Chapter 10
Viral-Mediated Gene Therapy for the Generation of Artificial Insulin-Producing Cells as a Therapeutic Treatment for Type 1 Diabetes Mellitus

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Introduction

Several approaches have been employed to develop cell and gene therapy strategies that generate artificial insulin-producing cells (IPCs) for potential therapeutic applications in the treatment of type 1 diabetes mellitus (T1D). The genetic engineering of functional IPCs necessitates a broad understanding of the pancreatic developmental process and the β-cell transcription factors that govern mature β-cell differentiation and function. To successfully obtain functional IPCs, the type of vectors utilized for gene transfer and the selection of a suitable target cell for subsequent differentiation into IPCs is of fundamental importance. Techniques for manufacturing IPCs include the de-differentiation and directed differentiation of autologous or allogeneic cells ex vivo followed by transplantation and the in vivo differentiation of target tissue via viral gene transfer. Ultimately, the goal is to construct IPCs that have the capacity to process, store and secrete insulin in response to and relative to the circulating blood glucose levels, whilst avoiding the administration of immunosuppressants and recurrent autoimmune destruction, thereby restoring normoglycaemia.

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Table 10.1 Criteria for comparing the suitability of potential target cells for T1D gene therapy

Characteristics	Target cell				
	Liver	Pituitary	Muscle	K cells	BMSC
Derived from endodermal origin	Yes	No	No	No	Yes
Endogenous β cell transcription factors	No	No	No	No	Yes
Glucose-sensing system	Yes	No	No	Yes	No
Proinsulin processing enzymes	No	Yes	No	Yes	No
Glucose-regulatable promoter	Yes	No	No	Yes	No
Secretory system	No	Yes	No	Yes	No
Autologous use	Yes	Yes	Yes	Yes	Yes
Allogeneic use	No	No	No	No	Yes

Selecting an Ideal Viral Vector for T1D Gene Therapy

The use of viruses as tools for the correction of genetic disorders due to their ability to infect and deliver genetic material to cells has paved the way for a number of promising cell and gene therapies. By engineering viral vectors that are replication deficient and efficiently transduce genes into target cells, key challenges encountered in the generation of successful therapies can be circumvented. Selecting a suitable viral vector is contingent on the nature of gene expression required (sustained or short-term expression), whether the vector is integrative or non-integrative, and the type of cells targeted for gene transfer (dividing or non-dividing) (Table 10.1). Ideally, the generation of artificial IPCs would utilise integrating viral vectors that offer long-term gene expression over the life of the patient, resulting in a persisting therapeutic benefit.

Retroviral Vectors

Retroviral vectors are derived from disabled murine viruses and are the most commonly used gene delivery vector due to their ability to integrate chromosomally and sustain expression of the transgene (Morgan and Anderson 1993). However, the risk of site-specific insertional mutagenesis near oncogenic locations could increase predisposition to tumour development, limiting their utility (Bushman 2007; Laufs et al. 2004). This feature of retroviral vectors was demonstrated in 1999 following the treatment of nine severe combined immunodeficiency (*Scid*) patients which led to the development of leukaemia in four of the patients (Cavazzana-Calvo et al. 2000). The primary disadvantage with retroviral gene transfer is the requirement for target cells to be in an active state of division for transduction; therefore, target tissues composed predominantly of non-dividing cells represent a challenge. A successful use of retroviral vectors to generate

IPCs was performed by Xu et al. (2007), who transduced bone marrow-derived mesenchymal stem cells (BMSCs) with an insulin gene under the control of the cytomegalovirus (CMV) promoter. Following transplantation into streptozotocin (STZ)-diabetic mice, they discovered that the transduced BMSCs expressed insulin and were able to maintain normoglycaemia for at least 42 days whilst evading autoimmune destruction.

