Adenovirus Transduction is Required for the Correction of Diabetes Using Pdx-1 or Neurogenin-3 in the Liver

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The regeneration of insulin-producing cells *in vivo* has emerged as a promising method for treating type I diabetes. Pancreatic duodenal homeobox-1 (Pdx-1), NeuroD, and Neurogenin-3 (Ngn3) are pancreatic transcription factors important for the development of insulin-producing cells in the liver. Other groups have demonstrated that adenoviral-mediated transgene expression of these transcription factors in the liver can reverse hyperglycemia in diabetic mice. We delivered Pdx-1 and Ngn3 to the livers of diabetic mice using adeno-associated virus (AAV) serotype 8, a vector that has been shown to result in non-toxic, persistent, high level expression of the transgene. We were unable to correct hyperglycemia in mice with streptozotocin-induced diabetes using AAV vectors expressing Pdx-1 and Ngn3. However, when we co-delivered these transcription factor expression cassettes in non-viral vectors with an irrelevant adenoviral vector, we were able to correct hyperglycemia in diabetic animals. Further studies demonstrated that an antigen-dependent immune response elicited by the adenoviral capsid together with the expression of a pancreatic transcription factor was required for restoration of serum insulin levels by the liver. Our results suggest that a host response to adenovirus in combination with expression of a pro-endocrine pancreas transcription factor is sufficient to induce insulin production in the livers of diabetic mice.

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INTRODUCTION

Diabetes is a debilitating disease that affects over 18 million people in the United States, over 6% of the entire population, resulting in direct and indirect costs of $132 million per year.[1] Type I diabetes, also known as insulin-dependent diabetes mellitus or juvenile-onset diabetes, results from autoimmune destruction of the insulin-producing beta cells in the pancreas. The standard treatment for this lifelong disease involves supplementing insulin through periodic injections or via an insulin pump. This is a major nuisance for diabetics, and even with the best compliance, substantial complications may occur. This necessitates the development of a safe, long-term treatment for type I diabetes.

Several strategies have been attempted to correct diabetes using gene therapy.[2,3] The delivery of insulin via gene therapy has been attempted by several groups with varying degrees of success, but the main caveat of this method is the difficulty in achieving tightly regulated and glucose-responsive expression of insulin.[4] It seems likely that the only method of achieving endogenous glucose-responsive secretion of insulin would be to have insulin secreted from normal insulin-producing beta cells present in the islets of Langerhans. Indeed, the transplantation of islets in humans has resulted in the successful lifelong correction of diabetes, but is limited by the extremely scarce number of islet donors.[5]

The regeneration of insulin-producing beta cells *in vivo* has emerged as a promising method for circumventing the problem of limited islet donors. Several key transcription factors have been identified that have been found to be crucial in the development of the pancreas and insulin-producing islets, including pancreatic duodenal homeobox-1 (Pdx-1), NeuroD, and Neurogenin-3 (Ngn3).[6,7] The lack of Pdx-1 expression early in development results in the complete absence of a pancreas in the adult mouse.[8] Ngn3 knockout mice also completely fail to develop islets, which suggests a crucial role for Ngn3 in islet development.[9] In addition, Pdx-1 delivery to the pancreas of diabetic mice using an adenovirus has been shown to induce the regeneration of islets *in vivo*, but was not sufficient to correct diabetes in mice.[10]

More recently, several groups have demonstrated the successful induction of islet neogenesis in the liver using these pancreatic transcription factors. This conversion of liver to pancreatic-like cells in adult mice is not entirely surprising, as the development of the liver and pancreas differ by only the

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expression of a few key genes, and are likely to derive from a common endodermal progenitor cell.[11] Ferber *et al.*[12] demonstrated that delivery of Pdx-1 to the liver of mice using an adenoviral vector resulted in insulin expression in the liver and correction of streptozotocin (STZ)-induced diabetes in these mice. Adenoviral-mediated delivery of Pdx-1 to the liver also resulted in the induction of exocrine and endocrine pancreatic gene expression, and long-term expression of insulin in the liver.[13] More recently, Kojima *et al.*[14] demonstrated that delivery of Pdx-1 to the liver using a helper-dependent adenoviral vector resulted in fulminant hepatitis and only a transient correction of STZ-induced diabetes in mice. They also demonstrated that delivery of a downstream factor of Pdx-1, NeuroD, in combination with betacellulin, was able to induce islet neogenesis in the liver and correct STZ-induced diabetes in mice, without leading to hepatitis. Transgenic mice ectopically expressing Pdx-1 in the liver also showed severe liver dysmorphogenesis owing to the initiation of both endocrine and exocrine pancreas differentiation.[15] Another group demonstrated that adenoviral delivery of MafA, a pancreatic beta cell-specific transcription factor, in combination with Pdx-1 and NeuroD, could induce insulin gene expression in the liver and ameliorate STZ-induced diabetes in mice, whereas delivery of a combination of both Pdx-1 and NeuroD resulted in only a slight correction of hyperglycemia in diabetic STZ-treated mice.[16]

