-

tors also led to an increased expression of *Pdx1* and *insulin* in mouse ES cell derivatives (Saldeen *et al*. 2006). However, single addition of growth factors participating in pancreas development might not fully recapitulate the complex developmental process. In order to circumvent this limitation, differentiating ES cells were also exposed to a complex biological environment generated by fetal pancreatic rudiments (Leon-Quinto *et al*. 2004; Brolen *et al*. 2005; Vaca *et al*. 2006), by regenerating adult mouse pancreas (Takeshita *et al*. 2006) or by mouse embryonic fibroblasts (Xu *et al*. 2006). Such treatments also resulted in improved pancreatic cell markers expression both *in vitro* and *in vivo*, and transplantation of antibiotic-selected cells with active insulin promoter reduced hyperglycaemia in diabetic mice.

LATEST GROUNDBREAKING ACHIEVEMENTS

Getting to the definitive endoderm: the "golden egg" finally found!

Despite the profusion of data pointing towards pancreatic markers expression by ES cell derivatives and correction of hyperglycaemia in diabetic models, it remained unclear whether differentiated cells were unequivocally comparable to true beta cells as far as ontology (origin from definitive endoderm, extra-embryonic visceral endoderm or ectoderm), insulin synthesis/content and glucose sensing are concerned. Many of the studies mentioned in the previous sections actually did not focus on the establishment of a definitive endoderm progenitor population as first step, owing to the lack of known mammalian endoderm inducing agents and selective extracellular markers for *in vitro* applications. These limitations principally contributed to the relatively slow progression in efficient generation of endodermal tissues from ES cells as compared to tissues of mesoderm or ectoderm germ layers for which established conditions were developed. However, there was sufficient *in vivo* genetic evidence to consider that definitive endoderm can be obtained from ES cells. Retinoid acid treatment of transgenic ES cells expressing GFP under *Pdx1* promotor indicated that the reporter-positive fraction expressed several endoderm markers (*Foxa2*, *Hnf4α*, *Hnf6*, *Hnf1β*) and low levels of *Ptf1a*, a marker of pancreas progenitors in early development (Micallef *et al*. 2005).

Taking advantage of the well-characterized effects of TGFβ signals in patterning zebrafish and *Xenopus* pluripotent embryonic cells towards the endoderm lineage, Kubo *et al*. supplemented transgenic (*Brachury*-GFP) mouse ES cells with high concentrations of activin-A (100 ng/ml) in serum-free conditions. They observed that up to 60% of EBs cells committed to definitive endoderm as evaluated by GFP, *Foxa2* and *Sox17* (*sex determining region Y*, *box-17*) expression on one hand, and further differentiation of GFP-positive cells into hepatic (Foxa2, albumin), intestinal (intestinal fatty acid binding protein (IFABP), muscin) and pulmonary (surfactant protein C (SftpC)) tissues on the other hand. Cells treated with lower concentrations of activin-A mainly gave rise to mesoderm-derived tissues even after initial purification for *Brachury* (*Bry*) expression (Kubo *et al*. 2004). In another transgenic cell line expressing GFP under *Goosecoid* (*Gsc*) promoter, a much lower concentration of activin-A (10 ng/ml) induced 65% and 93% positive cells by day 4 and 6 respectively. These cells showed strong upregulation of *Sox17*, *Gsc*, *Foxa2* and *Mixl1* (*mix homeobox-like 1*), but no expression of the visceral endoderm (*Sox7*, *Hnf4*, *Pthr1* (*parathyroid hormone receptor-1*)) or neuronal markers (*Sox1*, *Pax6*). Interestingly, the first Bry-positive and/or Gsc-positive cells were defined as *in vitro* primitive streak and mesendoderm equivalents that require both Wnt and TGFβ signaling for their induction, and that are capable of giving rise to both definitive endoderm and mesoderm derivatives upon differentiation (Tada *et al*. 2005; Gadue *et al*. 2006; Gouon-Evans *et al*. 2006). Further analysis of activin-induced cells indicated that definitive and visceral endoderm could be distinguished on the basis of *Gsc* and *Sox17* expression; that is Gsc(-) Sox17(+) for visceral endoderm and Gsc(+)Sox17(+) for definitive endoderm. Furthermore, definitive endoderm could be isolated from wild type ES cells treated with activin-A by sorting the double positive fraction for the extracellular proteins E-cadherin and CxCR4 (chemokine C-X-C motif receptor-4) (Yasunaga *et al*. 2005). Despite the fact that activin-A strongly induced endoderm from ES cells, it does not represent the native inducer of this germ layer *in vivo*. Another family member nodal, which is not yet commercially available in its potent form, normally assumes this function. To circumvent this drawback, ES cells were engineered to express tetracycline-regulated nodal. Upon differentiation, these cells were shown to generate definitive endoderm identified by CxCR4, VEGFR2 (VEGF receptor-2) and PDGFR-α (PDGF receptor alpha) cell sorting, as well as mesoderm cells. Visceral endoderm and neuroectoderm differentiation were severely repressed in this system and attenuation of nodal signaling after definitive endoderm establishment was a prerequisite for its further maturation into progenies expressing foregut (SftpC, albumin, tyrosine aminotransferase), midgut (Pdx1, glucagon, amylase) or hindgut (IFABP, villin) markers (Takenaga *et al*. 2007).

