Towards stem-cell therapy in the endocrine pancreas

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Many approaches of stem-cell therapy for the treatment of diabetes have been described. One is the application of stem cells for replacement of nonfunctional islet cells in the native endogenous pancreas; another one is the use of stem cells as an inexhaustible source for islet-cell transplantation. During recent years three types of stem cells have been investigated: embryonic stem cells, bone-marrow-derived stem cells and organ-bound stem cells. We discuss the advantages and limitations of these different cell types. The applicability for the treatment of dysfunction of β cells in the pancreas has been demonstrated for all three cell types, but more-detailed understanding of the sequence of events during differentiation is required to produce fully functional insulin-producing cells.

Diabetes and the call for stem-cell therapy

A major challenge in the treatment of diabetes (Box 1) is to provide patients with an insulin source that regulates glucose levels on a mandatory minute-to-minute basis. Conceivable approaches to achieve this goal consist of restoring an endogenous source and/or implanting an autologous- or non-autologous-derived source. At present, there are different strategies under investigation, such as (i) stimulation of regeneration of the residual islet cells in the diabetic pancreas, (ii) increase of the regenerative capacity by infusion of precursor cells [1–3] and (iii) islet-cell transplantation [4] (Box 2). The application of these approaches has been shown in both experimental animals and humans.

Studies that address the implantation of an autologous- or non-autologous-derived source for the treatment of diabetes have always been of limited clinical relevance due to shortage of sufficient supply of islet cells. This has been the main rationale for researchers to design ways to generate inexhaustible islet sources. In recent years, many have focused on making stem cells an inexhaustible source for islet cells (see Glossary). Stem cells are self-renewing progenitor cells that can differentiate into one or more specialized cell types. Stem cells have several features that qualify them as a potential inexhaustible source for islet cells. Theoretically, they have the capacity for unlimited replication and, if adequately differentiated, they can become fully mature and functional islet cells.

However, in practice fully functional islet cells have not yet been derived from stem cells.

Several strategies and different stem-cell sources for islet-cell substitution have been proposed. Not all have been equally successful. Here, we discuss the successes and failures of the different approaches in view of future clinical application. Also, we discuss the present insights in the developmental biology of the endocrine pancreas because this knowledge is mandatory for understanding and designing strategies to create fully functional islet cells from stem cells of both non-pancreatic and pancreatic origin.

Lessons from embryogenesis and organogenesis of the endocrine pancreas

The characteristic organization of the pancreas (i.e. the highly branched ductal structure) (Figure 1) is formed after the endodermal gut tube is created from the endoderm. The dorsal and the ventral pancreatic buds are pouched out from the endodermal epithelium. Subsequently, the ventral bud rotates towards the dorsal bud and fuses as the epithelium invaginates. Then, the pancreatic precursor cells differentiate into endocrine and exocrine structures [5], after which the various cell types are formed (Table 1).

The subsequent development of the endocrine pancreas is under tight control of several pancreas-specific transcription factors. Among these factors are pancreatic and

Glossary

Ectoderm: the outer germ layer of the embryo from which the epidermis, the nervous tissue and sense organs develop.
Embryogenesis: the development and growth of an embryo from the second week through the eight weeks following conception.
Endoderm: the inner germ layer of the embryo from which the gastrointestinal and digestive tract, and the lungs develop.
Euglycaemia: normal concentration of glucose in the blood. Also called normoglycaemia.
Hyperglycaemia: the presence of an abnormally high concentration of glucose in the blood.
Hypoglycaemia: an abnormally low concentration of glucose in the blood.
Mesoderm: the middle germ layer of the embryo from which the connective tissue, muscle, bone, the urinary and circulatory systems develop.
Organ-bound or endogenous stem cells: a small population of cells in each organ of the adult that are capable of continuous self-renewal and can differentiate into cells of the tissue they reside in.
Organogenesis: the formation and development of organs in living organisms.
Stem cells: self-renewing progenitor cells that can differentiate into one or more specialized cell types.
Transcription factors: proteins that regulate the activation of transcription of DNA in the eukaryotic nucleus.
Transdifferentiation: a process in which precursor cells differentiate into another cell type.

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Box 1. Diabetes mellitus

Diabetes mellitus is a chronic disorder that results from a deficiency of insulin. Insulin is produced by the β cells of the pancreas and regulates uptake of glucose from the blood into cells. Diabetes is manifested in two distinct forms: an absolute deficiency of insulin (type 1) and a relative deficiency of insulin (type 2) diabetes. Type 1 diabetes, also known as juvenile or insulin-dependent diabetes, results from the autoimmune destruction of β cells. Approximately 10% of all diabetics suffer from type 1 diabetes. The most common manifestation of diabetes is type 2 diabetes, also known as non-insulin-dependent diabetes. Type 2 diabetes results from insulin resistance, pancreatic β-cell dysfunction or both. The β cells seem to be unable to secrete the amounts of insulin that is required by the organism to stabilize glucose levels. This type of diabetes is often associated with obesity. It is usually sufficient to treat type 2 diabetic patients with a diet or medication to decrease insulin resistance.