Adenoviral and Adeno-Associated Vectors

Due to their ability to transduce dividing and non-dividing cells with high efficiency, adenoviral vectors have been studied intensively, particularly for the targeted therapy of cystic fibrosis (Zabner et al. 1993; Knowles et al. 1995). However, challenges associated with the administration of adenoviral vectors such as the development of immune responses against the viral capsid proteins, and in some cases the transgene itself, have limited their use in the clinic (Wold et al. 1999; McCaffrey et al. 2008; Nayak and Herzog 2010). In addition, adenoviral vectors transfer their genetic cargo episomally and subsequently offer only transient gene expression (Morgan and Anderson 1993; Volpers and Kochanek 2004). To address the immunogenicity of the viral capsid proteins, a "gutless" adenovirus was constructed where the genes encoding the common viral capsid proteins were deleted (Alba et al. 2005). Although the new generation vectors reduce immunogenicity, the administration of immunosuppressants is still required following treatment (Zhou et al. 2004).

Adeno-associated (AAV) viral vectors are becoming an attractive alternative for gene therapy as they are able to transduce both dividing and non-dividing cells, whilst also preferentially integrating their genetic cargo at a specific site on chromosome 19 (Muzyczka 1992). Despite possessing a limited gene cargo capacity of less than 5 kb, AAV vectors have been utilised to deliver the preproinsulin gene to livers of STZ-diabetic mice (Sugiyama et al. 1997), resulting in the transient stabilisation of blood glucose levels.

Lentiviral Vectors

Lentiviral vectors (LV) are derived from the human immunodeficiency virus (HIV) and are an attractive candidate for gene therapy as they are able to transduce both dividing and non-dividing cells (Yoon and Jun 2002). Due to their derivation from HIV, biosafety was a concern for their application as therapeutics; however, engineered deletions in the long terminal repeat (LTR) promoter reduce the possibility of producing replication competent virus and thereby improve safety (Zufferey et al. 1998). LV is at present the vector of choice for gene therapy within

our laboratory, and we have successfully used a lentiviral vector (HMD) to deliver furin-cleavable insulin (INS-FUR) to the livers of STZ-diabetic rats (Ren et al. 2007), non-obese diabetic (NOD) mice (Ren et al. 2013) and pancreatectomised Westran pigs (Gerace et al. 2013). In the rodent animal models, we observed liver to pancreas transdifferentiation characterised by spontaneous expression of β cell transcription factors, formation of insulin storage granules, normal glucose tolerance and permanent reversal of diabetes.

Selecting a Suitable Target Cell for T1D Gene Therapy

Somatic cell gene therapy for T1D was first performed in monkey kidney cells and fibroblasts (Laub and Rutter 1983; Iwata et al. 1993). However, due to their intrinsic lack of β cell characteristics, these cells were not able to produce biologically active insulin despite successfully transcribing and translating the insulin gene. Likewise, the targeting of muscle cells has been studied sparingly due to their lack of β cell characteristics. Nevertheless, a study utilising vascular smooth muscle cells transduced with INS-FUR was able to reduce blood glucose levels in spontaneously diabetic congenic BioBreeding (BB) rats for a period of 6 weeks (Barry et al. 2001). Of more success was the reversal of diabetes following the dual expression of insulin and glucokinase (GK) in the muscle of STZ-diabetic mice for >4 months (Mas et al. 2006). However, a suitable target cell for the production of functional artificial IPCs would possess characteristics similar to those of normal β cells (Table 2) such as a glucose-sensing system, proinsulin-processing enzymes and an exocytosis system (Zaret and Grompe 2008).

Endocrine Cells

Pituitary cells contain proinsulin-processing enzymes and secretory granules. In a study where a murine pituitary cell line (AtT20) was transfected with a recombinant plasmid containing a human preproinsulin cDNA (AtT20Ins-1.4), expression of biologically active insulin was demonstrated; however, glucose responsiveness was absent (Stewart et al. 1994). Upon the insertion of GLUT2 and glucokinase, the AtT20Ins-1.4 cells became glucose responsive; however, the secretion of adenocorticotropic hormone stimulated glucocorticoid synthesis which inhibited insulin function, and therefore limited their effectiveness (Hughes et al. 1993).