The data accumulated with serum-free activin-A or nodal treatment of mouse ES cells confirmed the conserved role of TGFβ signals in endoderm induction in lower vertebrates and extended it to rodents. When applied to human ES cell differentiation in presence or in absence of feeder cells, this treatment also led to the generation of definitive endoderm cells expressing *Gsc*, *Sox17*, *Foxa2*, *Mixl1*, and *Pdx1* but not *Sox7* (visceral endoderm) or *Sox1* (neuroectoderm). The induced cells were additionally purified to near homogeneity by CxCR4-positive cell sorting and after engraftment in SCID-mice, they generated intestinal (villin, caudal homeobox-2 (Cdx2)) and hepatic (hepatocyte-specific antigen) cell types (D'Amour *et al*. 2005; Yao *et al*. 2006). A four-day inhibition of the PI3K signaling in conditions that would otherwise support human ES cell self-renewal induced differentiation into mesendoderm and then to Sox17-positive CxCR4-positive definitive endoderm. This activity was actually related to a shift from combined self-renewal stimuli present in fibroblast-conditioned medium to a predominant TGFβ signaling in the absence of insulin-like growth factor 1 (IGF1) and insulin activities. When cultured in the presence of serum, activin-A could induce definitive endoderm from ES cells only when PI3K was inhibited. These findings shed light on the failure to produce definitive endoderm cells by initial reports that usually performed differentiation in 10% serum. It also explains why activin-based protocols require very low serum concentration or serum-free media to efficiently induce definitive endoderm (McLean *et al*. 2007).

The small chemical sodium butyrate has previously been applied to mouse ES cells monolayer cultures and appeared to induce differentiation of hepatic progenitors that further give rise to metabolically active hepatocytes and bile duct cells. However, these studies were not supported by a clear demonstration of initial definitive endoderm induction upon sodium butyrate treatment (Rambhatla *et al*. 2003; Sharma *et al*. 2006; Zhou *et al*. 2007). Nevertheless, owing to the common endodermal origin of liver and pancreas, short term ES cell exposure (24 hours) to sodium butyrate was tried and led to a strong induction of *Pdx1* transcripts as well as mesendoderm markers *Gsc*, *Bry* and *Sox17*. But, the resulting cells showed downregulated *Foxa2* expression, and upon protein analysis Pdx1 was mainly confined to the cytoplasm and its transcripts expression was lost as from day 4 (Goicoa *et al*. 2006).

By activating the same signaling pathway (SMAD) to generate definitive endoderm identified by different combinations of Gsc, Sox17, Foxa2, CxCR4 and E-cadherin, all these studies set a decisive landmark in the field of beta cell differentiation from ES cells. Nevertheless, one can already point out the large variations in the procedures followed by different groups concerning culture in monolayer or embry-

Table 3 Generation of ES cell-derived definitive endoderm and beta-like cells. After establishing conditions for derivation of definitive endoderm from mouse and human ES cells, further differentiation with factors known to regulate pancreas development *in vivo* results in relatively high proportion of Pdx1-positive progenitors and insulin-positive or C-peptide-positive beta-like cells. These cells however would represent immature fetal-like cells that need further maturation to become fully responsive to glucose stimulation. The table represents immunostaining data from different protocols applied to mouse or human ES cells.