Because type 1 diabetic patients are unable to produce insulin, it is necessary to treat them with exogenous insulin. Currently, most patients are treated with insulin therapy. However, treatment with insulin does not always result in euglycaemia. Both hypoglycaemic and hyperglycaemic episodes can occur. Episodes of hyperglycaemia are associated with various complications such as cardiovascular diseases, retinopathy or glaucoma, renal diseases, neuropathy and diabetic ketoacidosis. Tight glycaemic control by intensive insulin therapy reduces the probability of developing these complications. Unfortunately, however, also intensive insulin therapy is not without risks because it is associated with hypoglycaemic unawareness, which disables many patients.

Box 2. Transplantation of the pancreas or islets of Langerhans

The most widely used treatment for type 1 diabetes is intensive insulin therapy. However, this treatment cannot regulate glucose levels on a minute-to-minute basis and, therefore, exposes the patient to the risk of hyperglycaemic and hypoglycaemic episodes.

A different approach to achieve euglycaemia is to provide the diabetic patient with an endogenous source of insulin by transplantation of endocrine pancreatic tissue. There are two options: transplantation of the whole pancreas or only the islets of Langerhans. The first option, pancreatic organ transplantation, is an established treatment (>20 000 cases). At present, patient and graft survival rates almost equal those of more-conventional grafts such as kidney transplantation. A successful pancreas transplant provides almost normal glucose homeostasis, but it requires major surgery and life-long immunosuppressive medication. The side-effects of life-long immunosuppression together with the major surgical intervention do not make this option a suitable alternative for life-long insulin therapy. At present, the majority of hospitals provide combined pancreas and kidney transplantation only to diabetic patients with end-stage renal failure.

Islet transplantation, in contrast to pancreas transplantation, requires no major surgery and there are conceivable approaches that enable transplantation of islets in the absence of immunosuppression. Approaches currently under investigation are: immuno-isolation by encapsulation, immunotolerance and immunomodulation. The advantage of islet-cell transplantation is that it requires a minimally invasive procedure that can be repeated without major consequences for the recipient. The downside of this approach is that it requires 2–4 human donors per recipient. Islets disappear during isolation from the pancreas, but also during and after transplantation. This is not only due to immunological problems, but also to several viability and metabolic problems that are caused by ischaemic episodes in the immediate period after transplantation. Recent improvement in this transplantation technology is the administration of non-glucocorticoid immunosuppression (sirolimus, tacrlimus and daclizumab) by the group of Edmonton [40], which is associated with graft survival in most transplanted diabetic patients for up to 24 months. This improvement has led to a tremendous growth in the number of research groups that are involved in human islet transplantation. Nevertheless, because of donor shortage, islet transplantation cannot be performed on a world-wide basis. Therefore, at present many research groups are exploring new ways to generate insulin-producing cells that can be used for transplantation.

duodenal homeobox 1 (PDX1), neurogenin 3 (ngn3), paired box gene 4 (PAX4) and PAX6, islet-1 (ISL-1), β cell e-box transactivation 2 (BETA2) [also known as neurogenic differentiation 1 (NEUROD1)], v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF) family, NK2 transcription factor related, locus 2 (NKX2.2), and NK6 transcription factor related, locus 1 (NKX6.1). The most well-defined transcription factors and their expression are presented in Table 2 and some are described later in this review. These transcription factors regulate migration, differentiation and proliferation during development. Understanding the sequence, the signals for and the quantitative pattern of expression of these transcription factors is essential for designing *ex vivo* approaches for creating functional islet cells.

One of the most intensively studied genes is PDX1 (Table 2). During organogenesis, PDX1 is widely expressed in cells that eventually differentiate into endocrine and exocrine pancreatic cells, whereas in the adult pancreas the expression of PDX1 is restricted to β cells and δ cells [6]. It was shown that PDX1 is involved in islet-cell-specific expression of various essential genes for glucose metabolism, such as those that encode insulin and somatostatin [7,8]. The disruption of PDX1 in mice and humans results in the lack of development of the pancreas [9].

Also, ngn3 is crucial for the development of the pancreas [10]. Ngn3 is present in both the embryonic and postnatal pancreas, where it contributes to islet-cell renewal under physiological conditions [10]. Ngn3 expression leads to the expression of extracellular NOTCH ligands in precursor cells. Subsequently, this leads to the activation of NOTCH receptors, which is also crucial in the development of the pancreas [10].

The gene that encodes the basic helix–loop–helix (bHLH) BETA2 is associated with activation of the insulin (*INS*) gene [11]. During mouse embryogenesis, BETA2

