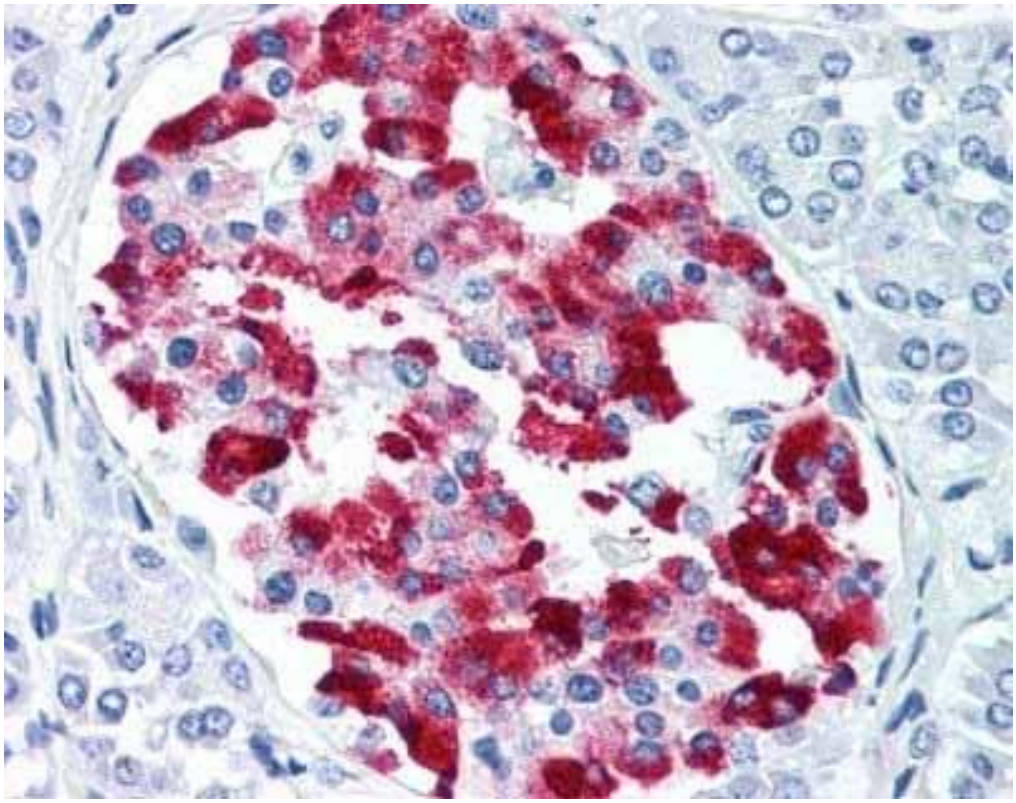
Potential new sources for β-cell replacement therapy in diabetes type 1 patients



Annemarie F. Penders
December, 2009

Front page: Islet of Langerhans with β-cells of human pancreas [Abcam]

Title	Potential new sources for β-cell replacement therapy in diabetes type 1 patients
Author	Annemarie Fenna Penders
Study	Master Biology of Disease Utrecht University
Department	Islet Transplantation Unit, Department of Nephrology, Leiden University
Supervisor	Dr. E.J.P de Koning (Leiden University) Dr. I.E.T. van den Berg (Utrecht University)
Date	December 11th, 2009

Index

Abstract

Long term complications in diabetes patients are a consequence of the constantly high blood glucose levels, hyperglycemia. In patients with type 1 diabetes hyperglycemia is caused by the absence of insulin production by β-cells, which are destructed.

With β-cell replacement therapy these destructed β-cells can be substituted by healthy β-cells, which are capable of producing insulin and thereby maintaining normal blood glucose levels. This procedure can be a potential cure for diabetes type 1. However, there is not enough cadaveric tissue available to help all current diabetes type 1 patients.

But, by identifying the origins of β-cells *in vivo*, these cells can be generated *in vitro* and contribute to an expanded mass. Thereby, a new source was discovered, induced pluripotent stem (iPS) cells, which can differentiate into insulin producing cells, after redifferentiation into an ES cell-like stage.

In this review potential sources of newly formed β-cells will be discussed, with focus on iPS cells. Also the safety issues that must be overcome before these cells can be used in β-cell replacement therapy for diabetes type 1 will be discussed.

Abbreviations

AP	=	alkaline phosphatase
BJ fibroblasts	=	neonatal human foreskin fibroblasts
BM	=	bone marrow
dbdb	=	diabetic mutant
dH1f, dH1cf	=	human ES-cell-derived fibroblast
DM	=	diabetes mellitus
EG cell	=	embryonic germ cell
EGF	=	epidermal growth factor
ES cell	=	embryonic stem cell
GLUT-2	=	glucose transporter-2
hAFF	=	human adult foreskin fibroblast
HDF	=	human dermal fibroblast
hEF	=	human embryonic fibroblast
HFF	=	human foreskin fibroblast
hFib2	=	adult dermal fibroblast
hFSF	=	human fetal skin fibroblast
ILC	=	islet-like cluster
iPS cell	=	induced pluripotent stem cell
Klf	=	Krüppel-like zinc finger
LIF	=	leukaemia inhibitory factor
MEF	=	mouse embryonic fibroblast
MRC5	=	primary fetal cells
MSC	=	mesenchymal stem cell
Ngn3	=	Neurogenin 3
NHDF	=	neonatal human foreskin fibroblast
NIC	=	nicotinamide
NOD	=	nonobese diabetic
PDX-1	=	pancreatic duodenal homebox-1
PP	=	pancreatic polypeptide
Px	=	partial pancreatectomy
sc	=	*subcutaneous*
SCID	=	severe combined immune deficiency
STZ	=	streptozotocin
TF	=	transcription factor
TRA	=	tumour rejection antigen
TTF	=	tail-tip fibroblast
VPA	=	valproic acid
WHO	=	World Health Organization

Introduction

According to figures provided by World Health Organization (WHO), in 1985 almost thirty million people suffered from diabetes, which increased to 177 million in 2000. WHO believes this number will reach an estimated number of 370 million in 2030.

Diabetes mellitus (DM), further called diabetes, is a degenerative disease, which is characterized by high levels of blood glucose caused by defects in insulin production, insulin action, or both. Diabetes type 1 and diabetes type 2 are the most prevalent forms of diabetes. Nowadays, focus of research and health campaigns is mainly aimed at patients with diabetes type 2, because concerns are rising about the age of onset of diabetes type 2, which is decreasing dramatically in industrialized countries. Despite this social focus on type 2 diabetes, further insights are needed in treatments of patients with diabetes type 1 because of long term complications. The focus of this review will be on β-cell (insulin producing cells of the pancreas) replacement therapy in diabetes type 1.

When diabetes patients have severe complications, caused by elevated blood glucose levels during life, transplantation can be an outcome. β-cell replacement therapy can also be a cure for these patients, when destructed β-cells become replaced by new healthy cells that are capable of insulin production, thereby preventing hyperglycemia. Although this seems promising, a major problem is a shortage of available donor tissue.

To solve this problem an alternative must be found. A possible solution lies in *in vitro* generation of β-cells from different sources, including replication of pre-existing β-cells [Dor et al., 2004], transdifferentation of acinar cells into islets [Lardon et al., 2004; Baeyens et al., 2005; Granger et al., 2009], islet neogenesis, or stem cells or progenitors that do not originate in the pancreas [Andersson et al., 2009]. The latter field focuses on replacement of lost or injured tissue with cells derived from mesenchymal stem cells (MSC) [Li et al., 2007; Da Silva Meirelles et al., 2008; Karnieli et al., 2007], bone marrow (BM)-derived cells [Hess et al., 2003; Ianus et al., 2003], and embryonic stem (ES) cells [Brolén et al., 2005; D'Amour et al., 2006; Jiang, W. et al., 2007; Jiang, J. et al., 2007; Kroon et al., 2008]. These sources are cells from which β-cells originate *in vivo*. Researchers are constantly looking for new possibilities. A study by Takahashi et al. [2006] offered a new perspective: iPS cells, characterized by properties similar to ES cells.

With focus on iPS cells, potential sources of newly formed β-cells will be discussed in this review. Before these cells can be used in β-cell replacement therapy for diabetes type 1, some safety issues must be overcome.

Diabetes and the role of β-cells

Diabetes is an endocrine disease, characterized by a shortage of the hormone insulin and thereby causing a rise in blood glucose levels. In diabetes type 1 insulin is not produced anymore as a consequence of destruction of β-cells. They are destructed by autoreactive T-cells, which characterises diabetes type 1 as an autoimmune disease. If no insulin is produced, glucose is not converted into glycogen and concentration glucose in blood rises, what is called hyperglycemia. In diabetes type 2, on the contrary, β-cells are not destructed and insulin is still produced but in a decreased and delayed way. Diabetes type 2 is characterized by insulin resistance; excreted insulin is not processed in an optimal way, and sensitivity is disrupted. So, although insulin is produced by β-cells, no glycogenesis occurs and concentration glucose in blood rises.

Next, function of β-cells will be discussed in more detail, as also complications of DM type I and possible treatments.