K cells, a type of endocrine cell found in the gut, possess many β cell characteristics and have drawn a significant attention as a potential target for gene therapy (Yoon and Jun 2002). An in vivo study targeting K cells with a construct of human insulin under the control of the glucose-dependent insulinotropic polypeptide (GIP) regulatory promoter resulted in the production of biologically active insulin and the restoration of normoglycaemia (Cheung et al. 2000). Despite this encouraging result, an efficient method of delivery to these cells is yet to be established.

Liver Cells

Considering liver cells are derived from the same endodermal origin as the pancreas (Zaret 2008), increasing effort has been focused on engineering hepatocytes to function as artificial IPCs. The possession of GLUT2 and glucokinase in hepatocytes permits a response to fluctuating glucose concentrations similar to that in normal β cells and hence their preferred use in gene therapy protocols for the treatment of T1D. Although hepatocytes do not contain proinsulin-processing enzymes and lack secretory granules, these functions can be induced in certain circumstances (e.g. via the expression of β cell transcription factors or INS-FUR) (Ren et al. 2007, 2013; Gerace et al. 2013; Tuch et al. 2003).

Mesenchymal Stem Cells (MSCs)

Within the last decade, the targeting of MSCs for genetic manipulation has become increasingly popular owing to their wide-ranging immunomodulatory properties and reported ability to elude immune detection (Gebler et al. 2012; Vija et al. 2009; Abdi et al. 2008; da Silva Meirelles et al. 2009). Due to the immunomodulatory capacities of MSC, their predominant use in the correction of diabetes has been in the form of transplantation of native MSCs intended to control immune responses (Lee et al. 2006; Ezquer et al. 2008). However, at the present time, MSCs are more commonly becoming utilised as target cells for the generation of artificial IPCs via chemically induced differentiation protocols or direct transfer of genetic material (Tang et al. 2004). With regard to the chemical induction of MSCs to generate IPCs, it has been shown that BMSCs cultured in high-glucose medium (Oh et al. 2004) or nicotinamide-enriched medium are inclined to differentiate into IPCs (Wu et al. 2007). Similarly, BMSCs subjected to a three-step chemically induced differentiation protocol produced glucose-responsive IPCs with high expression levels of *Pdx-1*, insulin and glucagon (Sun et al. 2007). On the other hand, BMSCs targeted for gene therapy with a retroviral vector containing the β cell transcription factor *Pdx-1* generated IPCs that reduced blood glucose concentrations after transplantation in STZ-diabetic/*Scid* mice and exhibited a normal glucose tolerance until 6–8 weeks post-transplantation (Karnieli et al. 2007).

Viral-Mediated Transfer of β Cell Transcription Factors

Currently, pancreas and islet transplantation are the only “cures” for diabetes mellitus. However, the limitations associated with the current therapeutic options necessitate the requirement for an alternative IPC that is also capable of evading recurrent immune destruction. During normal pancreatic development, islet cell differentiation is regulated by the expression of β cell transcription factors (Fig. 10.1), and during