Table 1. Pancreatic cells: their phenotypes and function

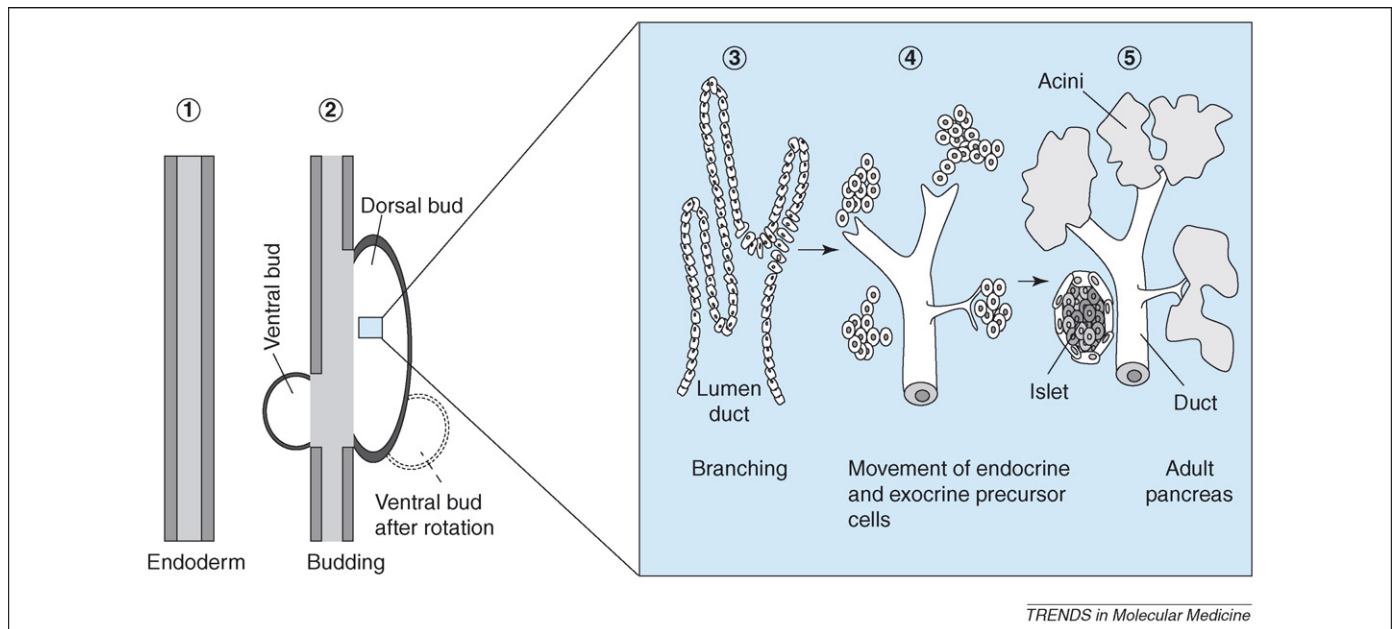| Pancreas	Cells	Function
Endocrine	α cells	Production of insulin for sustaining euglyceamia
	β cells	Secrete glucagon, which counteracts insulin hypoglycemic effects
	δ cells	Secrete somatostatin, which inhibits insulin secretion
	PP cells	Pancreatic polypeptide function remains unclear
	Ghrelin cells	Production of ghrelin can inhibit glucose-induced insulin release and stimulate glucagon secretion [70]
Exocrine	Acinar cells	Produce at least 22 digestive enzymes such as proteases, amylases, lipases and nucleases
	Duct cells	Produce non-enzymatic components of the pancreatic juice, including bicarbonate

Figure 1. Pancreas development. The human pancreas develops as two distinct buds, which subsequently fuse to form one organ. The dorsal pancreatic bud is the first to appear around day 26 of human development (E9.5 in rodents), as it buds from the endoderm close to the foregut (2). The ventral pancreatic bud arises less than a day later from the endoderm (E10.5 in rodents). One important component in the regulation of this program is the exclusion of the hedgehog gene family of signaling molecules. These molecules, sonic hedgehog (SHH) and Indian hedgehog (IHH), are involved in the promotion of intestinal differentiation. When SHH and IHH are downregulated, the dorsal and ventral pancreatic buds start to grow. During the fifth week of human development, the ventral pancreatic bud migrates around the foregut until the ventral bud comes into contact with the dorsal bud. By the beginning of the sixth week (E17 in rodents), both buds have fused (2). The dorsal and ventral buds grow into their surrounding mesoderm to form the main ducts. As the main ducts elongate, secondary ducts form and branch off; they, in turn, elongate and an additional generation of ducts branch off (3). By the beginning of the ninth week of human development, the tubules terminate in distinct clumps of cells: these will form the acini (4). By week 12, the interlobular ducts are established, forming the pattern of the future lobular structure of the pancreas. Differentiation of the primitive acini starts around week 12 (in rodents, the ductal structure and acini are clearly distinguishable at E14.5). By week 16, the first mature acini are formed and this process continues until birth. At further elongation and dilatation of the ducts, cell clumps that are distinct from those of the terminal structures bud off from the walls of the smaller branches: these will form the islets. The presumptive islet-cell clumps migrate away from the tubules into the stroma of the developing gland, while new clumps continue to form and bud off. The islets expand through proliferation of the islet-cell precursors and by the merging of cell clumps in close proximity. During week 10, α cells differentiate; during week 11, δ cells appear, followed by β cells two weeks later (in rodents, early endocrine cells appear from E18–19).

protein is expressed in a subset of pancreatic epithelial cells at embryonic day (E)9.5, whereas later on its expression is restricted to islets [11,12]. BETA2-deficient mice die 3–5 days post-partum owing to severe hyperglycaemia and islet anomalies [11].

PAX4 and PAX6 proteins are specifically expressed in the developing endocrine pancreas, and regulate differentiation and proliferation of islets [13]. During embryogenesis, PAX6 protein is present from E9 on a small subset of the pancreatic endoderm [13]. PAX4 expression is observed from E10 in the pancreatic bud [13]. At the end of development, PAX4 levels decrease, whereas PAX6 levels remain elevated [13]. Knockout studies have confirmed that PAX4$^{-/-}$ mice do not generate β cells and δ cells, whereas PAX6$^{-/-}$ mice are unable to form α cells [13].