Function and localisation of β-cells

The pancreas (Figure 1) is a gland, located behind the stomach, and has two main functions. It functions as an exocrine organ, by production of digestive enzymes, and as an endocrine organ, by the production of hormones. The adult pancreas is composed of three major tissues, including ducts that transport digestive enzymes, endocrine islets, and exocrine cells, like ducts and acinar cells, which produce digestive enzymes [Bonner-Weir et al., 2000; Jiang et al., 2007]. These endocrine islets are islets of Langerhans and they consist of β-cells, which produce insulin, α-cells for glucagon, δ-cells for somatostatin, ε-cells for production of ghrelin, and PP cells which produce pancreatic polypeptide [Murtaugh et al., 2007].

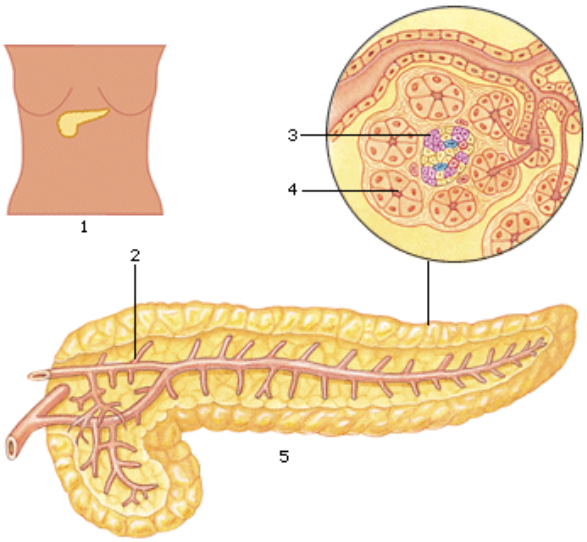


Figure 1: Pancreas

The pancreas is a gland located behind the stomach consisting of hormone producing cells. 1. location of pancreas 2. pancreatic duct 3. islet of Langerhans 4. acinar cells 5. pancreas [Medical Encyclopedia, 2006]

Short term and long term complications

The pathophysiology of untreated diabetes is characterized by high concentrations of glucose molecules in the blood in combination with low glucose levels in the cells. Low glucose levels in the cells lead to alternative ways to maintain their glucose levels: glycogenolysis, lipolysis, and protein breakdown. These activities lead to potassium depletion, hyperkalemia, hyperlipidemia, ketosis, and acidosis. Osmotic diuresis caused by high blood glucose levels can lead to dehydration. Elevated blood glucose levels lead to increased glycation of proteins with changes in protein function. Although presently there is no cure for diabetes [Holland et al., 2009], the disease is controllable via the injection with insulin, via a pump or via daily injections. A *subcutaneous* (*sc.)* injection with insulin replaces body's own insulin, restoring normal blood glucose level, which lies around five mmol/l. Thereby, hyperglycemia is prevented and the risk of long term complications are lowered. Even though normal blood glucose level is maintained with insulin, long term complications of elevated blood glucose levels are serious.

Diabetes can lead to number one cause of death worldwide: cardiovascular diseases. These days, diabetes is not a direct life threatening disease anymore when treated with insulin, as discussed earlier, and this treatment also increases life expectation of these patients. But this does not prevent diabetes patients suffering from long term complications, like atherosclerosis, kidney failures, neuropathy, retinopathy, and a high chance of infections at high age.

Transplantation and diabetes type 1

When long term complications of diabetes become life threatening for the patient, transplantation can be an option. This is presumably the case for type 1 diabetic patients with end-stage renal disease, in which pancreas as well as kidneys are transplanted. Yet, the introduction of the Edmonton protocol which improved transplantation of islets, gave an alternative for pancreas transplantation [Ryan et al., 2001]. A β-cell transplantation, in which β-cells are transplanted into type 1 diabetes patients to replace destroyed β-cells, provide healthy β-cells that are capable of producing insulin and thereby preventing hyperglycemia. So, β-cell transplantation may be a potential cure for diabetes.

But this transplantation is not without any risks and problems to overcome. To prevent rejection of the transplant, lifelong immunosuppressive drugs must be administered. But before patients must deal with these problems, the shortage of cadaveric donor tissue must be overcome. There are not enough pancreatic islets or pancreatic tissue available for the increasing demand at the moment. Even when β-cell replacement therapy can be implicated as a cure for diabetes type 1, the number of available donor tissue will never meet the increasing number of patients. It is also estimated that fifty to seventy percent of the transplanted islets will not survive during post transplantation period. Per transplantation around one million islets are needed, which represents nearly two billion β-cells, for a

successful transplantation [Lehman et al., 2007]. This high number combined with low functional capacity of the transplants implies the need of multiple islet donors for one patient.

To solve the problem of a shortage of available β-cells, an alternative must be found. For a possible treatment of diabetes with β-cell replacement therapy insight into the origin of β-cell is essential. When the *in vivo* origins of β-cells are known, these cells can be stimulated *in vitro* to differentiate into β-cells. The *in vitro* generation of β-cells from different sources can be a possible solution to expand the available β-cell mass, which will be described next.

Origin of β-cells

The pancreas originally was thought to be an organ with limited renewal, like liver, kidney, and salivary glands. Pancreatic cells, including β-cells, were believed to stop dividing after birth. But further research with pancreatic injury models suggested a regenerative capacity of the pancreas, when dividing cells were present in the diseased pancreas. These pancreatic injury models included partial pancreatectomy (Px) or pancreatic ductal ligation. To further explore the regenerative capacity of the pancreas, the origin of newly formed islets was determined. Furthermore, with the knowledge of sources of newly formed β-cells, these cells can be generated *in vitro* and used in β-cell replacement therapy.

Several studies have researched the origin of β-cells and reported that differentiation of new β-cells in the adult pancreas can occur from different sources, which are divided into extra-pancreatic and pancreatic sources. Figure 2 gives an overview of the potential pancreatic sources for β-cells, including replication of mature β-cells or pre-existing β-cells present in pancreas, transdifferentiation of acinar cells into β-cells, or neogenesis. Neogenesis is a process of regeneration and it describes budding of new islets from pancreatic progenitor cells which are located in or near pancreatic ductal epithelium, or ducts [Bonner-Weir et al., 2005].

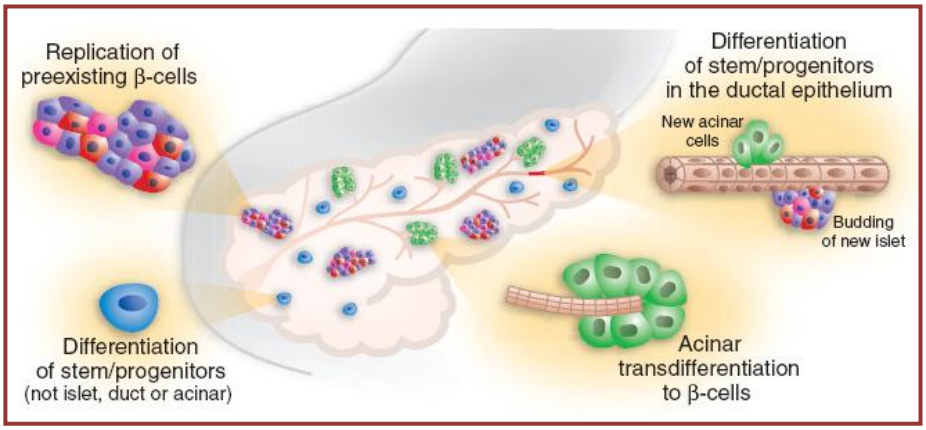


Figure 2: Potential sources for β-cells

The pancreas harbours sources that can differentiate into β-cells. These processes include replication of mature β-cells, and transdifferentiation of acinar cells into β-cells. β-cells can also arise from stem cells or progenitors that are not of pancreatic cellular origin. [Bonner-Weir et al., 2005]

Besides transdifferentiation, replication of pre-existing β-cells, or neogenesis, results indicated that newly formed β-cells originate from stem cells or progenitors that do not originate in the pancreas. This category includes MSC, BM-derived cells, and human ES cells.

The described experiments are mainly performed with animal models. However, it was found that human islets have a unique architecture and character, different from islets in rodents. But, in spite of this difference, mouse and other rodent models are still used and relevant in translational research.

Replication of mature β-cells

Two studies in the late sixties described the potential of differentiated β-cells to replicate. Although replication rate of these cells is very low, according to Logothetopoulos et al. [1966] and Like et al. [1969], an increase in cell numbers is the result of expansion of mature β-cells, which are located in islets of Langerhans in pancreas. With the use of diabetic mutants (dbdb) of C57 Bl/Ks mice, which are characterized by hyperglycemia, low concentration of insulin, and a decreased amount of β-cells, Like et al. [1969] showed that after a restricted diet, no ductal proliferation was present and the increase in β-cells was suggested to be caused by dividing mature β-cells. Dividing cells were stained and appeared to be located in interior and periphery of the pancreatic islets. 75 percent of these cells were β-cells, which was indicated by the presence of secretory granules.

