

## CHAPTER 2

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# Gene Therapeutics in Autoimmune Diabetes

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### Overview of Therapeutic Challenges in Type 1 Diabetes Mellitus (T1D)

A significant amount of resources has recently been devoted to the restoration of normal glycemic regulation in type 1 diabetic patients by transplantation of allogeneic islets of Langerhans. Despite the promise offered by this approach, logistical hurdles necessitate a comprehensive strategy aimed at different molecular and cellular determinants of the autoimmune pathology of type 1 diabetes. Developments in gene therapy permit the engineering of immune cells, islets, surrogate beta cells as well as the conditioning of the transplant recipient in order to facilitate allograft survival. More importantly, manipulation of subsets of immune cells also offers an opportunity to intervene prophylactically and within the short time-span between clinical diagnosis and complete beta cell mass destruction to restore some degree of normoglycemia. While outlining the issues that challenge the translatability of gene therapeutics to the diabetic clinic, we give an overview of the exciting potential that such gene therapeutic strategies can offer the clinician.

### *Molecular and Cellular Determinants of Type 1 Diabetes in Mouse and Man*

T1D is considered a classical autoimmune disease that is characterized by a breakdown in both central and peripheral tolerance. The breakdown in central tolerance leads to precursor pools of self-reactive T cells that escape into the periphery. In the periphery, an immune response against the pancreatic beta cells is initiated by as yet-unknown environmental triggers, ultimately leading to beta cell destruction and diabetes.<sup>1,2</sup> In the nonobese diabetic (NOD) mouse,<sup>3</sup> the most widely used animal model of T1D; the defect in peripheral tolerance is evident because the NOD suffers from spontaneous autoimmune diabetes.<sup>4,5</sup> However, a very important study by Markees et al<sup>6</sup> suggests that NOD mice have an inherent defect in peripheral tolerance, demonstrated by the inability of anti-CD40-treatment to induced tolerance to skin allografts, a tissue to which NOD mice have no known autoimmune reactivity. Furthermore, it has been demonstrated that NOD mice display enhanced immune responses and prolonged survival of lymphoid cells when immunized with nominal antigens.<sup>7</sup> These data support the paradigm that the NOD mouse may have peripheral tolerance defects that go

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beyond the manifestation of autoimmunity that also render these mice resistant to conventional allograft tolerizing strategies as well as exacerbated immune activation upon any antigenic challenge. Thus it appears that the "rules" governing peripheral tolerance in autoimmune prone individuals are vastly different or more rigorous than those that impact nonautoimmune individuals. Therefore if autoimmune-prone individuals have inherently stringent requirements for tolerance induction, then such recipients may require strategic design in therapies including, combinatorial therapies, in order to achieve successful treatment both prophylactically as well as for allograft transplant tolerance.

Studies conducted in the NOD mouse have determined that the infiltrate within the islets is composed of CD4<sup>+</sup>, CD8<sup>+</sup>, T lymphocytes, B-lymphocytes, macrophages, and dendritic cells.<sup>8-11</sup> The T cell subsets play an obligatory role in the initiation of the disease. In the NOD mouse it has been demonstrated that CD8 T cells are critical for the initiation of disease progression, while the CD4 population is indispensable for the mobilization of the mononuclear cell infiltrate. Although CD8<sup>+</sup> T cells are critical in the diabetes process, CD8<sup>+</sup> T cells isolated from stock NOD mice cannot independently initiate Type I diabetes.<sup>8,12,13</sup> Thus it appears that the CD8<sup>+</sup> T cell population that contributes to T1D development, in the stock NOD mouse, is dependent on helper functions provided by CD4<sup>+</sup> T cells.<sup>14</sup> This may be due to a CD4<sup>+</sup> dependent expansion of CD8<sup>+</sup> T cells to critical threshold levels, which can then initiate pathogenic effects. The CD4<sup>+</sup> and CD8<sup>+</sup> TCR transgenic mouse models that express a TCR from diabetogenic clones support this concept of critical expansion for initiation of disease. Both CD4<sup>+</sup><sup>15</sup> and CD8<sup>+</sup><sup>14</sup> TCR-transgenic strains can, independently of the either T cell subtype initiate diabetes. Clearly, the coordinate interaction of both the innate and adaptive immune response is necessary for the development of disease.