These somewhat conflicting results led us to further investigate the effect of long-term expression of Pdx-1 and a downstream factor of Pdx-1, Ngn3, in the livers of diabetic STZ-treated mice. For our studies, we chose to utilize adeno-associated virus (AAV) vectors to deliver these pancreatic factors to the liver, as AAV8 vectors have been shown to result in long-term and stable expression of the delivered transgene without toxicity at levels similar to that achieved with adenoviral vectors.[17,18] Interestingly, delivery of Pdx-1 and Ngn3 did not result in correction of STZ-induced diabetes in mice, which led us to suspect that some component of adenoviral transduction is necessary in combination with expression of Pdx-1 or Ngn3 in the liver to correct diabetes in mice, as all previous studies were performed using adenoviral vectors.

RESULTS

Synthesis of recombinant AAVs

To determine the effect of long-term expression of Pdx-1 and Ngn3 in the livers of mice *in vivo*, we constructed AAV serotype 8 vectors expressing Pdx-1 and Ngn3 under the transcriptional control of the elongation factor 1-α promoter, a promoter that has been shown to have high activity in the liver and does not experience silencing as seen with some viral promoters, such as the cytomegalovirus early promoter.[19,20] We chose AAV serotype 8 vectors for their low immunogenicity and high transduction efficiency and persistence in the liver, with transduction efficiencies comparable to that of adenoviral vectors.[18]

Administration of recombinant AAVs to diabetic mice

AAV8-Pdx-1 and AAV8-Ngn3 vectors were administered to 8- to 10-week-old C57Bl/6 mice made diabetic by a single dose of 150 mg/kg STZ. The mice were determined to be diabetic by blood glucose measurements at 1 and 2 weeks post-STZ administration, where both blood glucose levels were >300 mg/dl after a 6 h fast. The recombinant AAVs expressing Pdx-1 or Ngn3 were administered by a single tail vein injection at 2 weeks after STZ administration at a dose of 3×10^{11}–5×10^{11} particles, a dose sufficient to transduce approximately 20–30% of the hepatocytes in the liver.[18] AAV8-LacZ was also administered to a group of diabetic mice as a negative control.

Response of diabetic mice to AAVs expressing pancreatic transcription factors

Weekly fasting blood glucose measurements were performed to determine whether expression of Pdx-1 or Ngn3 from an AAV vector in the liver could induce hepatic insulin expression and correct hyperglycemia in STZ-treated diabetic mice. The blood glucose measurements showed no improvement in the diabetic condition of mice treated with AAV8-Pdx-1 or AAV8-Ngn3 (**Figure 1a** and **b**), and the mice continued to exhibit severe hyperglycemia

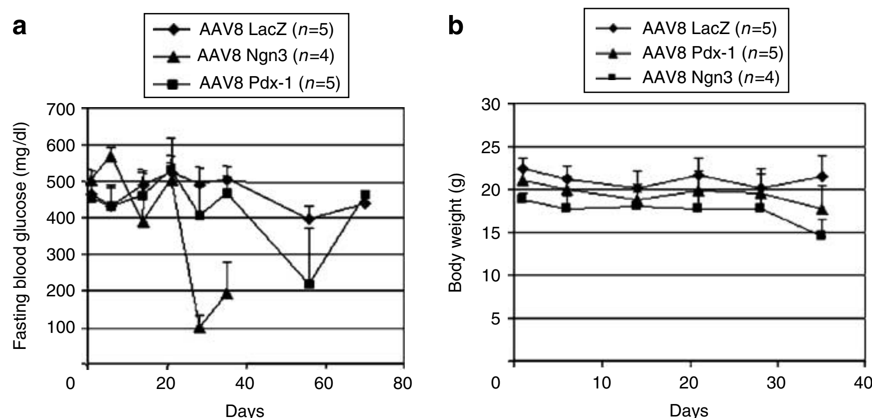

Figure 1 Blood glucose levels in diabetic animals treated with AAV-Pdx-1 or AAV-Ngn3. (**a**) Fasting blood glucose levels of STZ-treated diabetic mice injected with AAV8-Pdx-1 or AAV8-Ngn3. Male C57BL/6 mice were injected with 150 mg/kg STZ and injected 2 weeks later with the viruses. Glucose levels were determined after a 6 h fast from blood drawn from the retroorbital plexus. (**b**) Body weights of STZ-treated diabetic mice injected with AAV8-Pdx-1 or AAV8-Ngn3.