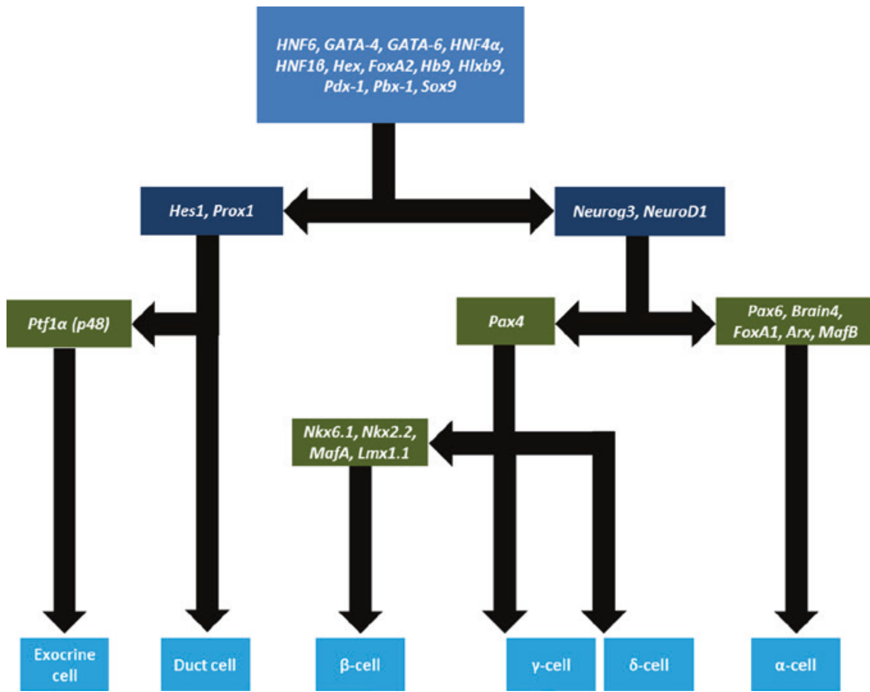

Fig. 10.1 Hierarchical representation of the transcription factors involved in pancreatic islet cell differentiation. Signals from the mesenchyme and notochord induce the early gut endoderm to form the pancreatic buds, where expression of *Pdx-1* then drives differentiation of the pancreatic precursor cells. Exocrine and endocrine differentiation are then specified by the expression of *Neurog3* and *Hes1*, respectively, with subsequent expression of *NeuroD1* maintaining the endocrine cell lineage. α-Cell and β cell differentiation is mediated by *Pax6* and *Pax4,* respectively. Final differentiation of β cells is governed by the expression of *Nkx2.2*, *Nkx6.1* and *MafA*

adult life, the transcription factors regulate the expression of pancreatic hormones. As a result, the utilisation of β cell transcription factors as mediators of IPC generation became of considerable interest as an alternative therapy for the treatment of diabetes mellitus. The production of IPCs for diabetes reversal via viral-mediated transfer of β cell transcription factors has been comprehensively examined in liver tissue as it is derived from the same endodermal origin as the pancreas (Zaret and Grompe 2008), making it more amenable to the transdifferentiation process.

Pdx-1

The direct in vivo delivery of the transcription factor *Pdx-1* to the livers of diabetic mice was studied by Ferber et al. (2000) as a potential method of correcting hyperglycaemia via the induced expression of insulin. The study reported the restoration of normoglycaemia in the mice for a period of 8 days as a consequence of

the expression of *Pdx-1* which induced insulin expression and secretion. However, uncontrolled transdifferentiation led to the undesirable development of exocrine tissue which resulted in hepatitis in the liver (Ferber et al. 2000; Kojima et al. 2003).

Exocrine differentiation following delivery of *Pdx-1* to the livers of STZ-diabetic mice was also reported by Kojima et al. (2003) and was presumably a consequence of the continuous expression of high levels of *Pdx-1*. Thus far, *Pdx-1*-mediated transdifferentiation from hepatocytes to pancreatic tissue has been accomplished on numerous occasions with varying degrees of success (Fodor et al. 2007; Ber et al. 2003; Wang et al. 2007; Sapir et al. 2005; Nagaya et al. 2009). Other target cells for direct delivery of *Pdx-1* include mouse pancreas via the bile duct (Taniguchi et al. 2003), rat intestinal epithelium-derived cells (IEC-6) (Yoshida et al. 2002) and primary duct cells (Noguchi et al. 2006). A combinatorial approach utilising the transcription factors *Pdx-1*, *Neurog3* and *MafA* successfully converted pancreatic exocrine cells into IPCs in vivo, providing support for the delivery of combinations of pancreatic transcription factors (Zhou et al. 2008). However, glucose tolerance in the animals differed significantly from normal.