The LIM-domain (named from cell lineage family member 11 (*LIN-11*), *ISL-1* and meiotic pachytene checkpoint protein 3 (*MEC3*) genes) protein ISL-1 is expressed in endocrine cells and in a subset of neurons in the adult rat [14]. During development, ISL-1 is present from E9 in the dorsal pancreatic epithelium of mice [15]. At E11, ISL-1 is detected in the ventral pancreatic epithelium, which is required for the differentiation of islet cells. In ISL-1 mutant mouse embryos, islet development is completely absent [15].

Also, MAF proteins are essential for the development of the endogenous pancreas. In adults and during embryogenesis, MAFA is a key activator of insulin expression

[16–18]. Although MAFB specifically regulates glucagon expression in adults [16], recently MAFB has been also observed at E12.5, when it is co-expressed with insulin [16]. This MAFB–insulin co-expression is persistent throughout subsequent development. In the postnatal pancreas, MAFB is downregulated in β cells, but not in α cells. In a later developmental stage (E18.5), only few PDX1- and MAFB-positive cells are present, which has been interpreted as differentiation into insulin-producing cells [16]. A follow-up study showed that in embryos MAFB is expressed before MAFA, suggesting that differentiation of β cells proceeds via a MAFB$^+$MAFA$^-$INS$^+$ intermediate cell into MAFB$^-$MAFA$^+$INS$^+$ cells [19]. These results indicate that MAFB has a dual role in embryonic differentiation of both β cells and α cells.

The NK homeodomain family is also involved in the development of islets. NKX2.2 protein expression is detected during early embryogenesis in all endocrine cell types [20], whereas in adult islets it is restricted to β cells, α cells and pancreatic polypeptide (PP) cells [21]. Knockout studies have shown that NKX2.2-null mutant mice develop severe hyperglycaemia [21] because β-cell precursors survive but fail to mature [21]. Another member of the NK-homeodomain family is the transcription factor NKX6.1. In the developing mouse pancreas, NKX6.1 protein expression is already observed at E10.5 [21]. From E15.5, the expression of NKX6.1 becomes restricted to insulin-producing cells, scattered ductal cells and periductal cells [21]. At later

Table 2. Summary of well-known transcription factors involved in the development of the pancreas[a]

Pancreatic cells	Transcription factors[b]	Function	Species	Expression in rodents	Refs
Endocrine progenitors	Ngn3	Directs differentiation of pancreatic precursor cells into endocrine lineages	Mouse, rat and human	Starting at E9.5; diminishes after birth; not present in adult	[10]
	PAX4	Formation of β and δ cells; represses glucagon transcription	Mouse, rat and human	Starting at E10; decreasing from late neonatal	[13]
	HNF6	Required for normal endocrine development. Appears to activate ngn3 expression	Mouse, rat and human	Starting at E9.5; neonatal; adult unknown	[71]
	ISL1	Early endocrine-cell differentiation	Mouse, rat and human	Starting at E9; decreasing from neonatal; in adult, restricted to islets	[15]
	BETA2/NEUROD	Islet growth and differentiation	Mouse, rat and human	Starting at E9.5; from E17.5 and in adults restricted to islets	[11]
	PAX6	Formation of α cells. Activates glucagon transcription	Mouse, rat and human	Starting at E9; present during neonatal; possibly present in adult	[13]
	NKX2.2	Necessary for β-cell precursors to express NKX6.1 and insulin	Rat and human	Early embryonic; decreasing in neonatal; in adult, restricted to islets	[72]
	NKX6.1	Final differentiation of β cells	Rat and human	Starting at E10.5 in whole pancreas; from E15.5 and in adults restricted to β cells	[21]
	MAFA	Controls insulin gene expression	Mouse, rat and human	Starting at E14; decreasing in neonatal; in adult, restricted to β cells	[73]
	MAFB	Formation of α and β cells	Mouse, rat and human	Starting at E15; decreasing in neonatal; in adult, restricted to α cells	[16]
	PDX1	Formation of β and δ cells	Mouse, rat and human	Starting at E8.5; neonatal; in adult, restricted to islets	[6]
Exocrine progenitors	HES1	Directs differentiation of pancreatic precursor cells towards exocrine lineages	Mouse and human	Starting at E9.5; decreasing in neonatal; in adult, not present	[74]
	PDX1	Formation of exocrine tissue	Mouse	Starting at E8.5; neonatal; in adult, restricted to islets	[6]
Mature β cells	LMX1.1	Insulin activation in mature β cells	Human	Not applicable	[75]
	HNF1α	Activation of insulin and PDX1	Mouse, rat and human	Not applicable	[76]
	HNF4α	Activation of HNF1α, insulin and SLC2A2	Mouse, rat and human	Not applicable	[77,78]
	PDX1	Important activator of insulin	Mouse, rat and human	Not applicable	[7,79]
	NKX2.2	Necessary for β-cell precursors to express NKX6.1 and insulin	Rat and human	Not applicable	[72]
	NKX6.1	Final differentiation of β cells	Rat and human	Not applicable	[21]
	BETA2/NEUROD	Insulin activation	Mouse, rat and human	Not applicable	[11]
	MAFA	Controls insulin gene expression	Mouse, rat and human	Not applicable	[73]
Mature α cells	NKX2.2	Necessary for β-cell precursors to express NKX6.1 and insulin	Rat and human	Not applicable	[72]
	MAFB	Controls glucagon expression	Mouse, rat and human	Not applicable	[16]
Mature PP cells	NKX2.2	Necessary for β-cell precursors to express NKX6.1 and insulin	Rat and human	Not applicable	[72]

[a]This table summarizes which transcription factors can be found in which precursor cells. Notably, some of these transcription factors remain expressed in mature endocrine cells.