and no improvement in glycemic control over mice treated with AAV8-LacZ for up to 2 months after AAV administration. The mice treated with AAV8-Pdx-1 or AAV8-Ngn3 also exhibited continued weight loss and polyuria, comparable to that observed in the control mice treated with AAV8-LacZ, suggesting no improvement in their diabetic condition. Expression of Pdx-1 and Ngn3 in the liver was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) (**Figure 8**). These results suggested that expression of Pdx-1 or Ngn3 from an AAV vector in the liver was not able to induce hepatic liver insulin expression or induce liver to pancreas transdifferentiation *in vivo*.

Immunohistochemistry of mice treated with AAV8-Pdx-1 or AAV8-Ngn3

In order to further investigate whether these vectors were able to induce liver to pancreas transdifferentiation *in vivo*, the livers of mice treated with AAV8-Pdx-1 and AAV8-Ngn3 were harvested, and insulin immunohistochemistry was performed on the liver samples. Insulin expression was not detected in the livers of mice treated with any of these vectors, whereas insulin was detected in pancreatic samples from untreated mice stained concurrently, further suggesting that these vectors were not able to induce liver to pancreas transdifferentiation in these mice (data not shown).

Liver function studies

Studies were also performed to determine whether or not the delivered transgenes were inducing liver damage in the mice treated with the AAVs. To determine whether liver damage was occurring in response to these vectors, aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), and bilirubin measurements were periodically determined from mouse serum samples. Mice treated with AAV8-Pdx-1 did not exhibit elevated levels of AST, ALT, or bilirubin, suggesting that expression of Pdx-1 in the liver does not cause liver dysfunction (**Figure 2a–c**). Interestingly, mice treated with AAV8-Ngn3 displayed severe jaundice and corresponding hyperbilirunemia, suggesting liver dysfunction in response to the delivered transgene.

Co-administration of Pdx-1 or Ngn3 with an irrelevant adenovirus ameliorates STZ-induced diabetes in mice

In order to study the possible role of adenovirus in correction of hyperglycemia in STZ-treated mice in conjunction with Pdx-1, we co-administered plasmids expressing Pdx-1 (pPdx-1) and pNgn3 under the control of the elongation factor 1-α promoter (the identical expression cassette used in the AAV studies), with an unrelated adenovirus expressing the human coagulation factor IX gene (AdVhFIX). Fifty micrograms of the pPdx-1 and Ngn3 were administered via hydrodynamic transfection, a technique resulting in transfection of approximately 30–40% of the hepatocytes.[21,22] Twenty-four hours after hydrodynamic transfection, 5×10^9 PFU of AdVhFIX, a dose sufficient to transduce 100% of the mouse hepatocytes, was administered via the tail vein. Adenoviral transduction was confirmed by detection of hFIX in the serum of injected mice. We then