A large body of evidence supports the concept that the antigen specific, T cell-mediated infiltration of inflammatory cells to the pancreas leads to the generation of reactive oxygen species (ROS), {superoxide, (O<sub>2</sub><sup>-</sup>), hydroxyl radical (·OH), nitric oxide (NO·) and peroxynitrite (ONOO·)}, and pro-inflammatory cytokines (TNFα, IL-1β and IFNγ).<sup>16-18</sup> Synergistic interaction between ROS and these cytokines results in the ultimate destruction of the pancreatic beta cells by both apoptotic and necrotic cell death. There is an extensive literature on the effect of free oxygen and nitric oxide radicals elaborated either by infiltrating immune cells or as a result of cytokine-induced beta cell-specific expression of enzymes generating these radicals (inducible nitric oxide synthase). Locally produced ROS are involved in the effector mechanisms of beta cell destruction.<sup>16-21</sup> In vitro, T cell and macrophage cytokines such as IFNγ, IL-1β and TNFα induce the production of ROS by beta cells, which leads to beta cell destruction.<sup>22</sup> This destruction may ultimately be caused by an apoptotic mechanism.<sup>23-27</sup> Beta cells engineered to over-express antioxidant proteins have been shown to be resistant to ROS and NO<sup>5,28-30</sup> Furthermore, stable expression of manganese superoxide dismutase (Mn-SOD) in insulinoma cells prevented IL-1β-induced cytotoxicity and reduced nitric oxide production.<sup>29</sup> Finally, others have shown that transgenic mice with beta cell-targeted over-expression of copper, zinc SOD or thioredoxin have increased resistance to autoimmune and streptozotocin-induced diabetes.<sup>31-33</sup> The protective effects demonstrated with the use of stable expression of antioxidant genes was recapitulated through the use of a superoxide dismutase mimic (a small molecule antioxidant) in an adoptive transfer system of autoimmune diabetes by a diabetogenic CD4<sup>+</sup> T cell clone.<sup>34,35</sup> These results are particularly exciting, as they are consistent with the previous reports where vector-mediated or transgenic over-expression of anti-oxidants protected beta cells. The ability to protect beta cells against CD4 mediated generation of pro-inflammatory cytokines and free radicals through the use of antioxidant therapy support the model of free radical generation as a pathogenic mechanism of T1D.

While less precise and specific data exist for the actual mechanism of beta cell destruction in humans, the body of evidence points to similarities between the etiopathology in the NOD mouse and humans. The chronic onset, the presence of a cellular inflammation, the transferability of diabetes and of protection by bone marrow transplantation and the immunosuppressibility by conventional pharmacologic agents. The genetics of the disease is multifactorial in humans and in the NOD mouse.<sup>36-38</sup> In humans, two loci (IDDM1 and IDDM2) have been confirmed to be in linkage with the disease. IDDM1 encompasses the HLA gene complex and it alone defines the most important risk factor. In humans the disease is associated with the inheritance of DR3/DR4 haplotypes (DR3: DQA1\*0501, DQB1\*0201 and DR4: DQA1\*0301, DQB1\*0302.<sup>39,40</sup> IDDM2 has been mapped to a variable number of tandem repeats (VNTR) polymorphism upstream of the insulin gene promoter which can determine thymic levels of insulin.<sup>41,42</sup> In fact, a recent study demonstrated that the number of active copies of insulin in a transgenic mouse can influence the degree of immune cell reactivity towards insulin, a putative autoantigen.<sup>43</sup> A number of other loci have demonstrated suggestive associations, but to date, none of these results have been replicated to establish significant linkage with the disease.<sup>44-46</sup>

A number of earlier hypotheses with some supporting evidence have been put forward to explain the possible mechanism of action of the environmental trigger including beta cell death secondary to virally-triggered inflammation, molecular mimicry, superantigens and diet.<sup>47-50</sup> What is certain is that at some point post-natally, the immune system of a genetically-predisposed individual is activated to chronically infiltrate the islets of Langerhans. While the initial phase of infiltration may not involve beta cell destruction, a number of studies in vivo and in vitro suggest that immune cells become able to render beta cells dysfunctional through the actions of cytokines they produce.

## Therapeutic Options

Other than insulin replacement by daily injections of the hormone, the only other clinically-acceptable means of insulin restoration remains islet transplantation.<sup>57-60</sup> Recent advances in understanding transplantation immunology in general and the process of insulinitis and the molecular/genetic bases of failure of central and/or peripheral mechanisms of tolerance to tissue-restricted antigens in particular have yielded a number of approaches for therapy and prevention of T1D. To manipulate the immune system in a prophylactic manner, cell and gene based modalities or a combination of both were tested. Therapeutic strategies strive instead to improve islet transplantation by improving insulin secretion, engraftment and most importantly, protection of the transplant from allogeneic immune rejection. In humans, islet grafts derive from allogeneic cadaveric donors. Stem/progenitor cells, alone or in engineered form are also potential candidates for beta cell replacement. Each approach has shown promise, but each approach has also demonstrated its limitations. New data suggest that a critical period between time of diagnosis and actual destruction of beta cell mass required for appropriate glycemic control (the so-called "honeymoon period"; see below) may be exploited immunologically to obviate the need of islet transplantation altogether. While antibody-based approaches are currently being tested, it is anticipated that emerging gene and cell therapies can overcome the safety and negative systemic effects associated with the antibody approach.

## Insulin Replacement: Islets or Surrogate Beta Cells

Novel immunosuppressive cocktails, culture in the presence of homologous serum proteins, minimization of time between pancreas procurement and islet processing combined with transplantation of a larger beta cell mass, were the most significant steps in improving islet transplantation outcome in the studies of Shapiro et al.<sup>61-64</sup> Although it is not clear which of

the parameters contributed the most to success, many factors still limit a large-scale diffusion of islet and beta cell replacement for type 1 diabetic patients. The need for chronic immunosuppression and for multiple donors as a source for islets remain the prime reasons or factors which impose a search for alternative ways of promoting islet cell allograft survival. Tolerogenic protocols, once successful, may allow the use of islet transplantation in young diabetic patients.

Gene transfer technology is such an option and a number of advances have been attained in animal models of islet allograft transplantation. Table 1 lists experiments in which significant prolongation of islet allograft or xenograft survival has been achieved.