A more recent article by Dor et al. [2004] described the genetic lineage tracing technique to discover a role of stem cells in postnatal β-cell proliferation. Yet, they found that mature β-cells appear to be the source of new β-cells in adults and after Px in mice. To determine the origin of newly formed β-cells, they heritably labelled differentiated β-cells in transgenic mice with a tamoxifen-inducible Cre/lox system. New β-cells derived from present mature cells were labelled in contrast to unlabelled β-cells derived from stem cells. Mice were injected at an age of six to eight weeks and were killed after two and a half, four, six, nine, or twelve months. They did not find negative labelled β-cells and could conclude that newly differentiated β-cells in adult mice were formed from β-cells present when mice were younger. With the removal of a part of the pancreas the hypothesis that stem or progenitor cells only arise and participate after stimulation, so called facultative stem cells, can be rejected since no β-cells were found derived from another source than mature β-cells [Dor et al., 2004].

Although Dor et al. [2004] have showed that newly formed β-cells originate from mature β-cells, they did not prove the absence of stem or progenitor cells. Facultative stem cells, discussed above, could maybe react to another kind of stimulus.

This proliferative capacity of β-cells *in vivo* can contribute to an increased β-cell mass *in vitro*. To gain further insight into possible implication of this process for patients with type 1 diabetes extended investigation is required. For instance, it is shown that diabetes type 1 patients still generate newly β-cells, but these cells are also attacked by autoreactive T-cells and destroyed.

Transdifferentiation

Baeyens et al. [2005] showed the possibility of adult rat exocrine cells to become insulin-producing cells cultured *in vitro* with epidermal growth factor (EGF) and leukaemia inhibitory factor (LIF). Exocrine cells transdifferentiated into mature β-cells, which were characterized by secretory granules. The β-cells were positively immunostained for C-

peptide, Pdx-1, and GLUT-2. Transdifferentiation describes the process of cells that revert to a dedifferentiated state and from this level differentiate in other phenotypes, including β-cells, as described by Lardon et al. [2004]. They found that acini were replaced by ductal complexes in an *in vivo* model of duct ligation-induced pancreas remodelling five days after ligation. This observation was supported by the findings that acinar cells transdifferentiated into ductal cell within five days in culture.

These finding are consistent with the results found by Song et al. [2004]. They described a so-called spontaneous transdifferentation of acinar cells into insulin-producing cells *in vitro*. Besides by transdifferentiation of acinar cells [Lipsett et al., 2002], β-cells can also arise from hormone producing islet cells, other than β-cells [Guz et al., 2001], or splenocytes [Kodama et al., 2003].

The process of transdifferentiation of non-insulin producing cells into β-cells has *in vitro* and *in vivo* implications for increasing the β-cell mass. However, other research groups found contrasting results and more research is needed to identify extracellular factors that are involved in β-cell differentiation, so that differentiation protocols into β-cells can be optimized.

Islet neogenesis

Islet neogenesis describes the process in which budding of new islets occur from pancreatic progenitor cells which are located in or near ducts [Bonner-Weir et al., 2005].
In 2000, a potential new source for *in vitro* β-cell generation was described. It appeared that ductal tissue could lose its specific duct phenotype and these ductal cells could revert to multipotent cells that became β-cells in culture. It was shown that newly formed β-cells were glucose responsive, since they produce insulin in pancreatic tissue. However, the amount of newly formed β-cells was limited and efficiency in differentiating cells was low, so the technique needed to be optimized [Bonner-Weir et al., 2000]. They also proved that during replication ductal epithelial cells regress to a less differentiated stage and act as progenitor cells and revert into β-cells in response to stimulating factors, which are not exactly known. They confirmed the model by results of a ninety percent Px in adult rats. They described the ductal cells as facultative progenitor cells [Bonner-Weir et al., 2004]. The presence of new islet formation by budding from exocrine ducts was also seen in a type 2 diabetes model [Butler et al., 2003]. Although the total β-cell mass is decreased in patients with type 2 diabetes, new islet formation from exocrine ducts was seen in these patients. They even report a fifty percent increase in β-cell volume in nondiabetic obese human. The decrease in β-cell-mass is due to an increase in β-cell apoptosis, which occurs in a higher rate than neogenesis.

These results are in contrast to what is described previously, in which is stated that new β-cells are only formed by self-replication after birth or after Px in mice [Dor et al., 2004]. They did not find any form of islet neogenesis. Yet, to rule out the presence of alternative sources marking techniques, like lineage tracing, can be used to identify the origins of newly

formed β-cells and to determine whether duct cells are potential progenitor cells [Bonner-Weir et al., 2005].

The study performed by Bonner-Weir et al. [2000] was reproduced and results supported the previously found results [Gao et al., 2003]. They describe the differentiation of pancreatic progenitor cells into endocrine cells in serum-free culture. Numerous small dense buds (CHIPS) were formed and transplanted under the kidney capsules of five nude mice, but only one engraftment continued to grow. Factors involved in the differentiation protocol were Matrigel for cell migration, laminin to promote β-cell differentiation in culture, nicotimanide (NIC) to promote development of endocrine cells, and FGF-7 [Gao et al., 2003].

When pancreatic ductal cells replicate, they express markers like Ipf-1/ Pdx-1, which are associated with islet differentiation, thereby confirming ductal epithelial cells as progenitors [Gao et al., 2003; Bonner-Weir et al., 2006].

The next step was obtaining highly purified pancreatic duct cells derived from human pancreatic tissue and test whether these cells could differentiate into insulin-producing cells *in vitro*. These cells were then transplanted under the kidney capsules of NOD-SCID mice and the presence of insulin positive cells was seen in the grafts. Despite this success these cells were thought to be immature, because of the presence of ductal markers. The existence of ductal progenitor cells in the pancreas followed by differentiation of these cells into functional β-cells was confirmed [Yatoh et al., 2007]. Likewise an article that provided first direct evidence for ductal progenitors as a potential source of newly formed β-cells. Differentiation of progenitor cells into new β-cells can be induced when Neurogenin 3 (Ngn3), an islet cell-specific TF in embryonic development, is activated [Xu et al., 2008].

Islet neogenesis appeared to be involved in islet cell regeneration after birth and after injury. Consequently, evidence is provided that pancreatic duct cells can serve as a source for β-cells, but this process needs an activating signal *in vivo* [Xia et al., 2009]. A future implication of this process is that, when the process is completely understood, it might be useful in expanding the β-cell mass by producing new β-cells [Bonner-Weir et al., 2006].

Non-pancreatic derived stem and progenitor cells

According to the studies described in the following, different types of stem cells can contribute to differentiation of new β-cells. Stem cells are divided into embryonic pluripotent and endogenous stem cells and figure 3 gives an overview of locations where these stem cells can be isolated.

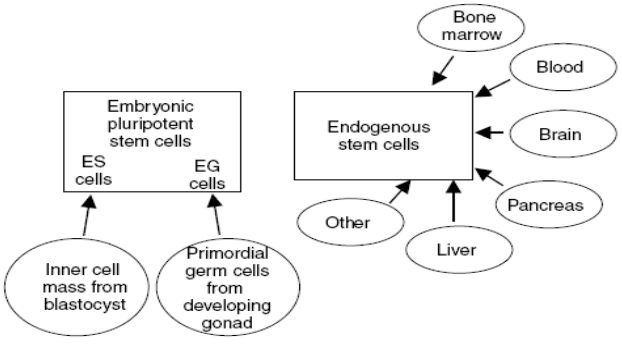


Figure 3: Various sources of stem cells

Stem cells can be divided into embryonic pluripotent stem cells, which are isolated from ES cells and embryonic germ (EG) cells, and endogenous stem cells. Endogenous stem cells can be isolated from different tissues, including BM, blood, brain, pancreas, liver, and other.
[Kaczorowski et al., 2002]

MSC, BM-derived cells, and ES cells will be discussed. ES cells provided a bridge to the discovery of iPS cells, which will be discussed later.

Mesenchymal stem cells

MSC or BM stromal cells are multipotent stem cells that differentiate into a variety of cell types. MSC become more interesting in research for cell-based therapies, since they have a high differentiation capacity and because of their lack of ethical issues and teratoma formation. But MSC characterization is still a little vague, since exact function and location of MSC *in vivo* are not well understood. But Da Silva Meirelles et al. [2008] provided evidence for a perivascular location for MSC; they are located as pericytes throughout the body. It also appeared that MSC not only exist in BM but in all organs.

High differentiation capacity of these cells provided the opportunity to become β-cells. Li et al. [2007] confirmed this hypothesis and showed that human MSC that express pancreatic duodenal homeobox-1 (PDX-1) can differentiate into insulin-producing cells. PDX-1 gene was introduced into human MSC via an adenoviral vector. PDX-1 is a transcription factor (TF) that plays a pivotal role in pancreas development and β-cell gene expression. The next step consisted in the introduction of these PDX-1^{+} cells into STZ-induced BALB/B mice. Hyperglycemic mice became euglycemic within two weeks and were stable for at least 42 days. Karnieli et al. [2007] also found that PDX-1 induced differentiation of human MSC into β-cells, thereby making therapeutical application of MSC in β-cell replacement possible.