As a result of the success of utilising *Pdx-1* as a mediator of pancreatic transdifferentiation, stem cells emerged as potential targets for gene transfer due to their regenerative capabilities and plasticity. As mentioned in "Mesenchymal Stem Cells (MSCs)" section, MSCs became of particular interest due to their unique immune-evading capabilities. MSCs from a variety of sources have been targeted for *Pdx-1* delivery, including BMSC (Karnieli et al. 2007; Sun et al. 2006; Limbert et al. 2011; Moriscot et al. 2005; Li et al. 2007, 2008), umbilical cord MSC (UC-MSC) (He et al. 2011) and adipose-derived MSC (AMSC) (Baer 2011; Lin et al. 2009; Kajiyama et al. 2010). A study by Miyazaki et al. (2004) targeted a murine embryonic stem cell (ESC) line (EB3) for *Pdx-1* gene transfer and found that it could be induced to differentiate into IPCs; however, the cells lacked expression of pancreatic genes in vivo; therefore, the cells would ultimately be of no therapeutic potential. Consequently, an effort was made to improve the generation of IPCs in a number of other ESC lines that could be of more benefit for the treatment of diabetes mellitus (Lavon et al. 2006; Vincent et al. 2006; Raikwar and Zavazava 2012).

Neurog3

The implications of *Neurog3* in specifying endocrine cell lineage suggest that it could potentially overcome the undesirable development of exocrine differentiation associated with the use of *Pdx-1* as a mediator of pancreatic transdifferentiation. Unfortunately, most studies employing the transfer of *Neurog3* have reported low levels of insulin production and a lack of glucose responsiveness (Noguchi et al. 2006; Kaneto et al. 2005; Wang et al. 2007; Song et al. 2007; Heremans et al. 2002). Adenoviral transfer of *Neurog3* and betacellulin to oval cells did result in the production of insulin and pancreatic transdifferentiation (Yechoor et al. 2009); however, the most effective use of *Neurog3*-induced transdifferentiation was in combination with other transcription factors (Zhou et al. 2008).

NeuroD1

Kojima et al. (2003) expressed *NeuroD1* and betacellulin in the livers of STZ-treated diabetic mice and showed a return to normoglycaemia for greater than 120 days, in addition to the expression of a number of pancreatic transcription factors. Most importantly, there was no evidence of any exocrine differentiation or hepatotoxicity. Further studies have also shown the ability of *NeuroD1* to strongly induce insulin expression, supporting its utility as a means for the production of IPCs (Noguchi et al. 2006; Yatoh et al. 2007).

Success utilising *NeuroD1* has also been reported within our laboratory, where the endocrine-specific transcription factor has been delivered to a genetically modified rat liver cell line (H4IIE) which does not endogenously express β cell transcription factors. Following the delivery of both insulin and *NeuroD1* to the H4IIE cell line, the cells were able to transcribe and translate insulin, process the translated protein into its mature form and package the mature insulin with storage granules (Swan MA 2009). After transplantation in NOD/*Scid* mice, the cells secreted insulin in response to increasing concentrations of glucose and restored normoglycaemia. Most importantly, they also displayed the hallmark characteristics of pancreatic transdifferentiation with expression of *Pdx-1*, *NeuroD1*, *Pax6*, *Nkx2.2* and *Nkx6.1*, in addition to rat insulin 1 and 2, glucagon, somatostatin, proconvertase 1 and 2 (PC1/2) and pancreatic polypeptide. Consequently, this study supports the already available evidence for the prospective use of *NeuroD1* to produce IPCs that are safe for use as a therapeutic for diabetes mellitus.