The main obstacle for a gene transfer-based approach is the choice of gene transfer vectors. Despite initial enthusiasm about the versatility of adenoviral vectors, their inherent immunogenicity raises a number of serious concerns in view of their possible application to engineer human islets for clinical use. The advent of lentiviral vectors appeared to alleviate some of the immunogenicity concerns, but lentivirus are not as efficient as adenoviruses in transducing intact human islets. Tables 2 to 4 list a number of gene transfer vectors as well as their pros and cons in the context of gene transfer to intact islets. However, an under-appreciated factor that very likely affects the success of islet engraftment is the metabolic status of the islets themselves following isolation and culture. There is no doubt that the time between organ retrieval and islet processing with the inherent intermediate steps including cold storage and enzymatic/mechanical digestion, affects islet yield, viability and function.<sup>35,65</sup> Furthermore the culture conditions prior to transplantation can crucially affect islet cells physiology and, consequently, the chance of successful engraftment. In general, the cessation of the oxygen supply to the pancreatic tissue at the time of donor organ harvesting, is known to trigger ischemic damage, free-radical mediated cell degeneration as well as initiation of apoptosis.<sup>66,67</sup> Also, the separation of the islets from the surrounding matrix and from the neighbor cells driven by the isolation procedure, further contributes to activate cell apoptosis.<sup>68,69</sup> Immediate-onset ischemia has been proposed to be an important determinant of acute and chronic allograft rejection.<sup>70</sup> In addition, organs carrying contaminating immune and a large number of endothelial cells or in which platelets have been trapped, will likely experience a so-called "cytokine storms", where the onset of apoptotic processes causes an abnormally large release of stored cytokines and other proinflammatory soluble mediators. Moreover a cycle is initiated whereby cytokines release can exacerbate the formation of reactive oxygen intermediates.<sup>71</sup> Presumably the combination of all these mechanisms predispose the islets to environmental damage both during culture and at the transplantation site, where inflammation is likely to occur shortly after implant even before allo-immune response initiates. Potential approaches to avoid this situation can include the perfusion of the organs with solutions containing chemical inhibitors of apoptosis (ZVAD-fmk) as well as anti-apoptotic genes like bcl-2, bcl-xL, and enzymes that break down, or prevent, the formation of free-radicals such as catalase, thioredoxin, heme-oxygenase-1 and superoxide dismutase.<sup>72-77</sup> Some of these anti-apoptotic proteins fused to protein-transduction domains can successfully prevent apoptosis and significantly improve islet yield and survival following isolation.<sup>34,65,78</sup> We and others have also shown that the inclusion of synthetic mimetics of free-radical scavengers seem to prevent islet degeneration possibly limiting the initiation of apoptotic processes.<sup>34,35</sup> Islets also take up oligonucleotides quite efficiently (unpublished observations). Knowledge of the primary transcripts whose protein products are involved in apoptosis activation or suppression of insulin production can be targeted with antisense oligonucleotides during the isolation procedure.

Oligonucleotide therapy offers a simple and convenient method to interfere with not only gene expression, but also with transcription using short double-stranded decoys containing binding sites for specific transcription factors involved in inflammatory responses, like NF- $\kappa$ B and STATs. Soluble binding proteins and ligand-binding domains of chemokines can also be considered potential tools with which primary islet dysfunction can be prevented. Chemokines are potent immunoattractants fairly resistant to degradation and are sequestered by proteoglycans

**Table 1. Target genes for the therapy of diabetes****A. Genes which promote islet allo-/xenograft survival in vitro and in vivo and/or beta cell survival in culture****Anti-apoptotic genes**

bcl-2<sup>74,75,234-236</sup>  
 bcl-xL<sup>78,237</sup>  
 heme oxygenase-1<sup>72,73,238</sup>  
 dominant negative protein kinase C delta<sup>239</sup>  
 dominant negative MyD88<sup>240</sup>  
 IGF-I<sup>241</sup>  
 IkappaB alpha super-repressor<sup>242</sup>  
 Hsp70<sup>243</sup>  
 A20<sup>244</sup>  
 PEA-15<sup>78</sup>  
 catalase<sup>245,246</sup>  
 manganese superoxide dismutase<sup>247</sup>  
 I-kappaB kinase inhibitor<sup>248</sup>

**Cytokines:**

IL-4<sup>249</sup> (although one report demonstrated no protection<sup>250</sup>)  
 interleukin-1 receptor antagonist protein<sup>251</sup>  
 IL-12p40<sup>252</sup>  
 viral IL-10<sup>253</sup>  
 IL-10<sup>254</sup> (one report did not show protection<sup>250</sup>)  
 TGF-beta<sup>254</sup> (one report showed negative results<sup>255</sup>)

**Immunoregulatory genes:**

Indoleamine 2,3-dioxygenase<sup>256</sup>  
 CTLA-4Ig<sup>257</sup>  
 Fas ligand<sup>258</sup> (although in a number of reports Fas ligand was not protective:<sup>259</sup>)  
 adenoviral E3 genes<sup>260</sup>