Bone marrow-derived cells

Endogenous stem cells, cells that are part of the adult body, differ from ES cells in that they have a limited proliferation capacity *in vitro* and they are not pluripotent, two of the characteristic advantages of ES cells. But, since these endogenous stem cells are part of the adult body, they can be used for autologous transplantation [Kaczorowski et al., 2002].

BM-derived stem cells are capable of repairing tissue after damage. This is shown in mice with pancreatic injury, induced by STZ injection. Mice became hyperglycaemic, but when they received GFP^{+} BM-derived cells, mice became euglycemic within seven days. Instead of turning into insulin producing β-cells, BM-derived cells became vascular endothelial cells. These cells are believed to secrete factors that induce differentiation into β-cells, thereby restoring euglycemia. It is thought that BM-derived cells can contribute to β-cell regeneration, but therefore they need a signal, like pancreatic injury, before these cells are recruited from BM to site of injury [Hess et al., 2003].

In contrast, other research stated that BM harbours cells that can differentiate into almost all cells of the body. They made use of the CRE-LoxP system to analyze cells derived from transplanted BM and they found that these cells became β-cells, which expressed insulin in a glucose-responsive manner, GLUT-2, and TF specific for normal β-cells. Although euglycemia was restored, they could not exclude the possibility that BM-derived cells directly differentiate into β-cells. Furthermore, it is possible that BM-derived cells enter an intermediary stage and then respond to signals and differentiate into β-cells. It is also possible that BM-derived cells reside in BM during development and home to the pancreas in response

to signals. These extra-pancreatic progenitors would then be the same as pancreatic progenitors [Ianus et al., 2003; Lee et al., 2003].

So, the exact mechanism by which BM-derived cells contribute to β-cell repair and if these β-cells do derive from the BM still requires further investigation.

Embryonic stem cells

Although previously described methods seem promising as a new tool in producing more β-cells, certain obstacles must be overcome before these methods are optimized and can be used as a therapy for people with diabetes. In most described methods researchers made use of terminally differentiated cell types (somatic cells), cells which were found to differentiate into β-cells *in vivo*. It appeared to be difficult to guide these cells to β-cells *in vitro*, partly because not all factors, involved in this differentiation process, are known.

In contrast, ES cells, which have pluripotent characteristics, contribute to the germ line and can differentiate into all cell types of the body. These ES cells can be directed through a process that mimics pancreatic development to produce β-cells. Other characteristics of ES cells are their ability to proliferate well in culture [Kaczorowski et al., 2002], their almost indefinitely dividing capacity, self-renewability, and that they can be genetically manipulated to avoid rejection by immune system of the host.

At the end of last century it was made possible to do research with human ES cells [Thomson et al., 1998]. This was a useful discovery, because this leaves out the extrapolation step from animal to human. It also appeared that there is a difference in need of growth factors between human and mouse ES cells [Brons et al., 2007; Tesar et al., 2007].

Brolén et al. [2005] induced human ES cells to differentiate into β-cells *in vitro*, but instead of β-cells, human ES cells differentiated into pancreatic and endocrine progenitors. When these progenitors were transplanted under the kidney capsule of five- to seven-weeks-old female SCID mice, together with mouse embryonic pancreas, differentiation into insulin positive cell clusters occurred. These cells had features similar to normal β-cells, in that they produced insulin and they were positive for β-cells TF, such as Isl1, Pdx1, and Foxa2. It appeared that extracellular cues are needed before differentiation into β-cells can occur and these cues were provided by the kidney and co-transplanted pancreas.

With a five stage process that mimics *in vivo* pancreatic organogenesis, human ES cells can be converted into endocrine cells, which produced all pancreatic hormones, [D'Amour et al., 2006]. These stages describe development of definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm and endocrine precursors, and development of hormone-expressing endocrine cells. β-cells generated from human ES cells had a high insulin content and contained secretory granules. But as these cells were not fully responsive to glucose, the researchers believed the cells were immature. Although they have proven β-cells can be differentiated from human ES cells, the lack of *in vivo* results provided no further evidence [D'Amour et al., 2006]. This protocol was further worked out. Cells produced during stage four were transplanted into mice in which they differentiated into functional β-cells, which expressed β-cell-like TF, processed pro-insulin, and which had secretory

granules. When these mice were made diabetic by injection with STZ, human β-cells survived and protected the mice against hyperglycemia [Kroon et al., 2008]. In a slightly different differentiation protocol islet-like clusters (ILC) were formed within thirty days out of human ES cells. This protocol shows similarity with the earlier discussed five-stage protocol in that *in vivo* development of β-cells is mimicked. However, they used a different combination of growth and differentiation factors, including Activin A, EGF, bFGF, and sodium butyrate. In combination with a longer differentiation time and the fact that the protocol was performed in suspension culture, this probably led to the results that newly formed β-cells were able to produce insulin in response to a high blood glucose level [Jiang, J. et al., 2007].

A step further was the transplantation of human ES derived β-cells under the renal capsules of STZ-treated four-to six-weeks-old BALB/c male nude mice [Jiang, W. et al., 2007]. With a mix of factors, including Activin A, all-trans RA, bFGF, and NA in DMEM/F12, they were able to differentiate human ES cells into insulin-producing cells. The next step was the implementation of β-cells into mice and it was proven that β-cells were glucose responsive and able to produce insulin, as thirty percent of the treated mice became euglycemic again for at least six weeks. The protocol is not optimal, partly because of presence of pancreatic exocrine cells in the induced ILC, which can have a negative influence on β-cells. Consequently, purification of the newly formed β-cells is needed for improvement. Protocols described by Jiang, W. [2007] and Jiang, J. [2007] have in common that they both made use of serum-free culture medium.

Previously described studies have proven the potential use of ES cells as a source for β-cells. But ES cells also have their difficulties, like the identification of different human ES cell lines. It is not known how far these cell lines differ in their outcome. ES cells are also genetically unstable, thereby increasing the risk of genomic instability. Transplantation of ES derived β-cells in animals can form teratomas, caused by undifferentiated cells. Teratomas are tumours that consist cells of all three germ layers, including entoderm, mesoderm, and ectoderm. Another problem, which is not unique for ES derived cells, is that transplanted cells acquire the disease of the host. So, in the case of diabetes, the β-cells also become attacked by autoreactive T-cells. Also important, since the cells are not autologous, patient needs immunosuppressive drugs.

Summarizing, although use of human ES cells appears promising for production of enough β-cells for transplantation, it can do no harm to look for other possibilities to produce β-cells *in vitro* for transplantation. The variable results in studies investigating potential sources of β-cells suggest a need of an optimal technical approach, and the presence of other potential sources of β-cells cannot be ruled out.

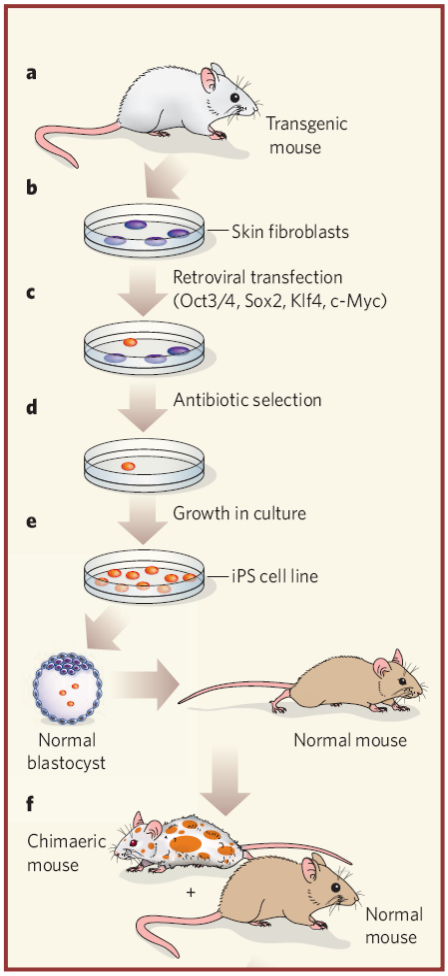
That is what Takahashi et al. [2006] must have thought when they started reprogramming cells. Their recent discovery has led to a new potential source of β-cells: iPS cells.

Induced pluripotent stem cells

Nuclear reprogramming is described as the reversal of the differentiation state of a mature cell to an undifferentiated state, characteristic for embryonic cells. Four strategies, including nuclear transfer, cellular fusion, exposure to oocyte cell extracts, and culture-induced reprogramming, are reviewed [Hochedlinger et al., 2006]. In these different processes of reverting somatic cells to pluripotent cells, the somatic cells in the first three processes need oocytes or parts of oocytes before they can turn into pluripotent cells. However, the culture-induced cells turn into pluripotent cells without the use of oocytes, but instead only need a specific combination of growth factors [Kanatsu-Shinohara et al., 2004; Guan et al., 2006]. Although it appears promising, the genomic imprinting is not balanced, and ES cells are not able to contribute broadly to living chimeras.

Recently another type of pluripotent cells was generated: iPS cells.