Pax4

Pax4 is a lower-hierarchy transcription factor that is essential for directing β cell differentiation and therefore is a suitable candidate for the generation of IPCs. Liew et al. (2008) demonstrated that human ESCs engineered to overexpress *Pax4* have an enhanced propensity to form putative β cells. A study supporting this finding showed that IPCs created from mouse ESCs via the overexpression of *Pax4* and selected for nestin expression were proficient in restoring normoglycaemia for 14 days (Blyszczuk et al. 2003). However, the propensity for ESCs to form teratomas limits their potential for clinical application (Stachelscheid et al. 2013; Hentze et al. 2009).

Nkx6.1

The disruption in the differentiation of β cells in mice has been demonstrated by knockouts of the *Nkx6.1* transcription factor, implicating it as an essential part of the β cell developmental pathway. As a result, it has the potential to be

a successful mediator of IPC generation. However, ectopic expression of *Nkx6.1* alone has been shown to poorly induce the expression of vital upper-hierarchy β cell transcription factors. Only upon co-expression with *Pdx-1* are the upper-hierarchy transcription factors expressed, which subsequently leads to insulin expression and glucose-responsive insulin release (Gefen-Halevi et al. 2010). As a result of the poor capacity of *Nkx6.1* to induce the expression of the full repertoire of β cell transcription factors, it is not a promising choice for the generation of IPCs.

Viral-Mediated Transfer of Insulin

As mentioned in section "Lentiviral Vectors", our laboratory has successfully employed the use of lentiviral vectors to transfer insulin to hepatocytes as an alternative therapeutic strategy for the treatment of T1D. The development of exocrine differentiation associated with liver-directed gene therapy using *Pdx-1* (Ferber et al. 2000; Kojima et al. 2003) has never been observed in our studies, presumably due to the diverse choice of genes utilised for gene delivery. In the human liver cell line (Huh7), which endogenously expresses β cell transcription factors, we were able to induce the development of insulin storage granules and glucose-responsive insulin secretion following the delivery of the insulin gene (Huh7ins). Diabetes in NOD/*Scid* mice was corrected following transplantation of the Huh7ins cells (Tuch et al. 2003).

Insulin Transfer in Rodent Models

Permanent reversal of diabetes in STZ-diabetic rats (Ren et al. 2007) and spontaneously diabetic NOD mice (Ren et al. 2013) following liver-directed lentiviral delivery of INS-FUR has been demonstrated within our laboratory. In these rodent models, we showed the spontaneous expression of the essential upper-hierarchy β cell transcription factors (*Pdx-1*, *Neurog3* and *NeuroD1*), some lower-hierarchy β cell transcription factors (*Pax4* and *Nkx2.2*) and the development of glucose-responsive insulin secretion (Nathwani et al. 2011; Lisowski et al. 2014; Apelqvist et al. 1999; Sommer et al. 1996). Permanent reversal of diabetes was characterised by normal intravenous glucose tolerance tests in the STZ-diabetic rat and NOD mouse study (Fig. 10.2). The insulin-secreting liver cells and NOD mouse livers engineered to express insulin generated within our laboratory are also resistant to the detrimental effects of β cell cytotoxins and proinflammatory cytokines that play a principle role in the pathogenesis of T1D (Ren et al. 2013; Tabiin et al. 2001, 2004; Tuch et al. 1997).