**B. Other gene/cell therapy approaches to prevent/abrogate autoimmunity and/or promote islet allo-/xenograft survival****Bone marrow transplantation/chimerism induction**<sup>183,188-191,222,261-266</sup>**Antigen-presenting cell transfer**

class I MHC<sup>267</sup>  
 autologous dendritic cell transfer<sup>193</sup>

**Co-stimulation blockade**

soluble ICAM-1-Ig<sup>268</sup>  
 CTLA-4Ig<sup>269-273</sup>  
 OX40Ig<sup>272</sup>

**Cytokines**

IL-10<sup>274-277</sup>  
 IL-4<sup>275,278</sup>  
 soluble IFN-gamma receptor<sup>279,280</sup>  
 TGF-beta<sup>281</sup>  
 VIL-10<sup>277</sup>

**Autoantigen transfer**

GAD<sup>282,283</sup>

**Others**

adenovirus E3 proteins<sup>284</sup>  
 orally-administered putative autoantigens (insulin, GAD)<sup>285-287</sup>  
 CD152<sup>288</sup>

**Table 2. Gene vectors which transduce islets (with references)**

Plasmid DNA	<sup>257,289-291</sup>
Adenovirus	<sup>244,250,252,253,258,260,292-299</sup>
Adeno-associated virus	<sup>74,75,89,91,241,242,251,256,300-304</sup>
MoLV retrovirus	<sup>305</sup>
Lentivirus	<sup>249,306,307</sup>
Herpes simplex virus	<sup>234,235</sup>
Cationic liposomes	<sup>290,291,295</sup>
Peptide fusion domains	<sup>78,248,308</sup>

**Table 3. Properties of gene transfer vectors with applicability to islet gene transfer**

Vector Type	Stable Transduction	Cell Cycle Requirements	Immunogenicity	Islets Transduced?
Plasmid DNA	No	Dividing/non-dividing	No	Mouse/human
Adenovirus	No	Dividing/non-dividing	Yes	Mouse/human
Adeno-associated virus	Possibly	Dividing/non-dividing	Minor	Human
MoLV-based retrovirus	Yes	Dividing	No	(Mouse/human) Very poor
Lentivirus	Yes	Dividing/non-dividing	No	Mouse/human
Herpes simplex type-1 virus	No	Dividing/non-dividing	Inherent toxicity	Human
Cationic liposome	No	Dividing/non-dividing	No	Human
Peptide fusion domains	No	Dividing/non-dividing	No	Human/mouse

on the endothelium.<sup>79,80</sup> Chemokines promote endothelial adhesion in addition to their chemotaxin properties.<sup>79,80</sup> Virally-encoded proteins have been identified which bind chemokines and could be a means of achieving chemokine blockade.<sup>81,82</sup> This blockade can easily be attained using peptide transduction domains fused to recombinant proteins or short oligonucleotides, especially if administered during procurement and reperfusion of the donor pancreas. However, long-term expression of some of these molecules may have a greater effect on graft survival once stable gene expression is achieved. This necessitates the use of gene vectors that can deliver the therapeutic gene with the objective of expression for the entire lifetime of the recipient.

Injection of animals with a number of vectors like adenovirus<sup>83-88</sup> and adeno-associated virus<sup>89-92</sup> encoding proinsulin under the control of a number of promoters including CMV, insulin, PEPCK and L-pyruvate kinase has resulted in correction of hyperglycemia. In many instances, however, the effect appears to have been transient. This approach suffers from the potential immunogenicity of the virus and in many cases precludes a second dosing due to the generation of neutralizing antibodies. Other issues are related to choice of promoter, which in the instance of L-pyruvate kinase demonstrates slow kinetics, although one study with this promoter was able to achieve relatively rapid responses to glucose.<sup>90</sup> Finally, many tissues do not express the necessary proteinases which process proinsulin into the potent bioactive insulin.

**Table 4. General characteristics of gene delivery vehicles**

Vector Type	Pros	Cons
Plasmid DNA	Easy to engineer, grow and purify; multicistronic variants easy to engineer	Poor persistence, non-specific cell targeting, poor tissue diffusion
Adenovirus	Choice vector for pilot proof-of-principle experiments; High titers easily obtained; almost all cells and tissues are transducible; cell retargeting is possible	Immunogenic in vivo; non-stable transduction
Adeno-associated virus	Site-specific, stable integration achievable, almost absent immunogenicity; many cell types transducible	Time for transgene expression can be on the order of days
MoLV-based retrovirus	Stably integrating vector in rapidly-dividing cells; cell-type retargeting possible; good titers obtainable	Subject to chromosomal position—effect sensitivity of—as well as methylation and cytokine effects on gene expression
Lentivirus	Non-immunogenic, stably integrating; Choice vector for non-dividing, non-cycling cells; good titers obtainable; Data support absence of replication-competent-recombinant vector particles in stocks	Clinical safety concerns with HIV-1-based vectors
Herpes simplex type-1 virus	Large genome available for multiple large size cistrons; good persistence in many cell types; cell-type retargeting possible	Inherent toxicity
Cationic liposome	Easy to manipulate to deliver plasmid DNA to almost all cells and tissue. Non-immunogenic; cell-type non-specific, cell-type retargeting possible	Poor control of diffusion kinetics
Peptide fusion domains	Many cell-types transducible; High-level protein/peptide import; intact proteins/peptides delivered; not subject to gene regulation; targeting of specific proteins possible; high-level peptide production easily achievable; no reported immunogenicity	Short half life; subject to proteolytic degradation; large amounts require some time to generate