Generation of iPS cells



Takahashi et al. [2006] hypothesized that the factors that are responsible for pluripotency of oocytes can contribute to the reprogramming of somatic cells into ES-like cells. They started with 24 candidate genes, and found that a combination of four specific factors was enough to generate iPS cells from mouse embryonic and adult fibroblast cultures by retroviral transduction. These four factors are Oct3/4, Sox2, c-Myc, and Klf4. Figure 4 gives an overview of how iPS cells are generated. Fibroblasts are derived from a transgenic mouse. The isolated cells are transfected with retroviruses that carry the four TF, Oct3/4, Sox2, Klf4, and c-Myc. After drug selection cells resemble ES cells are isolated and expanded into cell lines. A further step is injection of these iPS cells into blastocysts to create chimeric mice.

Figure 4: Generation of iPS cells

a. Transgenic mice were generated expressing a drug-selectable marker to verify pluripotency.
b. Fibroblasts were isolated and
c. retrovirally transfected with Oct3/4, Sox2, Klf4, and c-Myc.
d. Antibiotic selection of transfected cells.
e. ES-like cells were isolated and expanded into iPS cell lines.
f. Injection into blastocysts leads to birth of chimeric mice.
[Adapted from Rossant, 2007]

Donor cells for generation of iPS cells

To generate iPS cells, different type of cells are used as donor cells. First experiments, which will be discussed later, were performed with mouse derived cells, which was followed by human somatic donor cells. Both mouse and human fibroblast cells, which can easily be obtained, were most used as donor cells. Also hepatic cells, B lymphocytes and gastric epithelial cells could be converted into iPS cells [Aoi et al., 2008; Hanna et al., 2008].

Different cells give rise to diverse iPS cell lines with their own characteristics. This is also the case for similar donor cells, suggesting a change in the differentiation process. The question is what are the best donor cells and what is the best differentiation process.

Function and role of reprogramming factors

Three groups were formed to divide the 24 candidate genes, the first group included TF specifically expressed in ES cells, the second group included ES cell-related tumour and growth-related TF, and the third group included not yet fully characterised ES-cell related TF. Oct3/4 and Sox2 were part of the first group, and c-Myc and Klf4 are tumour and growth-related TF.

These factors each play a different role in the reprogramming process into iPS cells. The reprogramming process can be divided into three phases, including the initial phase, the intermediate phase of partially reprogrammed cells, and the final phase of fully reprogrammed cells [Sridharan et al., 2009].

Two research groups have shown that this process is not random, but that TF follow defined steps in a gradual process [Brambrink et al., 2008; Stadtfeld et al., 2008]. The initial phase involves the repression of somatic-specific genes towards an ES-like gene expression pattern, of which c-Myc is the promoter. c-Myc functions in differentiation, cell growth, and proliferation by activating the transcription of cyclin-E, thereby promoting the transition from G1 to S-phase. c-Myc is related to the maintenance of pluripotency and self-renewal of ES cells. This is because the TF is a downstream target of the LIF (leukaemia inhibitory factor)/STAT3 and the Wnt signalling cascade. c-Myc is also a characteristic player in most human cancers. The other three factors, Oct3/4, Klf4, and Sox2 are needed for activation and regulation of pluripotent state. Oct3/4 is a pivotal player in maintenance and self-renewal of pluripotent cells, and thereby promotes differentiation. Sox2 acts in combination with Oct3/4 to activate ES-cell associated genes in ES cells [Boyer et al., 2005]. Like Oct3/4 and Sox2, Nanog is a TF responsible for pluripotency in ES cells, but this TF appeared not to be required for reprogramming of cells into iPS cells [Boyer et al., 2005]. Also Klf4 acts together with Sox2 and Oct3/4 to upregulate a set of target genes and is required for self-renewal in ES cells. Besides acting as a tumour-suppressor by activating p21, Klf4 can act, like c-Myc as an oncogene by down-regulating p53. Although only co-binding of Sox2, Oct3/4, and Klf4 is needed for fulfilling reprogramming, c-Myc plays an important role in inducing reprogramming, and absence of c-Myc leads to inefficient reprogramming [Sridharan et al., 2009].

As described above, the four TF have different functions in how they contribute to the manipulation of somatic cells into pluripotent cells. The exact process and how these factors act and interact are not yet entirely clear. Figure 5 gives an overview of a hypothetical model of these actions in the reprogramming process.

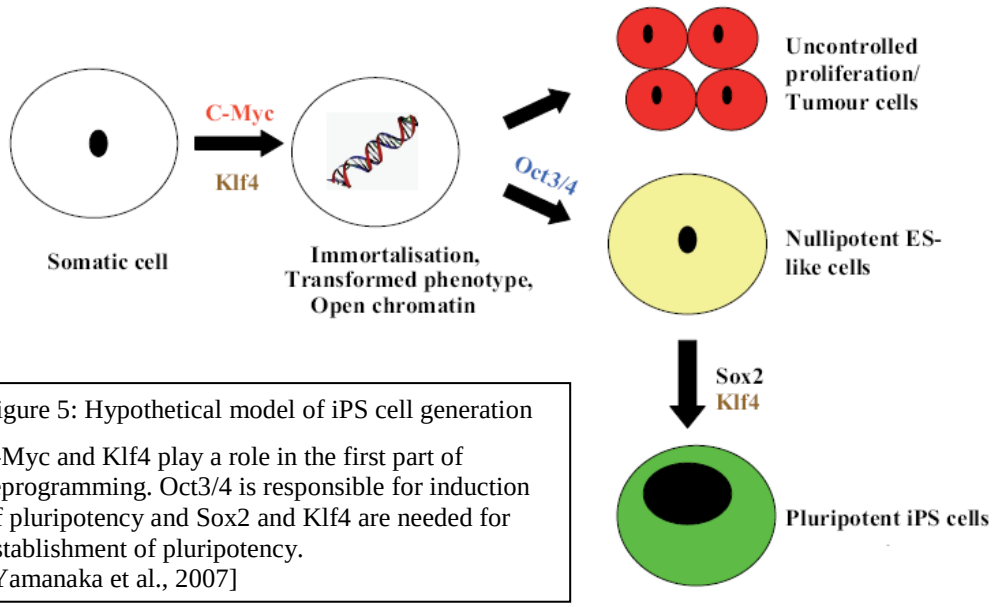


Figure 5: Hypothetical model of iPS cell generation

c-Myc and Klf4 play a role in the first part of reprogramming. Oct3/4 is responsible for induction of pluripotency and Sox2 and Klf4 are needed for establishment of pluripotency.
[Yamanaka et al., 2007]

TF each turns on a different set of genes, and it has been observed that these upregulated genes are similar to the genes upregulated in ES cells [Sridharan et al., 2009]. This is the desired result, to modulate cells that are almost exactly like ES cells. However, a well defined balance must be reached between the various TF before reprogramming can occur, since c-Myc is and Klf4 can act as an oncogene. An alternative is the identification of substitute factors that also can induce reprogramming, which will be discussed later.

Safer and improved cell reprogramming

The generation of iPS cells by Takahashi et al. [2006] was a breakthrough in reprogramming of cells. They were the first to show results of pluripotent cells induced from somatic cells. Because they were the first, they still had some problems to overcome, including low frequency of iPS cell derivation. Less than one percent of somatic cells in which the four TF were induced became pluripotent. Furthermore, tumour formation, and viral integration of the TF posed barriers to effective cell reprogramming.

In the following years other research groups repeated the experiment with the same resulting outcome, and tried to improve the reprogramming process by overcoming the hurdles in different ways.

Selection markers

No living chimeric mice were born when the first generated iPS cells were injected into blastocysts [Takahashi et al., 2006]. This may be explained by the fact that their Fbx15-selected iPS cells were only partially reprogrammed. That is why other groups made use of other selection markers which are more associated with pluripotency, like Nanog or Oct3/4.
Maherali et al. [2007] generated iPS cells from mouse embryonic fibroblasts (MEF) with Nanog as selection marker. After an extended period of time (seven days instead of three days with Fbx15 selection), iPS cell lines were generated. These Nanog selected iPS cells showed similarities with ES cells, like pluripotency, which was confirmed after teratoma formation. Also chimeric mice were born after injection of iPS cells into blastocysts, confirming the generation of fully programmed cells, probably due to the longer infection period.

Compared to this study, in which not many differences were found between Fbx15- and Nanog-selected iPS cells, another study did. When Nanog was used as a selection marker, iPS cells had more similarities with ES cells in gene expression and DNA methylation patterns. Again chimeric mice were formed when Nanog-selected iPS cells were injected into blastocysts from C57BL/6-pseudo-pregnant mice [Okita et al., 2007]. These results were confirmed by another research group, which used Nanog selection for iPS generation, but also Oct4. It appeared that with Oct4 as selection marker more iPS cells were induced per colony, suggesting that Oct4 is more associated with pluripotency [Wernig et al., 2007].

The newly formed Nanog and Oct4 selected iPS cells by these groups were improved in that they were fully reprogrammed and able to produce living chimeric mice.