[b]Abbreviations: HES1, hairy of enhancer of split 1; HNF, hepatocyte nuclear factor; LMX1.1, LIM homeobox factor 1.1.

stages and in the adult pancreas, NKX6.1 protein becomes restricted to insulin-producing cells [21]. Disruption of NKX6.1 leads to blockage of β-cell neogenesis [21].

Adequate knowledge of the expression pattern of transcription factors is not only essential for stem-cell research, but also for understanding the pathophysiology of diabetes. This is illustrated by the discovery of six human genes called the maturity onset diabetes of the young (MODY) genes, which are responsible for the onset of a subtype of diabetes type 2 [22]. Among these *MODY* genes, there are genes that encode transcription factors carrying mutations. These genes are *MODY-4*, a mutated PDX1 gene, and *MODY-6*, a mutated BETA2 gene.

Stem cells for islet-cell substitution
The embryonic stem cells
Different sources of stem cells have been proposed for the production of β cells. The application of embryonic stem cells (ESCs) in regenerative medicine is currently under investigation [23]. ESCs can be derived from blastocyst and differentiate *in vivo* into cells of endoderm, mesoderm and ectoderm origin and, thereafter, into cells that are specific for the various tissues in the body (Figure 2a). Murine and human ESCs can generate embryoid bodies that contain cells with a β-cell-like phenotype *in vitro* [24–27]. Differentiated ESCs can also lower blood glucose levels in rodents [28,29]. Various groups have indicated that the number of insulin-producing cells during *in vitro* differentiation can be enhanced by overexpressing PAX4, PDX1 or NKX6.1 in ESCs [29–31]. An increase in the number of differentiated β cells can also be accomplished by culturing ESCs with differentiation factors. Murine ESC-derived embryoid bodies that are grown in the presence of 15% foetal calf serum (FCS), followed by serum-free conditions, generate a population of cells that express insulin and PDX1 [32]. Also Segev *et al.* [27] reported enhanced expression of β-cell-specific genes in ESCs after manipulation of culture medium.

The potential application of ESCs for producing β cells has brought optimism into the field of diabetic research. However, there is a downside that also requires some considerations. Despite enormous progress in the understanding of the developmental biology of β cells, there are still gaps in the knowledge of the processes that are involved in the differentiation process of ESCs into β cells. It has been suggested that β cells derived from ESCs do not represent *bona fide* embryonic or foetal phenotypes, but rather they are aberrant products from misregulated or scrambled differentiation programs [33]. The lack of knowledge of normal differentiation is probably also responsible for the fact that at the moment products of stem cells that show many features of senescence such as multiple fragments of chromosomes are generated [34]. Moreover, evidence that the newly differentiated β cells produce insulin in a normal biphasic way is still lacking. Not only functional problems can be observed in differentiated ESCs, but also the change of culturing malignant cells during the differentiation process should be considered as a threat. ESCs closely resemble embryonal carcinoma cells, which can form teratocarcinomas. It has been shown that animals develop tumours when they are transplanted with ESC-derived insulin-producing cells [35]. Therefore, it follows that it is advisable to perform detailed efficacy studies on ESCs before proposing these cells as a suitable source of insulin-producing cells in the treatment of diabetes.

Not only ESCs but also umbilical cord blood cells can differentiate into islet cells [2,36–38]. Freshly isolated cells of the umbilical cord express nestin and ngn3 proteins, whereas they express the mRNA of nestin, cytokeratin 8 (CK8), CK-18 and CK-19, in addition to the β-cell-specific transcription factors ISL-1, PAX4 and ngn3 [2]. The applicability of the umbilical cord blood cells for the treatment of diabetes has been shown in various animal studies in which infusion of these cells lowered blood glucose levels in diabetic mice [36–38]. Moreover, it was demonstrated that the infused cells differentiated into insulin-producing cells [37,38]. Therefore, umbilical cord blood cells deserve careful consideration as a source of cells of the endocrine pancreas.

Bone-marrow-derived stem cells
The bone-marrow-derived stem cells (BMSCs) have been the subject of haematopoietic studies for many years and are, therefore, the best characterized stem-cell source. BMSCs are multipotent, capable of self-renewal and well known as a source of stem cells for blood cells (Figure 2b).

However, they can also differentiate into other cells, because BMSCs can migrate towards a site of damage and differentiate under the influence of factors from the microenvironment (e.g. cell–cell, cell–extracellular matrix interactions and growth factors) [39,40]. The process in which precursor cells differentiate into another cell type is called transdifferentiation [40,41]. Besides transdifferentiation, BMSCs cells can fuse with neighbouring specialized cells to substitute damaged cells [42]. This, however, is probably a rare event in the pancreas [43].