ES cells	DE induced + differentiated	DE cells	Pdx1+ cells	Ins+ cells	References
Mouse	2.5d serum, then 3.5d SF; or 7d SF + ActA (100 ng/ml)	40-60% [(a)]	-	-	Kubo *et al.* 2004
	RA (10 μM) at d4, EBs culture till d8	yes	1% [(a)]	-	Micallef *et al.* 2005
	6d SF + ActA (10 ng/ml), Col-IV coating, ML	93% [(a)] 60% [(b)]	-	no	Tada *et al.* 2005
	6d SF + ActA (10 ng/ml), Col-IV coating, ML	96% [(a)] 92% [(b)] 30% [(c)]	-	-	Yasunaga *et al.* 2005
	2d SF; 2d ActA (25 ng/ml) / Wnt3a (100 ng/ml); 2d ActA (10 ng/ml)	92% [(a)]	-	no	Gadue *et al.* 2006
	2d SF-N2-B27, 4d ActA (50 ng/ml)	70% [(a)]	-	-	Gouon-Evans *et al.* 2006
Human	5d ActA (100 ng/ml), 0-2% serum, ML	80% [(b)] 72% [(d)]	-	-	D'Amour *et al.* 2005
	1d Wnt3a (25 ng/ml) + ActA (100 ng/ml), 3d ActA, 4d FGF10 + cyclopamine, 3d + RA, 4d exendin-4, >6d IGF1 + HGF	yes	yes	7.3%	D'Amour *et al.* 2006
	9d CDM-N2-B27 + ActA (100 ng/ml)	>80% [(e)]	>80%	-	Yao *et al.* 2006
	7d ActA (4 nM) + NaBut, 3d EGF (20 ng/ml) + FGF2 (2 ng/ml) + Noggin (100 ng/ml), 7d EGF + Noggin, 7d IGF2 ± NA	80% [(b)] 70% [(c)]	24%	4% [(f)]	Jiang *et al.* 2007a
	2d CDM-ITS, 4d ActA (50 ng/ml), 4d RA (1 μM), 3d FGF2, 5d FGF2 ± NA	yes	25%	15% [(f)]	Jiang *et al.* 2007b
	5d LY294002 in self-renewal settings; or 5d serum + LY294002 + ActA (100 ng/ml)	70% [(c)]	-	-	McLean *et al.* 2007
	10d Nodal overexpression, 4d aggregation in 10% FBS	84% [(b)]	5.4%	-	Takenaga *et al.* 2007

Abbreviations: [(a)], *Bry*, *Gsc* or *Pdx1* reporter expression; [(b)], E-cadherin or CxCR4 cell sorting; [(c)], *Sox17* transcript or reporter expression; [(d)], co-localisation Sox17 and Bry, [(e)], co-localisation Sox17 and Foxa2; [(f)], C-peptide staining; CDM, chemically defined medium containing ITSFn; DE, definitive endoderm; ML, monolayer culture; NaBut, sodium butyrate; RA, retinoic acid; SF, serum-free.

oid bodies formation, total length of exposure to activin receptor ligands (from 4 to 10 days), concentration of activin-A (between 10 and 100 ng/ml), addition of ITSFn, N2 or B27 supplements and the use of small molecules such as retinoic acid and sodium butyrate (**Table 3**). It would be interesting to compare all these protocols in the same ES cell line in order to examine how equivalent they are.

From definitive endoderm to pancreatic beta-like cell: enough done, but much still to be done!