Viral integration

It has been proven by several groups that viral integration is not required for iPS cell generation. Instead of viral integration, non-integrating adenoviruses expressing TF can be used. iPS cells, induced from fibroblasts and hepatocytes with the described method, show similarities with ES cells like DNA demethylation and pluripotent gene expression. Pluripotency was demonstrated when iPS cells were injected into SCID mice, which resulted in teratoma formation exhibiting cell types of the ectoderm, mesoderm, and entoderm [Stadtfeld et al., 2008]. Another alternative for viral integration is the implementation of plasmids. The cDNA of Sox2, Oct3/4, Klf4, and c-Myc was built into two plasmids which were transfected into MEF, thereby inducing iPS cells. When these cells were injected into nude mice, teratomas were formed and chimeric mice were born after injection of iPS cells into blastocysts [Okita et al., 2008]. A third possibility to overcome viral integration is by the use of valproic acid (VPA), which is a small-molecule chemical. Results showed formation of iPS cells from human fibroblasts, induced by the combination of VPA, Oct4, and Sox2. VPA replaced reprogramming factors c-Myc and Klf4 and no viral integration was needed [Huangfu et al., 2008].

iPS generation without the use of viral integration represents a further step towards regenerative medicine and might yield the possibility to replace all TF in that manner one day.

Alternative combinations of TF

As an oncogene c-Myc can induce tumour formation. Thus, before iPS cells can be used in therapeutic applications, it must be made certain that no tumour formation occurs. In order to optimize the reprogramming process, other research groups have generated iPS cells without c-Myc, or with other combinations of TF.

Park et al. [2007] repeated the experiment performed in 2006 with Oct3/4, Sox2, Klf4 and c-Myc as TF, but used fetal, neonatal and adult human fibroblasts as donor cells for iPS cell generation. ES-cell like iPS cells were formed, cells expressed ES markers, like SSEA3, SSEA4, NANOG, OCT4, Tra-1-81, and Tra-1-60, and even colonies were formed when either Klf4 or c-Myc was eliminated. This proves the essential role of Oct3/4 and Sox2 in reprogramming, although with three instead of four TF efficiency decreased. They also used a different combination of TF and added NANOG and LIN28 to the two essential TF. This was confirmed by Yu et al. [2007] who showed that the combination of Oct3/4, Sox2, Nanog, and Lin28 is enough to induce pluripotent cells from human somatic cells with an improved efficiency due to expression of Nanog.

An alternative for reducing the chance of tumour formation is to replace c-Myc with the family member n-Myc, which is less tumorigenic. It was seen that MEF, after expression of Oct3/4, Sox2, Klf4, and n-Myc, via infection with lentivral vectors, were reprogrammed into pluripotent cells. When these cells were injected into immune deficient mice, teratomas were formed [Blelloch et al., 2007]. More research is needed to test whether n-Myc is an efficient substitute for c-Myc.

Another research group started to test the combination of two extra TF, namely p53 siRNA and UTF1. This new combination increased efficiency of iPS generation by more than 200 times. Although efficiency decreased when c-Myc was removed, c-Myc appeared dispensable in iPS cell reprogramming. Eventually, they tested the combination of Oct3/4, Sox2, Klf4, and UTF1 in reprogramming, and the iPS cell lines were positive for teratoma formation [Zhao et al., 2008].

As stated above, Sox2 and Oct3/4 are essential and indispensable in the process of generating iPS cells, which is also apparent in a more recent study. iPS cells were induced from MEF with Oct3/4, Sox2, and Esrrb. Esrrb is an orphan nuclear receptor that is involved in reprogramming by upregulating genes involved in pluripotency and self-renewal. iPS cells had ES-like characteristics and chimeric mice were born, when these cells were injected into blastocysts and implemented into pseudo-pregnant F1 mice [Feng et al., 2009].

Table 1a and 1b in the legend give an overview of discussed experimental studies investigating the generation of iPS cells.

Applications and challenges of β-cell replacement therapy

Diabetes is characterized by an inadequate functional mass of β-cells, which leads to a diminished or even absent production of insulin. Transplantations are nowadays performed on diabetes type 1 patients with severe complications. But it could be possible to cure these patients with a β-cell replacement therapy by substituting their destructed β-cell mass. Here comes in the problem; there is a shortage of available donor tissue and the entire diabetes type 1 population is too large to guarantee cure of all patients. However, with the right source of newly formed β-cells a pancreas or kidney transplantation becomes unnecessary, thereby preventing a major surgery.

New β-cells can be formed by transdifferentiation of acinar cells, replication of mature β-cells or pre-existing β-cells present in pancreas, or via neogenesis. Another group of potential sources are the stem cells or progenitors, which are of non-pancreatic cellular origin. Human ES cells, which are part of the second group, are closest to therapeutic application. These cells are pluripotent and able to form all cells of the body, including insulin producing cells. But, these cells are highly unstable thereby increasing the risk of genomic instability, teratomas can be formed, the cells are not body-own, and research with hES cells raises ethical concerns.

Inspired by an article by Cowan et al. [2005], which describes reprogramming of human ES cells by fusion with human body cells, iPS cells were generated *in vitro* as a new potential source of β-cells. The discovery of iPS cells was a major breakthrough, because they have the characteristics of ES cells, and are also body-own. One of the applications of iPS cells is that they can be used in cell-based therapy, like for diabetes type I. iPS cells are pluripotent and thus can form all cells of the body, including β-cells which can be used in transplantations. This was seen when ILC were generated from iPS cells with Oct3/4, Sox2, Klf4, and c-Myc. These ILC contained C-peptide-positive and glucagon-positive cells and were glucose sensitive [Tateishi et al., 2008].

Another purpose of iPS cell generation is the possibility to study the progress of the disease. This was seen in the generation of T1D-specific iPS cells, with Oct3/4, Sox2, and Klf4, derived from adult fibroblast from patients with DMI. Next step was differentiation of these T1D-iPS cells into insulin-producing cells. But this process must be further optimized, as efficiency is very low [Maehr et al., 2009]. When donor cells are derived from a diabetes patient, the generated iPS cells can be used to study the progress of and processes involved in the disease. With this knowledge new therapies can be tested or discovered.

iPS cell research is quite young and other researchers are working on repeating and, more importantly, optimizing the reprogramming process. For an optimal differentiation, growth and differentiation factors are chosen based on current knowledge on pancreas development. But when are the newly formed β-cells ready for clinical application? These cells must meet certain requirements before they can be used in β-cell replacement therapy in diabetes type 1 patients. The requirements include that newly formed β-cells must resemble human β-cells as

much as possible. They should be able to detect blood glucose levels and to produce insulin in a glucose-dependent manner to maintain normal blood glucose levels. The cells should survive an environment where they are exposed to high blood glucose levels, until they produce insulin and normal blood glucose levels are reached.

Although iPS cells differentiated into β-cells looks promising, it must overcome some limitations, including viral integration of TF and tumour formation. Some alternatives are given for viral integration, like plasmids or small molecules. An incomplete reprogramming of somatic cells to iPS cells can result in impaired differentiation of iPS into β-cells which can lead to tumour formation. Tumour formation can also be initiated by either TF c-Myc or Klf4, which are both oncogenic. These factors must be replaced before generated cells can be used in human.

When iPS derived β-cells are ready for clinical application, a suicide gene can be build in as safety mechanism. When cells turn out to be still tumorigenic, this gene can be activated and it causes the cell to go in apoptosis.

When all above mentioned problems are overcome, and newly formed β-cells can transplanted into diabetes type 1 patients, we have to keep in mind that the immune system is not changed. This means that transplanted β-cells can also be a target of the autoreactive T-cells.

Concluding remarks

β-cell replacement therapy can be used as a cell-based therapy, whereby destructed cells in diabetes type 1 patients are substituted by healthy β-cells, which are capable of producing insulin in a glucose-dependent manner. The shortage of available donor tissue can be circumvented by the *in vitro* production of β-cells from *in vivo* origins, as previously showed by the described mouse and human data. The generation of iPS cells has some advantages over the other sources. However, before implication of these cells can be realized, safety issues must be overcome, like viral integration and increased risk of tumour formation. *In vitro* generated β-cells from iPS cells increase the available mass for replacement therapy. The iPS cells are also useful for studying progress of and processes involved in diabetes type 1, when iPS cells are generated from patient-derived cells, and possible treatments can be tested or discovered. Although these future implications look promising, researchers are constantly looking for new approaches in finding an ultimate cure for diabetes type 1.

Acknowledgements

I would like to thank Dr. Eelco de Koning from Leiden University for the time he was available and for his help with the structure and contents of my thesis.

I also would like to thank Dr. Inge van den Berg from Utrecht University for being second reviewer of my thesis.

Annemarie Penders

References

Andersson, E.R. and Lendahl, U. *Regenerative medicine: a 2009 overview.* Journal of Internal Medicine 266: 303-310 (2009).

Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T. and Yamanaka, S. *Generation of pluripotent stem cells from adult mouse liver and stomach cells.* Science 321: 699-702 (2008).

Baeyens, L., De Breuck, S., Lardon, J., Mfopou, J.K., Rooman, I. and Bouwens, L. *In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells.* Diabetologia 48: 49-57 (2005).

Blelloch, R., Venere, M., Yen, J. and Ramalho-Santos, M. *Generation of Induced Pluripotent Stem Cells in the Absence of Drug Selection.* Cell Stem Cell 1: 245-247 (2007).