Infusion of bone-marrow cells can restore chemically induced diabetes in mice [1,3]. Notably, however, it has not been indisputably shown that BMSCs differentiate into β cells. A recent study described engraftment of endogenous bone marrow in damaged islets [44]. However, the engraftment did not generate cells that express insulin or β-cell-specific transcription factors such as PDX1 or NKX6.1 [44]. This suggests a supportive role of BMSCs in regeneration rather than direct substitution of damaged cells. Other studies do not corroborate the above-mentioned findings, because they could not show a single endogenous BMSC type in the injured pancreas [45]. In spite of conflicting findings, the role of BMSCs in regenerative processes *in vivo* remains the subject of study because the *in vitro* findings are promising [3,46]. BMSCs that are cultured *in vitro* can produce *de novo* insulin and express insulin and solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2), when challenged with high glucose [3,46].

In summary, current research suggests that BMSCs do not differentiate into β cells *in vivo*. However, it has been shown that BMSCs can support pancreatic growth *in vivo* and can be manipulated *in vitro* to differentiate into β cells.

Organ-bound or endogenous stem cells
It is generally accepted that the pancreas in the adult has a small population of cells that are capable of continuous
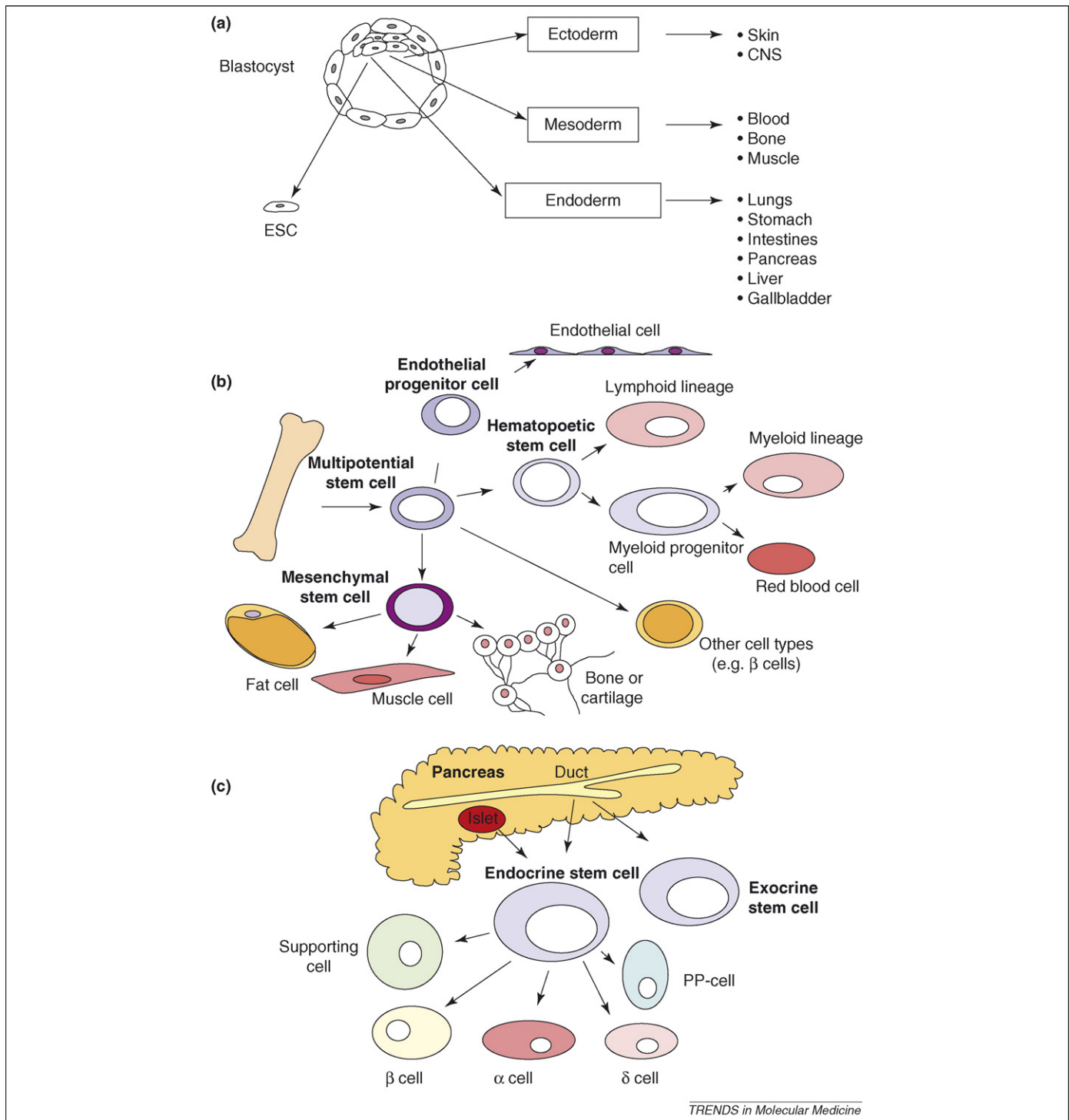
Figure 2. The embryonic, bone-marrow-derived and organ-bound stem cells. **(a)** Embryonic stem cells can derive from the embryoblast. The embryoblast is the inner cell layer of the blastocyst. The outer cell layer of the blastocyst is the trophoblast. The embryoblast forms the three germ layers: ectoderm, mesoderm and endoderm, which develop into the various tissues, as shown in the figure. The trophoblast develops into the placenta. **(b)** Bone-marrow-derived stem cells (BMSCs) are multipotent stem cells that can differentiate into multiple cell lineages. A well-known line of stem cell, which differentiates from the multipotent stem cell, is the haematopoetic stem cell line, which can differentiate into lymphoid and myeloid progenitor cells. These cells can further differentiate into cells of the lymphoid lineage (immune cells such as T cells, B cells and natural killer (NK) cells involved in adaptive immune responses) and myeloid lineage (blood cells and phagocytes involved in innate immune responses). Other lines of stem cells are MSCs and endothelial progenitor cells. BMSCs can also differentiate into other cells or tissues such as probably β cells. **(c)** Organ-bound stem cells are unspecialized cells or cells with some but not all features of specialized cells. These cells can renew themselves and differentiate into fully specialized cells of the organ they reside in. It is assumed that pancreatic stem cells reside in the ducts and in the islets of Langerhans. Cells from ducts can differentiate into exocrine and endocrine cells. Stem cells in the islets can differentiate into endocrine cells and possibly also into supporting cells such as endothelial cells. The function of these cells is plausibly to replace damaged cells in case of organ damage.