Establishment of common *in vitro* conditions required for definitive endoderm induction in both mouse and human ES cells opened new perspectives in the search for new sources of shortened islets of Langerhans. Similarly, chronic diseases affecting other endoderm-derived organs might benefit from this advancement (**Table 1**). Human ES cells differentiated to definitive endoderm by exposure to activin-A were soon shown to sequentially give rise to *in vitro* equivalents of primitive gut tube (*Hnf1β*, *Hnf4*), posterior foregut (*Hnf6*, *Pdx1*, *Hlxb9* (*homeobox HB9*)), pancreatic endoderm and endocrine precursors (*Ngn3*, *Pax4*, *Nkx6.1*, *Nkx2.2*), and finally hormone expressing cells (7% insulin-producing cells). The transition through these stages required subsequent exposure to media cocktails containing FGF10, cyclopamine, retinoic acid, exendin-4, IGF1 or hepatocyte growth factor (HGF) which are signaling molecules with recognized function in pancreas development. Although these cells contained sufficient insulin and released C-peptide in response to several secretagogues, they were not stimulated by glucose; a phenotype that classifies them as fetal immature beta-cells (D'Amour *et al*. 2006). Worthy to note, this study found that inhibition of hedgehog signaling with cyclopamine during primitive gut tube and posterior foregut stages is mandatory for the generation of insulin-producing cells. Indeed, activin induction of definitive endoderm is associated with upregulation of hedgehog signaling, resulting in repression of the leading pancreas marker *Pdx1*, which can be rescued by supplementation of hedgehog interacting protein (Mfopou *et al*. 2007). In the same line, transcription of *insulin1* and *insulin2* genes by mouse ES cells derivatives was recorded upon activin-A and retinoid acid treatment of 4 days-old EBs in the context of absent hedgehog induction. These data are strongly consistent with the widely studied effects of hedgehog ligands on pancreas development, though this phenomenon was usually neglected in early studies (Mfopou and Bouwens 2007; Nakanishi *et al*. 2007). Following 4 days serum (20%), 4 days activin-A (30 ng/ml) and 2 days retinoic acid (10 μM) treatment of human ES cells, pancreatic progenitors co-expressing nestin and Pdx1 were generated. When plated in ITSFn-supplemented medium, they gave rise to 35% Pdx1-positive cells that lost nestin expression. Despite low transcripts levels detected by PCR, no pancreatic hormone expression was identified after extended culture *in vitro*, but transplantation of these cells in diabetic mice resulted in insulin-producing cell differentiation and progressive lowering of blood glucose over 4 weeks (Shim *et al*. 2007).

A combination of activin-A and sodium butyrate was found to improve endoderm induction in human ES cells as compared to activin-A alone. Further exposure of re-aggregated cells to EGF, FGF2 and noggin generated Pdx1-positive pancreas progenitors that were further differentiated into islet-like clusters expressing insulin (2-8% C-peptide positive cells), glucagon and somatostatin after culture in nicotinamide and IGF2-containing medium. A much simplified protocol involving monolayer culture of human ES cells in chemically defined medium (50% F2 nutrient mixture, 1% ITS supplement) with activin-A induction of definitive endoderm and sequential exposure to retinoic acid, FGF2, combined FGF2 and nicotinamide resulted in 15% C-peptide positive cells that normalized blood glucose in 30% of transplanted diabetic nude mice (Jiang *et al*. 2007a; Jiang *et al*. 2007b).

CONCLUDING REMARKS AND PERSPECTIVES

More than twenty years after the first derivation of embryonic stem cells, they continue to fascinate the scientific community with regards to their self-renewal properties, their wide differentiation potential *in vitro* or *in vivo*, and more interesting their prospective use as surrogates of shortened cells needed in transplantation therapies. As far as diabetes cell therapy is concerned, a plethora of studies pointed to the possible expression of pancreatic transcription factors and hormones including insulin by progenies of ES cells. The first- and second-generation studies lacked a formal proof that differentiated cells were of endodermal origin and not representing extra-embryonic visceral endo-

derm or neuroectoderm cells that share some markers with the pancreas including insulin. These critics also apply to third-generation studies in which use was made of several growth factors with recognized functions in embryonic pancreas development. The major breakthrough in this field came with the efficient generation of definitive endoderm, the germ layer from which pancreas normally develops *in vivo*. This advance also confirms the crucial role played by TGFβ signaling in endoderm induction, which now encompasses several species from lower vertebrates to mammals. Although much has already been done to derive pancreas progenitors from ES-derived definitive endoderm, it is believed that adequate implementation of complex developmental biology findings related to pancreas development will further push the field forward, so that matured glucose-responsive beta-cells could be obtained with high efficiency to satisfy clinical needs. Although the latest strategies proposed rely on certain specific aspects of pancreas development, it is difficult to reconcile all the data because of major variations in cell treatments as well as subtle technical issues. Presently, no study reports on efficiency above 15% insulin-positive cells and it is not totally clear whether definitive endoderm and/or pancreas progenitors established *in vitro* from ES cells would spontaneously give rise to similar percentages in the absence of "pro-endocrine" cocktails used so far. As suggested from the study by D'Amour *et al*. (2006), the question is to know whether early step of endoderm induction is the only and most important condition that has driven present observations. To this end, fine-tuning of proposed strategies is required; with special focus on Notch signaling which to our opinion has just marginally been explored. Once a fully developmental-based, efficient, reproducible and universally accepted protocol is established for generation of beta-cells from ES cells, attention will need to be paid to issues of tumor formation and immune rejection by transplanted patients. But before we get there, we might probably need equivalent milestones as from ES cell discovery to date.

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