Bonner-Weir, S., Taneja, M., Weir, G.C., Tatarkiewicz, K., Song, K., Sharma, A. and O'Neil, J. *In vitro cultivation of human islets from expanded ductal tissue.* PNAS 97: 7999-8004 (2000).

Bonner-Weir, S., Toschi, E., Inada, A., Reitz, P., Fonseca, S.Y., Aye, T. and Sharma, A. *The pancreatic ductal epithelium serves as a potential pool of progenitor cells.* Pediatric Diabetes 5: 16-22 (2004).

Bonner-Weir, S. and Weir, G.C. *New sources of pancreatic β–cells.* Nature Biotechnology 23: 857-861 (2005).

Bonner-Weir, S. and Sharma, A. *Are there pancreatic progenitor cells from which new islets form after birth?* Nature 2: 240-241 (2006).

Boyer L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G. Gifford, D.K., Melton, D.A., Jeanisch, R., and Young, R.A. *Core transcriptional regulatory circuitry in human embryonic stem cells.* Cell 122: 947-956 (2005).

Brambrink, T., Foreman, R., Grand Welstead, G., Lengner, C.J., Wernig, M., Suh, H. and Jaenisch, R. *Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells.* Cell Stem Cell 2: 151-159 (2008).

Brolén, G.K.C., Heins, N., Edsbagge, J. and Semb, H. *Signals From the Embryonic Mouse Pancreas Induce Differentiation of Human Embryonic Stem Cells Into Insulin-Producing β-Cell-Like Cells.* Diabetes 54: 2867- 2874 (2005).

Brons, I.G.M., Smithers, L.E., Trotter, M.W.B., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A. and Vallier, L. *Derivation of pluripotent epiblast stem cells from mammalian embryos.* Nature 448: 191-195 (2007).

Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A. and Butler, P.C. *β-Cell Deficit and Increased β-Cell Apoptosis in Humans With Type 2 Diabetes.* Diabetes 52: 102-110 (2003).

Cowan, C.A., Atienza, J., Melton, D.A. and Eggan, K. *Nuclear Reprogramming of Somatic cells After Fusion with Human Embryonic Stem Cells.* Science 309: 1369-1373 (2005).

Da Silva Meirelles, L., Caplan, A.I. and Nardi, N.B. *In Search of the In Vivo Identity of Mesenchymal Stem Cells.* Stem Cells 26: 2287-2299 (2008).

D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A, Kroon, E., Carpenter, M.K. and Baetge, E.E. *Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells.* Nature Biotechnology 11: 1392- 1401 (2006).

Dor, Y., Brown, Y., Martinez, O.I. and Melton, D.A. *Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation.* Nature 429: 41-46 (2004).

Feng, B., Jiang, J., Kraus, P., Ng, J., Heng, J.D., Chan, Y., Yaw, L., Zhang, W., Loh, Y., Han, J., Vega, V.B., Cacheux-Ratabouls, V., Lim, B., Lufkin, T. and Ng, H. *Reprogramming of fibroblast into induced pluripotent stem cells with orphan nuclear receptor Esrrb.* Nature Cell Biology 11: 197-203 (2009).

Gao, R., Ustinov, J., Pulkkinen, M., Lundin, K., Korsgren, O. and Otonkoski, T. *Characterization of Endocrine Progenitor Cells and Critical Factors for Their Differentiation in Human Adult Pancreatic Cell Culture.* Diabetes 52: 2007-2015 (2003).

Guan, K., Nayernia, K., Maier, L.S., Wagner, S., Dressel, R., Ho Lee, J., Nolte, J., Wolf, F., Li, M., Engel, W. and Hasenfuss, G. *Pluripotency of spermatogonial stem cells from adult mouse testis.* Nature 440: 1199-1203 (2006).

Granger, A. and Kushner, J.A. *Cellular origins of β-cell regeneration: a legacy view of historical controversies.* Journal of Internal Medicine 266: 325-338 (2009).

Guz, Y., Nasir, I. and Teitelman, G. *Regeneration of pancreatic beta cells from intra-islet precursor cells in an experimental model of diabetes.* Endocrinology 142: 4956-4968 (2001).

Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine, E.J., Cassady, J.P., Foreman, R., Lengner, C.J., Dausman, J.A. and Jaenisch, R. *Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency.* Cell 133: 250-264 (2008).

Hess, D., Li, L., Martin, M., Sakano, S., Hill, D., Strutt, B., Thyssen, S., Gray, D.A. and Bhatia, M. *Bone marrow-derived stem cells initiate pancreatic regeneration.* Nature Biotechnology 21: 763-770 (2003).

Hochedlinger, K. and Jaenisch, R. *Nuclear reprogramming and pluripotency.* Nature 441: 1061-1067 (2006).

Holland, A.M., Elefanty, A.G. and Stanley, E.G. *Pancreatic differentiation from pluripotent stem cells: tweaking the system.* Cell Research 19: 395-396 (2009).

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E. and Melton, D.A. *Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds.* Nature Biotechnology 26: 795-797 (2008).

Ianus, A., Holz, G.G., Theise, N.D. and Hussain, M.A. *In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion.* The Journal of Clinical Investigation 111: 843-850 (2003).

Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G. and Majumdar, A. *Generation of Insulin-Producing Islet-Like Clusters from Human Embryonic Stem Cells.* Stem Cells 25: 1940-1953 (2007).

Jiang, W., Shi, Y., Zhao, D., Chen, S., Yong, J., Zhang, J., Qing, T., Sun, X., Zhang, P., Ding, M., Li, D. and Deng, H. *In vitro derivation of functional insulin-producing cells from human embryonig stem cells.* Cell Research 17: 333-344 (2007).

Kaczorowski, D.J., Patterson, E.S., Jastromb, W.E. and Shamblott, M.J. *Glucose-responsive insulin-producing cells from stem cells.* Diabetes Metabolism Research and Reviews 18:442-450 (2002).

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., Toyoshima, M., Niwa, O., Oshimura, M., Heike, T., Nakahata, T., Ishino, F., Ogura, A. and Shinohara, T. *Generation of pluripotent stem cells from neonatal mouse testis*. Cell 119: 1001-1012 (2004).

Karnieli, O., Izhar-Prato, Y., Bulvik, S. and Efrat, S. *Generation of Insulin-Producing Cells from Human Bone Marrow Mesenchymal Stem Cells by Genetic Manipulation.* Stem Cells 25: 2837-2844 (2007).

Kodama, S., Kühtreiber, W., Fujimura, S., Dale, E.A. and Faustman, D.L. *Islet Regeneration During the Reversla of Autoimmune Diabetes in NOD Mice.* Science 302: 1223-1227 (2003).

Kroon, E., Martinson, L.A., Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., Cunningham, J., Agulnick, A.D., D'Amour, K.A., Carpenter, M.K. and Baetge, E.E. *Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo.* Nature Biotechnology 26: 443-452 (2008).

Lardon, J., Huyens, N., Rooman, I. and Bouwens, L. *Exocrine cell trasdifferentiation in dexamethasone-treated rate pancreas*. Virchows Archive 444: 61-65 (2004).

Lee, V.M. and Stoffel, M. *Bone marrow: An extra-pancreatic hideout for the elusive pancreatic stem cell?* The Journal of Clinical Investigation 111: 799-801 (2003).

Lehman, R., Zuellig, R.A., Kugelmeier, P., Baenninger, P.B., Moritz, W., Perren, A., Clavien, P., Weber, M. and Spinas, G.A. *Superiority of Small Islets in Human Islet Transplantation.* Diabetes 56: 594-603 (2007).

Li, Y., Zhang, R., Qiao, H., Zhang, H., Wang, Y., Yuan, H., Liu, Q., Liu, D., Chen, L. and Pei, X. *Generation of Insulin-Producing Cells From PDX-1 Gene-Modified Human Mesenchymal Stem Cells.* Journal of Cellular Physiology 211: 36-44 (2007).

Like, A.A., Chick, W.L. *Mitotic division in pancreatic beta cells.* Science 163: 941-943 (1969).

Lipsett, M. And Finegood, D.T. *Beta-cell neogenesis during prolonged hyperglycemia in rats.* Diabetes 51: 1834-1841 (2002).

Logothetopoulos, J. Bell, E.G. *Histological and autoradiographic studies of the islets of mice injected with insulin antibody.* Diabetes 15: 205-211 (1966).

Maehr, R., Chen, S., Snitow, M., Ludwig, T., Yagasaki, L., Goland, R., Leibel, R.L. and Melton, D.A. *Generation of pluripotent stem cells from patients with type 1 diabetes.* PNAS 106: 15768-15773 (2009).

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K. and Hochedlinger, K. *Directly Reprogrammed Fibroblasts Show Global Epigenetic Remodleing and Widespread Tissue Contribution.* Cell Stem Cell 1: 55-70 (2007).

Murtaugh, L.C. *Pancreas and beta-cell development: from the actual to the possible.* Development 134: 427-438 (2007).

Okita, K., Ichisaka, T. and Yamanaka, S. *Generation of germline-competent induced pluripotent stem cells.* Nature 448: 313-317 (2007).