### Beta Cell Surrogates

Surrogate beta cells offer an alternative to intact islet transplantation and direct injection of proinsulin-expressing vectors. A variety of cell types including fibroblasts, muscle, neuroendocrine cells and hepatocytes have been engineered to produce insulin.<sup>93-97</sup> Despite these exciting data, a very recent manuscript considers alternative hypotheses for the observations made and cautions in favor of very stringent experimentation to make the conclusion that nonbeta cells can produce and express insulin.<sup>98</sup> In contrast, the most notable advances have been made using engineered hepatocytes.<sup>85,87,88</sup> Hepatocytes are particularly attractive because they can easily engraft in the liver, and because they possess identical glucose-sensing molecules as the pancreas (e.g., GLUT2, GK). Furthermore, one can exploit a number of hepatocyte gene promoters which are sensitive to glucose, in order to engineer insulin transgenes to be glucose concentration-sensitive. Despite a number of promising approaches exploiting a number of glucose-regulated promoters,<sup>85,90,99-106</sup> much more work is needed to make hepatocytes into

fully surrogate beta cells. The first feature that a hepatocyte is missing to properly act like a beta cell surrogate is the ability to respond to glucose in a sufficiently rapid fashion, as rapid as that characteristic of beta cells. Second, the liver-specific glucose-sensitive promoters have elements that respond to hormonal and metabolic signals which can impede, attenuate or abrogate the desired objective of tight glucose regulation. For example, instances of hyperglucagonemia which is to be expected in the absence of functional endogenous beta cells in diabetics, will most likely attenuate or repress the LPK promoter as well as other promoters such as glucokinase.<sup>88,107,108</sup> Third, glucose-dependent trans-activation of the LPK promoter requires GK-dependent phosphorylation of glucose, an activity that is insulin-dependent<sup>103</sup>). Other promoters have been suggested, such as that of phosphoenolcarboxykinase (PEPCK), but this promoter is activated by glucagon and inhibited by insulin, which may not result in the desired kinetics of physiological gluco-regulation.<sup>109,110</sup> It is possible that a combination of promoter elements from different glucose-responsive hepatic genes may be needed to create an optimal synthetic promoter to drive hepatic insulin expression in a true glucose-sensitive fashion.

In an entirely different approach, tissue-specific promoters have been exploited to engineer cells to express insulin in cells that are not targets of autoimmune destruction. Lipes et al have expressed insulin in the anterior pituitary gland of NOD mice under the control of the pro-opiomelanocortin promoter. Insulin was expressed, stored into secretory granules and exhibited regulated secretion. Moreover, transplantation of transgenic anterior pituitary tissue to NOD mice was able to partially restore normoglycemia without any signs of immune rejection.<sup>111,112</sup> It was not clear however, if in these cells, insulin secretion was glucose concentration-dependent. More recently, an ingenious approach harnessing intestinal K-cells as surrogate glucose-responsive insulin producers was demonstrated. In this approach, transgenic mice expressing human insulin under the control of the gastrointestinal inhibitory peptide (GIP) promoter were generated. These mice expressed and secreted insulin from intestinal K cells in which the GIP promoter is active. Insulin secretion in these mice was glucose-responsive and was maintained following streptozotocin treatment, indicating that the K-cells were spared the effects of streptozotocin.<sup>113</sup> These data suggest that it may be feasible to target the intestinal cells with vectors encoding the GIP-Insulin transgene, or by ex vivo engineering intestinal cells in which glucose-sensitive promoters are driving insulin expression. However, an effective means of gene delivery to these cells needs to be developed for in vivo gene therapy, as these cells are present in the crypts of the gut, significantly impeding access to viral transduction.

### Stem and Progenitor Cells

The considerable genetic manipulations that are required to convert nonbeta cells into efficient glucose-sensing, insulin-secreting cells have led other investigators into considering means of expanding adult or neonatal beta cells or of harnessing the developmental potential of islet precursor cells and of embryonal stem cells. However, despite the culture conditions and manipulations, commitment to beta cells and insulin production has not always been consistent.<sup>114-119</sup> Much excitement has also surrounded observations that adult stem cells from bone marrow or from other tissues could "transdifferentiate" into a number of other lineage-different cell types. Such stem cells have been described and sometimes physically isolated in the nervous system, pancreas, epidermis, mesenchyme, liver, bone, muscle and endothelium. Hematopoietic stem cells, in some studies were proven able to yield endothelial, brain, muscle, liver and mesenchymal cells. In some studies, hematopoietic cells could also be generated from neuronal or muscle stem cells (reviewed in ref. 120). A number of issues however, have tempered the enthusiasm with which these observations were initially greeted. The contamination of hematopoietic stem cells with mesenchymal precursors, or the programming by growth factors in culture, and more recently, the phenomenon of fusion of stem cells with tissue cells and false



positives due to insulin in the culture media are perhaps the most important variables to better test.<sup>98,120,121</sup> Recent developments, however, strengthen the belief that mesenchymal cells in bone marrow may be a multipotent source of cells.<sup>122-124</sup> This characteristic can be exploited, however there are no data on whether such cells can be differentiated along the islet and beta cell lineage. Clearly, the ability to manipulate blood-borne progenitors into the beta cell lineage should provide a significant breakthrough for surrogate beta cell technology as insulin replacement.