self-renewal and can differentiate into cells of the pancreas. These organ-bound stem cells (or pancreatic stem cells) have received a lot of attention of the scientific community during the past five years because they hold several advantages over the other sources. They combine the ability for prolonged proliferation with an already partial differentiation towards an endocrine phenotype. This might facilitate a more readily and less complicated

differentiation strategy towards islet cells than that of other sources. Also, these cells can be the target of specific clinical therapies to stimulate endogenous stem cells to induce compensatory growths [47]. At least two populations of pancreatic stem cells have been described in the pancreas (Figure 2c). The first group of stem cells consists of ductal cells and acinar cells. It has been suggested that these cells are pancreatic ductal epithelium cells that express the ductal marker CK-19 and PDX1 [48]. These cells can expand and differentiate into endocrine cells [49]. The second group of stem cells consists of islet-derived stem cells. The evidence for the existence of an islet-derived population of stem cells comes from studies that show a population of insulin-containing cells reappearing in the islets after total destruction of the β-cell mass with streptozotocin [47]. Few studies have addressed the identification of residential islet-bound stem cells [50,51]. These studies have recognized several distinct populations, as will be discussed in the following sections.

For many years, nestin-positive cells have been proposed to represent a population of islet-derived stem cells [51]. It has been shown that nestin-positive islet-derived progenitor cells (NIP cells) express the ATP-binding cassette (a stem-cell marker) [50] and have an extended capacity to proliferate *in vivo*. Also, NIP cells that are derived from the islets can differentiate *in vitro* into cells with liver, pancreatic exocrine and/or ductal, and endocrine phenotypes [51]. After differentiation, the cells were shown to produce insulin, glucagon, glucagon-like peptide-1 (GLP-1) and the transcription factor PDX1 [51]. Nestin, however, is not only present in the endocrine pancreas, but also in ductules [52]. These ductal nestin-positive stem cells also have features of β-cells precursors such as high levels of PDX1 [52,53]. Unfortunately, studies on nestin-positive cells have not been carried forward because it has been shown in some studies that nestin in the pancreas is only expressed in cells that are not destined to become β cells [54–56]. However, recent evidence suggests that there are different populations of nestin-positive cells in the pancreas [57]. In summary, nestin seems to be a functional protein that, under specific circumstances, is expressed in β-cell precursors but it might also be present in other cell types [57]. In our opinion, nestin is not a suitable marker for endocrine stem cells, but might be an important functional protein that is present in a wide variety of stem-cell sources. It is therefore mandatory to investigate further the role of nestin in β-cell precursors because its expression pattern suggests an essential function in the maturation of precursor cells.

The pancreas also contains a population of cells that carry the hepatocyte growth factor (HGF) receptor – that is the c-met receptor [58]. In the pancreas, HGF has been suggested to be a potent regulator of development of β-cell function, differentiation and proliferation [59]. It has been long assumed that the c-met-positive cells in the pancreas are the oval hepatic cells [58]. However, recent studies suggest that c-met is not specific for the liver and is also present in other tissues [58]. In the neonatal mouse pancreas, c-met-positive cells reside in acinar tissues, but not in the islets [60]. Also, some cells are present in ducts, but these cells do not have an endocrine or acinar phenotype.

In the adult pancreas, c-met is mainly expressed in and around the ducts, vascular endothelium and cells around acinar tissues [58]. Moreover, it has been shown that c-met-positive colonies derived from c-met-positive cells contain cells that express several markers for endocrine lineage cells and that these cells can differentiate into endocrine cells *in vivo*.

A third population of cells in the pancreas that qualifies for residential islet-bound stem cell is the c-kit-positive cells. C-kit is known to be a universal marker for stem cells. Binding of stem-cell factor to the c-kit receptor leads to cell proliferation, cell survival and chemotaxis [61]. The c-kit receptor is expressed on various cells and has been recently shown to be expressed in the prenatal and postnatal rat pancreas [62]. Co-localization studies in the postnatal day 7 (P7) rat pancreas demonstrated that c-kit is expressed in the periphery of islets and co-localize with glucagon-positive cells [62]. Studies on epithelial monolayers that are derived from postnatal rat pancreatic islets demonstrate the presence and increase of c-kit-expressing cell number [63]. These epithelial monolayers express c-kit and developmental transcription factors such as PDX1 and ngn3 during a culture period of eight weeks. After further differentiation, insulin and glucagon are weakly expressed, whereas c-kit mRNA disappears [63]. Although, the role of c-kit in the development of β cells remains to be further elucidated, these studies show that c-kit-positive stem cells have features of endocrine cells precursors and can be observed in islets.