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T. and Yamanaka, S. *Generation of Mouse Induced Pluripotent Stem Cells Without Viral Vectors.* Science 322: 949-953 (2008).

Park, I., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W. and Daley, G.Q. *Reprogramming of human somatic cells to pluripotency with defined factors.* Nature 451: 141-146 (2007).

Rossant, J. *The magic brew.* Nature 448: 260-261 (2007).

Ryan, E.A., Lakey, J.R., Rajotte, R.V., Korbutt, G.S., Kin, T., Imes, S., Rabinovitch, A., Elliott, J.F., Bigam, D., Kneteman, N.M., Warnock, G.L., Larsen, I., Shapiro, A.M. *Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol.* Diabetes 50: 710-719 (2001).

Song, K.H., Ko, S.H., Ahn, Y.B., Yoo, S.J., Chin, H.M., Kaneto, H., Yoon, K.H., Cha, B.Y., Lee, K.W. and Son, H.Y. *In vitro transdifferentiation of adult pancreatic acinar cells into insulin-expressing cells.* Biochemical and Biophysical Research Communications 316: 1094-1100 (2004).

Sridharan, R., Tchieu,J., Mason, M.J., Yachechko, R., Kuoy, E., Horvath, S., Zhou, Q. and Plath, K. *Role of the Murine Reprogramming Factors in the Induction of Pluripotency.* Cell 136: 364-377 (2009).

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G. and Hochedlinger, K. *Induced Pluripotent Stem Cells Generated Without Viral Integration.* Science 322: 945-949 (2008).

Takahashi, K. and Yamanaka, S. *Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors.* Cell 126: 663-676 (2006).

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. *Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors.* Cell 131: 861-872 (2007).

Tateishi, K., He, J., Taranova, O., Liang, G., D'Alessio, A.C. and Zhang, Y. *Generation of Insulin-secreting Islet-like Clusters from Human Skin Fibroblasts.* Journal of Biological Chemistry 283: 31601-31607 (2008).

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L. and McKay, R.D.G. *New cell lines from mouse epiblast share defining features with human embryonic stem cells.* Nature 448: 196-199 (2007).

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. *Embryonic Stem Cell Lines Derived from Human Blastocytes.* Science 282: 1145-1147 (1998).

Wernig, M., Meisnner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E. and Jaenisch, R. *In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state.* Nature 448: 318-324 (2007).

Xia, B., Zhan, X., Yi, R. and Yang, B. *Can pancreatic duct-derived progenitors be a source of islet regeneration?* Biochemical and Biophysical Research Communications 383: 383-385 (2009).

Xu, X., D'Hoker, J., Stange, G., Bonne, S., De Leu, N., Xiao, X., Van De Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D., Bouwens, L., Scharfmann, R., Gradwohl, G. and Heimberg, H. *β Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas.* Cell 132: 197-207 (2008).

Yamanaka, S. *Stategies and new developments in the generation of patient-specific pluripotent stem cells.* Cell Stem Cell: 39-49 (2007).

Yatoh, S., Dodge, R., Akashi, T., Omer, A., Sharma, A., Weir, G.C. and Bonner-Weir, S. *Differentiation of Affinity-Purified Human Pancreatic Duct Cells to β-Cells.* Diabetes 56: 1802-1809 (2007).

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A. Ruotti, V., Stewart, R., Slukvin, I.I. and Thomson, J.A. *Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells.* Science 318: 1917-1920 (2007).

Zhao, Y., Yin, X., Qin, H., Zhu, F., Liu, H., Yang, W., Zhang, Q., Xiang, C., Hou, P., Song, Z., Liu, Y., Yong, J., Zhang, P., Cai, J., Liu, M., Li, H., Li, Y., Qu, X., Cui, K., Zhang, W., Xiang, T., Wu, Y., Zhao, Y., Liu, C., Yu, C., Yuan, K., Lou, J., Ding, M. and Deng, H. *Two Supporting Factors Greatly Improve the Efficiency of Human iPSC Generation.* Cell Stem Cell 3: 475-479 (2008).

Medical Encyclopedia, 2006

Legend

Table 1a: Overview of experimental studies investigating generation of iPS cells

	Takahashi et al., 2006	Takahashi et al., 2007	Maherali et al., 2007	Wernig et al., 2007	Blelloch et al., 2007	Yu et al., 2007	Okita et al., 2007
Model	Fbx15$^{\beta geo/\beta geo}$mice	Caucasian female, mouse	Mouse	Mouse	rosa26/βgeo;oct4/ gfp mice	Human, mouse	C57BL/6 mice
Donor cells	MEF, mouse adult fibroblast (TTF)	HDF, HFLS, BJ cells	MEF	MEF, TTF	MEF	IMR90 fetal fibroblasts + human foreskin fibroblasts	MEF
TF	Oct3/4, Sox2, c-Myc, Klf4	Oct3/4, Sox2, c-Myc, Klf4	Oct4, Sox2, c-Myc, Klf4	Oct4, Sox2, c-Myc, Klf4	Oct4, Sox2, Klf4, n-Myc	Oct4, sox2, Nanog, Lin28	Oct3/4, Sox2, Klf4, c-Myc
TF transfection	Retroviral	Retroviral	Retroviral	Retroviral	Lentiviral	Lentiviral	Retroviral
Period of transfection	2-3 weeks	25-30 days	~3 weeks	20 days	11 days	12-20 days	~12days
Efficiency	Low	Low	Low	0.05-0.1%	0.05-0.1%	Improved by Nanog expression	0.001-0.03%
ES cell similarities/ ES cell markers	Myb, Kit, Gdf3, Zic3	Telomerase activity, large nuclei, SEA-3,-4, TRA-1-60, -81, Nanog	SSEA1, CD9, AP activity, similar DNA methylation	Similar DNA methylation + expression profile, AP activity	AP activity, similar morphology	Telomerase activity, SSEA-3,-4, TRA-1-60 ,-81	Similar morphology and gene expression, ES-like proliferation
Teratoma/ EB/ chimeric mouse formation	Teratomas/ chimeric embryos	Teratomas/ EB	Teratomas/ newborn chimeric mice	Teratomas/ newborn chimeric mice	Teratomas/ chimeric embryos	Teratomas/ EB	Teratomas/ newborn chimeric mice
Supplementary results	Selection marker Fbx15	Differentiation into neural + cardiac cells	Selection marker Nanog	Selection markers Oct4 + Nanog	No drug selection	-------	Selection marker Nanog

Table 1b: Overview of experimental studies investigating generation of iPS cells

	Park et al., 2007	Zhao et al., 2008	Huangfu et al., 2008	Tateishi et al., 2009	Feng et al., 2009	Maehr et al., 2009
Model	$Rag2^{-/-}/\gamma c^{-/-}$ mice, human	Mouse, human	Mouse, human	$Rag2^{-/-}$/gamma-$C^{-/-}$ mice, human	Mouse	Caucasian T1D patient, mouse
Donor cells	dH1f, dH1cf, MRC5, BJ cells, MSC	BJ cells, hEF, hFSF, hAFF, MEF	Primary human fibroblasts/ BJ cells/ NHDF	HFF-1	MEF	Human adult skin fibroblasts
TF	Oct4,Sox2, Klf4, c-Myc	Oct4, Sox2, Klf4, UTF1	Oct4, Sox2 VPA	Oct4, Sox2, Klf4, c-Myc	Oct4, Sox2, Esrrb	Oct4, Sox2, Klf4
TF transfection	Lentiviral	Lentivrial	Retroviral + small-molecule inhibitor	Retroviral	Retroviral	Retroviral
Period of transfection	14-21 days	35 days	~1 month	16, 23, 30 days	14 days	~4 weeks
Efficiency	~0.1%	100 times higher	~1%	Extremely low	Low	Low
ES cell similarities/ ES cell markers	AP activity, TRA-1-60, -81, SSEA3,SSEA 4, Oct4, Nanog	SSEA4, TRA-1-60, -81, Nanog, similar gene expression, AP activity	GDF3, Oct4, Nanog, Sox2, similar gene expression	AP activity, Sox2, Oct4, TRA-1-60, SSEA4	AP activity, Nanog, SSEA1, similar gene expression	AP activity, Oct4, SSEA4, Nanog, Sox2, TRA-1-60, -81
Teratoma/ EB/ chimeric mouse formation	Teratomas	Teratomas/ EB	Teratomas/ EB	Teratomas	Teratomas/ newborn chimeric mouse	Teratomas
Supplementary results	-------	-------	Differentiation into dopaminergic neurons + cardiomyocytes	Differentiation into insulin-producing islet-like clusters	-------	Differentiation into insulin producing, glucose responsive cells

AP = alkaline phosphatase
BJ = neonatal human foreskin fibroblast
dH1f = human ES-cell-derived fibroblast
hAFF = human adult foreskin fibroblast
hEF = human embryonic fibroblast
hFib2 = adult dermal fibroblast
hFSF = human fetal skin fibroblast
MEF = mouse embryonic fibroblast
MRC5 = primary fetal cells
MSC = mesenchymal stem cells
NHDF = neonatal human foreskin fibroblast
TTF = tail-tip fibroblast