Despite the current controversy and the serious ethical issues raised by cloning technology, it is likely that therapeutic cloning, under strict and defined conditions, will find its place in stem cell therapies.<sup>125-127</sup> In this regard, one possible means of propagating beta cells or progenitors while avoiding the complications involved with the immune response could entail the removal of DNA or nucleus from somatic cells of a patient, transfer it into an enucleated embryonal stem cell and its expansion into an appropriate beta cell lineage. While this remains highly speculative at present, the rapid pace of basic work in this area, despite restrictions, will likely yield insight into such manipulations.

Immortalization of islet cells with a beta cell phenotype has been attempted and successfully achieved. Insulin production, however, seems to be linked to terminal differentiation of the cell, an event normally reached with growth arrest. This problem has so far limited the utility of cell immortalization. Also, this approach carries with it the possibility of oncogenic transformation.<sup>128-131</sup>

Although still controversial, there are data indicating that mature human beta cells can be induced to replicate under the effects of hepatocyte growth factor (HGF).<sup>132-134</sup> The limitation of this approach, however, rests on the loss of differentiation of the induced beta cell along with a substantial decrease in insulin production.<sup>135</sup> Conditional replication of nonhuman beta cells has been achieved by placing the SV-40 T antigen under the control of an inducible promoter.<sup>129</sup> In these studies, beta cells were able to replicate and to maintain differentiated function under inducible conditions. No data exist on whether such an approach is feasible in human beta cells.

Propagation of islet precursor cells with subsequent genetic manipulation to commit them to the beta cell lineage and ultimately to beta cells has also been considered.<sup>118,136</sup> To become feasible, this approach, however, requires a more complete understanding of the hierarchy of master regulatory transcriptional genes. Depending upon the cell type, PDX-1 over-expression can impart onto it a beta cell or a beta-cell-like phenotype.<sup>137-139</sup> Indeed, Ferber et al demonstrated that adenoviral gene transfer of a PDX-1 gene into liver resulted in insulin-expressing cells, although it was not clear if these cells were glucose-sensitive and were actually secreting the insulin in a timely fashion.<sup>140</sup> Other important transcriptional regulators associated with differentiation of ductal epithelial cells into endocrine islet cells include the HNF family of transcription factors, PAX-4 and PAX-6, NeuroD/B2, Nkx 2.2 and Nkx 6.1.<sup>141-143</sup> Along with intracellular determinants, precursor cells require signalling from their environment to differentiate appropriately. A variety of polypeptide growth factors including insulin-like growth factors I and II,<sup>144-148</sup> prolactin,<sup>149</sup> placental lactogen,<sup>150,151</sup> parathyroid hormone-related peptide,<sup>152,153</sup> and to a limited extent, TGF-alpha,<sup>154-156</sup> can promote pancreatic cell growth and islet cell proliferation. Hart and colleagues have produced evidence suggesting that fibroblast growth factor (FGF) signalling is important for beta cell generation.<sup>157,158</sup> Strategies aimed at engineering beta cell progenitors from pancreatic ductular epithelium with FGF in the presence of a permissive PDX-1 expression could promote expansion of beta cell progenitors or a differentiation of progenitors into a prebeta cell lineage.

Another class of factors has been identified whose expression and production is associated with pancreatic regeneration.<sup>159-161</sup> The Reg secreted protein, in particular, promotes increases

in beta cell mass in rats that had undergone pancreatectomy.<sup>162-164</sup> The expression and secretion of another molecule that belongs to the Reg family of proteins, termed INGAP (islet neogenesis associated protein), is upregulated in hamster islets where neogenesis was artificially induced.<sup>165,166</sup> The precise role of INGAP on beta cell proliferation and function, however, remains unclear.

Bonner-Weir and colleagues have shown that it may be feasible to derive beta cell cluster buds from exocrine pancreatic tissue from which originate the ductular epithelial cells destined to become endocrine pancreatic islet cells.<sup>167</sup> This approach is exciting in that mature, nonendocrine tissue of the pancreas need not be wasted during the process of islet isolation, but can be used in defined culture systems to generate islet progenitor cells for further manipulation, genetic or hormonal.

Thus, taken together, the transfer of combinations of genes encoding soluble and intracellular differentiation factors to stem/progenitor cells could become feasible once their precise role in the pathway of commitment and differentiation to beta cells becomes clearer. However, beta cells have a limited life-span in vitro. To what extent apoptosis or senescence play a role in this is uncertain. Nonetheless, a better understanding of cell cycle control in beta cells or neonatal islet cells could lead to the discovery of molecules that could be exploited, in a conditional manner, to promote growth in vivo and maintenance or extension of life-span, both in vitro and in vivo. Possible means include the transfer of cyclin-dependent kinases, pro-replication and mitotic factors and/or telomerase, to promote expanded cell life-span, all under regulatable promoters. Such an approach could achieve the expansion of semi-committed or fully committed islet precursor cells, or early beta cells. Combined with xenogeneic donor manipulation, these interventions could provide an almost limitless supply of beta cells for transplantation. The recent success in knocking in a nonfunctional  $\alpha 1,3$ -galactosyltransferase in order to generate a transgenic pig deficient for this enzyme, may forecast the inclusion of modalities in which transgenic porcine islets can be used instead of allogeneic human islets for transplantation.<sup>168-170</sup> The importance of this breakthrough is underscored by the fact that the major target of xenoreactive antibodies which promote an acute rejection of porcine tissues is the epitope that is synthesized by this enzyme. While this is the major porcine xenoantigen, it is almost certain that other minor porcine epitopes will contribute, perhaps not to acute rejection, but to delayed or chronic xenograft rejection and these are challenges that must be surmounted in the future.