Sources of non-pancreatic organ-bound stem cells

Not only pancreas-derived cells, but also extrapancreatic organ-bound stem cells can differentiate into islet cells. Recently, liver cells [41,64–66], human adipose-tissue-derived mesenchymal stem cells (MSCs) [67], gut stem cells [68] and splenocytes [69] have been shown to have this ability. The rationale to study extrapancreatic sources is that organ-bound stem cells from organs such as the adipose tissue can be harvested more easily. Extrapancreatic precursor cells might also be available in higher amounts than precursor cells in the pancreas. Strategies to induce this transdifferentiation from extrapancreatic sources are similar to those from ESCs. Cultured hepatic stem cells from rodents or humans differentiate into a β-cell phenotype in a high-glucose environment. Also, overexpression of the transcription factor PDX1 [66] and the combination of the overexpression of BETA2 and addition of betacellulin to the medium [41,65] is a successful approach to promote differentiation into a β-cell phenotype. Unfortunately, there are still no functional studies that show biphasic insulin release upon glucose challenge in these cells.

Future directions

Researchers have shown the applicability of embryonic stem cells, bone-marrow-derived stem cells and organ-bound stem cells for the treatment of diseased endocrine pancreas. However, they have also shown several challenges (Box 3). The most important challenge is to produce fully functional insulin-producing cells with a normal biphasic insulin release and the ability to regulate blood

Box 3. Outstanding questions

Despite the progresses that have been made in the knowledge and technologies to differentiate stem cells into mature β cells, several challenging questions still need to be answered.
- What are the requirements to manipulate stem cells to become fully functional insulin-producing β cells?
- Which biological factors are involved in the end-stage differentiation of a precursor into a glucose-responsive β cell?
- How can senescence and genetic disorders such as unlimited proliferation in stem cells be avoided?

glucose in a minute-to-minute dynamic fashion. In our opinion, to achieve this goal it is mandatory to gain insights into the basic biology of differentiation towards insulin-producing cells. Studies in which novel transcription factors have been overexpressed in precursor cells, which then differentiated into an endocrine phenotype, have shown that this is a successful approach [27,31]. Without doubt there are functional genes to be identified that are responsible for the induction of the glucose-activated insulin response.

Here, we have illustrated the importance of endogenous stem cells for clinical application. Although this is a new stem-cell source, it provides several advantages over other systems. These cells might be applied autologously because they can be of pancreatic or extrapancreatic origin. Also, because these cells are already partially differentiated they can readily differentiate into fully functional β cells. These endogenous stem cells are present in the ductal system of the pancreas, possibly in the endocrine pancreas and in other organ structures. Conceivable applications of endogenous stem cells are (i) enhancement of the regenerative capacity of the endocrine pancreas in non-autoimmune diabetes by stimulation of differentiation using trophic factors or (ii) infusion of these cells into diabetic patients. Also, these cells might be applied in islet transplantation to increase the regenerative capacity of the graft in the ischaemic period between transplantation and full vascularization of the graft. Also, ex vivo islet-cell generation might be an option but, up to now, there are no studies that show an unlimited proliferation of endogenous stem cells.

A potential pitfall that requires careful consideration is that many cultured stem cells show not only features of senescence but also of genetic disorders such as unlimited proliferation, leading to tumour formation. Technologies should be designed to remove these cells. Another challenge is to overcome autoimmune destruction when stem cells are proposed for treatment of type 2 and type 1 diabetes. The exogenously differentiated β cells are phenotypically identical to β cells and, therefore, prone to autoimmunity.

Concluding remarks

Many have described the potentials and benefits of stem cells for the treatment of diabetes. Several approaches have been described and reviewed. Stem cells might increase the regenerative capacity of the diabetic pancreas [1,3] and they might also be used as a source for islet cells to solve donor shortage in islet transplantation [26]. These applications have been extensively reviewed during recent years. Fundamental biological processes, however, have gained not enough attention because there is still not much understanding of which mechanisms are involved in the differentiation into fully functional insulin-producing β cells. A strict sequence and pattern of expression of transcription factors is required for producing islet cells with normal biphasic insulin responses. At present, it is difficult to stimulate this process exogenously. Therefore, progress has not yet met clinical expectations. This however does not mean that progress is lacking. During recent years, many new insights in the developmental biology of the pancreas and improved culture technologies have made clinical application of stem cells a realistic option. Stepwise analysis of expression patterns of transcription factors has provided information of the sequence of events that result in β-cell formation and also enables quantification of differentiation towards β cells. Subsequently, there have been studies on exogenous manipulation of the expression of transcription factors in stem cells to promote differentiation of stem cells towards islet-cell phenotypes. However, there is still a gap in the knowledge on the developmental biology of β cells, leading to the inability to produce fully functional β cells from stem cells in vitro. Therefore, more knowledge and step-by-step scientific research is required. Nevertheless, stem cells are a promising alternative source for grafting of novel β cells.

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