Due to the success shown by the viral delivery of INS-FUR within our laboratory, a number of successive studies utilising viral delivery of INS-FUR showing amelioration of hyperglycaemia in rodent models were reported (Han et al. 2011; Tatake

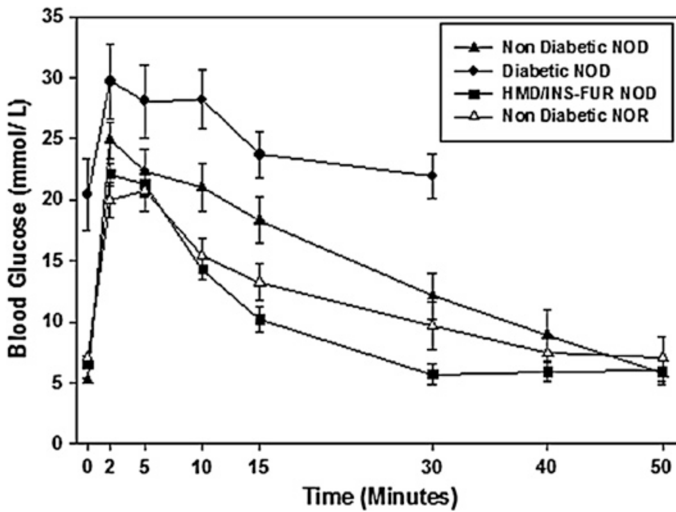

Fig. 10.2 Plasma glucose levels following an intravenous glucose tolerance test (IVGTT) in diabetic non-obese diabetic (NOD) mice treated with furin-cleavable human insulin (INS-FUR) within a lentiviral vector (HMD). An IVGTT was performed on non-diabetic NOD (12–16 weeks), non-obese-resistant (NOR) mice and HMD/INS-FUR-treated NOD mice, 5 months after reversal of diabetes. ($n = 5$, data were examined by one-way analysis of variance after log transformation of data and expressed as the mean ± SEM). [Reproduced from Ren et al. (2013)]

et al. 2007; Tudurí et al. 2012; Hsu et al. 2008). In contrast to our research, Elsner et al. (2012) applied lentiviral delivery of INS-FUR to the liver of diabetic rats and observed reversal of hyperglycaemia; however, pancreatic transdifferentiation and development of secretory granules were absent. Their gene therapy approach also lacked closely regulated control of blood glucose observed within normally functioning β cells, therefore requiring islet supplementation for clinical application.

Insulin Transfer in a Porcine Model

In order to more appropriately translate the rodent studies performed within our laboratory to the clinical setting, we also reversed diabetes in a large animal model that more closely resembles human physiology. Reversal of diabetes following liver-directed lentiviral delivery of INS-FUR was characterised by the expression of β cell transcription factors and normal glucose tolerance (Gerace et al. 2013). Unexpectedly, the complexity of applying the surgical procedure (which isolates the liver from the circulation) in a large animal model led to difficulties in reproducing the successful reversal of diabetes. This suggested that translation of the surgical procedure in the clinical setting may also raise a number of challenges; therefore, it would likely be of more benefit to transplant cells modified ex vivo so as to overcome the surgical obstacles.

Conclusion

Viral-mediated gene transfer of β cell transcription factors and insulin represents an alternative approach to generating artificial IPCs for the treatment of T1D. Currently, one of the challenges facing the translation of these potential alternative cell therapies to the clinic is the generation of sufficient quantities of IPCs on a large scale. The success of liver-directed gene therapy in the past decade is nowadays becoming overshadowed by the greater understanding of the regenerative and therapeutic potential of stem cells. It is understandable that the emerging cell and gene therapy approaches are targeting stem cells for ex vivo modification as they overcome the surgical difficulties associated with in vivo gene therapy. The source of stem cells is also to be considered, as autologous cell therapies would require considerable effort to generate a single therapy. In addition, the increased likelihood of developing a full repertoire of β cell autoantigens from autologous cell modification would increase susceptibility of the grafts to recurrent autoimmunity. As discussed, the success of generating IPCs via gene therapy that are functionally equivalent to normal β cells is closely related to the choice of β cell transcription factor, viral vector and gene promoter, as unwanted exocrine differentiation can lead to tissue destruction. Ideally, targeting of an allogeneic source of cells that are capable of circumventing the autoimmune response for ex vivo gene therapy and subsequent differentiation into IPCs would overcome these limitations.

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