### ***The Gene Vehicles: Viral, Nonviral and Cellular***

An appreciable amount of work has focused on using viral vectors to infect intact islets in culture prior to transplantation into recipients to impede the allogeneic rejection (reviewed in Giannoukakis et al<sup>171,172</sup>). The excitement generated by these studies, however, was tempered by the appreciation that permanent allograft survival was generally not achieved. Often, to explain this limited success, investigators invoke the immunogenicity of the particular vector used, although recent evidence suggests that the quality of the islets may be more crucial than the vector choice in determining the presence and grade of inflammation in and around the graft.<sup>65</sup> Tables 2 to 4 list the vectors that have been used to date to transduce intact human islets as well as their pros and cons. The list indirectly demonstrates that no "ideal" vector yet exists. New technology including small interference RNA (siRNA),<sup>173</sup> adeno-associated virus inverted terminal repeat (AAV ITR)-based plasmids,<sup>174,175</sup> novel classes of lentivirus (EIAV, FIV),<sup>176-182</sup> lentivirus-herpesvirus hybrids and other viral vectors, is in development, but their efficiency has yet to be reported in the context of intact islet transduction. Equally unknown is the degree to which these vectors can contribute to post-transplantation inflammation.

Cell therapy constitutes an alternative approach to induce tolerance to alloantigens. Allogeneic bone marrow transplantation, with or without the addition of immunoregulatory antibodies (blocking CD28:B7 and CD40:CD40 ligand interactions), has been the choice of many investigators to promote allogeneic islet transplantation in mouse models of autoimmune diabetes.<sup>183-192</sup> In some instances, permanent allograft survival has been reported in prediabetic mice (permanent in the sense that the recipient maintained normoglycemia at the time it was last tested). It is not clear however, if these strategies would work equally well in an already-diabetic individual. A number of studies attempted to promote the activity of regulatory immune cells by dendritic cells. This novel and rational approach, however, may require multiple administrations to maintain a sufficient level of activity.<sup>193,194</sup> Combinations of these approaches, including gene-engineered dendritic cells expressing a variety of immunosuppressive molecules have shown promise in allograft survival<sup>195-201</sup> and are awaiting rigorous testing in the context of islet allograft transplantation. Considering successes and failures, it is perhaps fair to conclude that while gene vectors and cells alone may not have yet supported permanent islet allograft survival, their utility cannot be yet dismissed as many important parameters have still to be evaluated, including combinative approaches. In fact, very few studies have attempted to engineer islets expressing more than one immunoregulatory transgene at a time. This is an important aspect of the problem to consider since the immune response against the transplant (and perhaps the vector) may involve more than one pathway.

## Prevention Strategies

In order to prevent the disorder, one must be able to first identify with a sufficient degree of confidence individuals who are at very high risk for developing type 1 diabetes. While inheritance of susceptibility alleles at loci linked to and/or associated with the disorder is an important risk factor, it alone cannot guarantee that the individual will in fact become diabetic. This is the main reason for the ongoing debates on prevention based on genetic screening.<sup>39,202</sup> While outright prevention based only on genetic screening may not be yet acceptable, other strategies which fall inside the realm of "prevention" can be acceptable. There are data indicating that newly-onset diabetics still possess adequate beta cell mass to sustain normoglycemia if the autoimmune inflammation can be promptly controlled.<sup>203-209</sup> The time between diagnosis and elimination of beta cell mass adequate to sustain normoglycemia has been termed the "honeymoon" period. One can exploit immunoregulatory networks to promote hyporesponsiveness of autoaggressive immune cells in this period as a viable means of improving or restoring normoglycemia. Supporting this approach are the studies where treatment of prediabetic and/or overtly-diabetic NOD mice with an anti-CD3 antibody restored normoglycemia in a substantial portion of mice for a sustained period of time.<sup>210</sup> Very recently, human trials using the same approach also seem quite promising.<sup>211</sup> Although clinical diabetes onset has most often been associated with beta cell death, it is possible that the low levels of insulin production are due to the effects of cytokines which modulate their production. If this is the case, this process can be reversed.<sup>212-218</sup> Some data strongly suggest that suppression of the activity of the insulinitic cells by the induction of immune hyporesponsiveness in clinically-diabetic individuals may promote either beta cell neogenesis and/or rescue of the cytokine-suppressed beta cells in the insulinitic environment.<sup>192</sup>

Inherent in this philosophy is the ability to promote T1D-specific autoantigen tolerance or T1D-specific autoantigen immune hyporesponsiveness. To achieve this, one can target genes and/or cells to the thymus, or one can manipulate the peripheral immune effectors using cells alone or gene-engineered cells. The evidence suggesting that a preventive approach manipulating the thymic environment of antigen presentation is possible was initially obtained by generating transgenic NOD mice with different H2 genes. Mice carrying H2 transgenes

conferring resistance did not develop diabetes.<sup>219,220</sup> Additionally, diabetes in the NOD mouse was also prevented by thymic inoculation of soluble islet antigens in the form of cellular lysates or by expression of putative beta cell autoantigens in the thymus.<sup>219,221</sup> Could this approach be clinically-applicable? Recent data on plasticity of bone marrow stem cells<sup>120,122-124</sup> seem to imply that culture conditions could be defined in which bone marrow progenitors could be propagated towards "thymic" antigen-presenting cells. These cells could be engineered using a number of viral or nonviral vector methods (gene vectors to be described in a later section) to present autoantigen. These cells could then be injected into the host where they could eventually populate the recipient thymus. To obviate the problems associated with graft versus host disease in an allogeneic context, one could envisage the use of hematopoietic stem cells propagated from peripheral blood precursors of the recipient. Preliminary evidence seems to suggest that the newly-generated insulin-generating cells may not have the same phenotypic makeup of normal beta cells and because of this characteristic, they may be able to escape the recurrence of preexisting autoimmunity.

A number of studies have shown that allogeneic bone marrow transplantation into NOD or BB rats with the aim of inducing a state of chimerism can also prevent diabetes and facilitate allo- and xenograft islet transplantation.<sup>183-192,222</sup> While the mechanisms are believed to involve central and peripheral tolerance, the applicability of this approach in humans is impeded by the use of very high radiation conditioning of the recipient. The need for complete or partial myeloablative treatment and of allogeneic donors could be obviated by genetically-engineering peripheral blood-derived autologous hematopoietic stem cells with transgenes promoting the induction and activity of immunoregulatory networks. Independently of the means utilized to abrogate autoimmunity, a state in which the diabetic patient is free of autoreactive T-cells and their assault on pancreatic beta cells is optimal to allow or promote the rescue or regeneration of enough insulin-secreting cells in the endogenous pancreas. This may allow physiologic euglycemia. Alternative measures to control the glycemia during the possibly long recovery period must also be implemented.

Although considered potent immunostimulators, dendritic cells (DC) have recently been shown to possess tolerogenic characteristics under defined conditions. DC tolerogenicity manifested as the suppression of T cell activation, has been documented in tumor, allo-, and auto-immunity.<sup>223</sup> The conditions that can yield tolerogenic DC include UV irradiation, as well as exposure to CTLA-4Ig, TGF-beta or IL-10.<sup>224-226</sup> How a tolerogenic DC acts to suppress immunoreactivity is not completely understood, but may involve the promotion of anergy of T-cells that come into contact with DC, a shift from TH1 to TH2-type responses, apoptosis of the autoreactive T-cells or the induction of regulatory cells including regulatory T-cells and NK-T cells.<sup>223,227-231</sup> With the aim of establishing a durable tolerogenic state in the recipient of an allogeneic transplant, myeloid DC have been genetically modified using adenoviral and retroviral vectors encoding CTLA-4Ig, TGF-beta and IL-10 in the mouse.<sup>224-226</sup> CTLA-4Ig-expressing DC significantly prolong allograft survival, can induce alloantigen-specific T cell hyporesponsiveness, and display enhanced survival in nonimmunosuppressed, allogeneic hosts.<sup>225</sup> The *in vivo* presentation of alloantigens by donor or recipient DC in the absence of costimulation along with local production of immunosuppressive molecules like TGF-beta, could likely promote the inhibition of anti-donor reactivity and promote tolerance induction without causing any major systemic immunosuppression. DC engineered to express vIL-10 following retroviral gene transfer produce high levels of vIL-10 *in vitro*, exhibit marked reduction in cell surface MHC and costimulatory molecule expression, decrease T cell allostimulation and promote the induction of T cell hyporesponsiveness.<sup>224</sup> Genetically-engineered DC may be used to prevent islet allograft rejection, since they are able to manipulate anti-donor and/or autoantigen immunoreactivity. If recent observations showing islet-specific molecule gene

expression in peripheral lymphoid organs can be confirmed in antigen presenting cells<sup>232</sup> like bone marrow-derived dendritic cells (Machen et al manuscript submitted), one can envision infusing autologous DC engineered ex vivo to lack costimulatory capability, but also express islet-specific genes (e.g., GAD65 or insulin), into prediabetic or early-onset diabetic patients, with the objective of inducing autoantigen-specific hyporesponsiveness. In fact, DC have been treated ex vivo with oligodeoxyribonucleotide decoys to NF- $\kappa$ B, an important maturational transcriptional mediator in DC, and injected into an allogeneic host. These DC were able to prolong the survival of an allogeneic heart.<sup>233</sup> It is likely that this and other transcriptional pathways in APC could be exploited by decoy nucleotide strategies to present autoantigen in the absence of costimulatory signals or in the presence of death ligands to silence or kill autoreactive T-cells.

## Conclusion

While pharmacologic agents will no doubt continue to be discovered to promote safer immunosuppression, insulin sensitisation and enhancement of insulin output, diabetes mellitus will continue to be a challenging disorder in which these agents could be applied. Gene therapeutics, however, will take advantage of the knowledge of the underlying stage and severity of diabetes and will very likely be patient-specific. Nonetheless, a targeted approach, which is offered by gene medicines, is certainly much better than the systemic effects and toxicities that many drugs in use today are associated with.

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