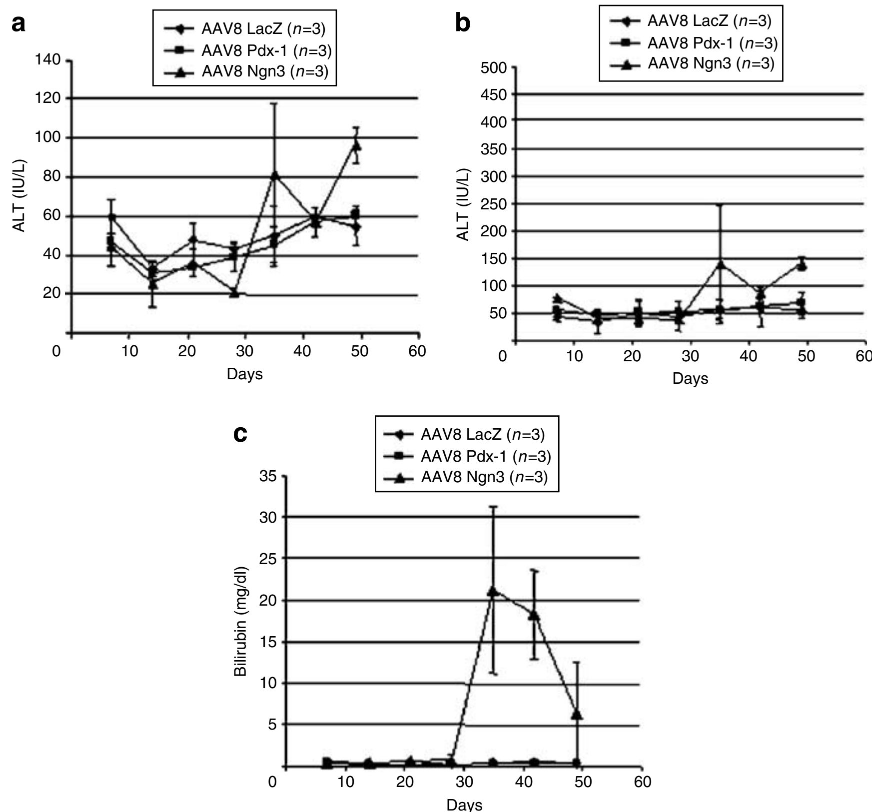

Figure 2 Liver injury parameters in treated mice. (a) Serum ALT, **(b)** AST, or **(c)** bilirubin levels from STZ-treated diabetic mice treated with AAV8-Pdx-1 or AAV8-Ngn3.

monitored the fasting blood glucose levels of these mice and compared them with mice treated with only pPdx-1 or Ngn3.

STZ-treated diabetic mice treated with pPdx-1 in combination with AdVhFIX showed a dramatic improvement in hyperglycemia and body weight, with the mice exhibiting normal fasting glycemic levels within about 4 weeks of the combination treatment (**Figure 3a** and **b**). A similar effect was seen with mice treated with both pNgn3 and AdVhFIX (**Figure 4a** and **b**). These mice displayed normal fasting blood glucose levels within about 4 weeks of treatment and gained significantly more weight than mice treated with either pNgn3 or AdV alone. Diabetic mice treated with only adenovirus, pPdx-1, or pNgn3 alone did not show any improvement in blood glucose, suggesting that adenoviral transduction as well as expression of a transcription factor was required for the correction of hyperglycemia.

Functional studies on mice treated with Pdx-1 or Ngn3 and an irrelevant adenovirus

Glucose tolerance tests performed on mice treated with pNgn3 and AdVhFIX showed that they displayed near-normal glucose response profiles in response to a bolus of glucose injected intraperitoneally (**Figure 5a**). In addition, these mice secreted insulin at significantly higher levels than mice treated with pNgn3 alone, albeit at slightly lower levels than normal non-diabetic mice (**Figure 5b**). Histological examination of the livers of mice showed signs of significantly altered liver morphology in mice treated with both pNgn3 and AdVhFIX, but not pNgn3

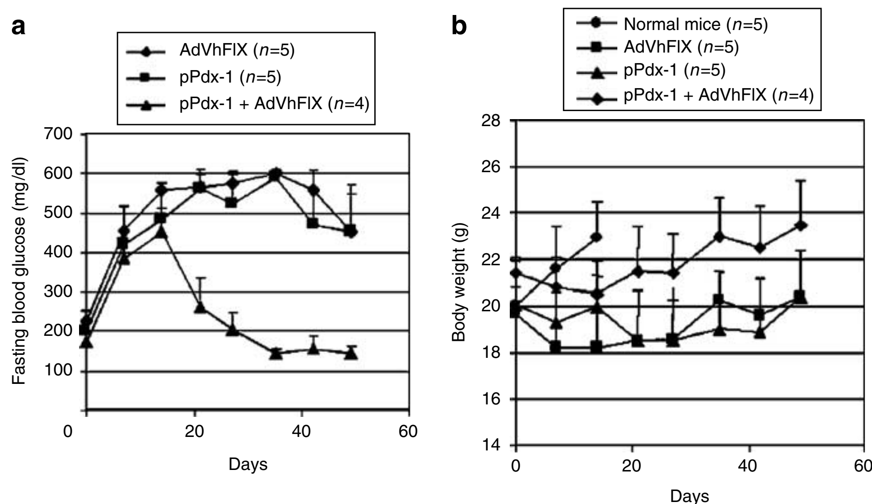

Figure 3 Blood glucose and growth parameters in STZ-diabetic mice undergoing various gene transfer protocols. (**a**) Fasting blood glucose levels of STZ-treated diabetic mice treated with pPdx-1/AdVhFIX. Male C57/BL6 mice were injected with 150 mg/kg STZ and injected 2 weeks later with pPdx-1 via hydrodynamic transfection. After 24 h, 5×10^9 PFU of AdVhFIX were injected through the tail vein. Glucose levels were determined after a 6 h fast from blood drawn from the retroorbital plexus. (**b**) Body weights of STZ-treated diabetic mice injected with pPdx-1/AdVhFIX.

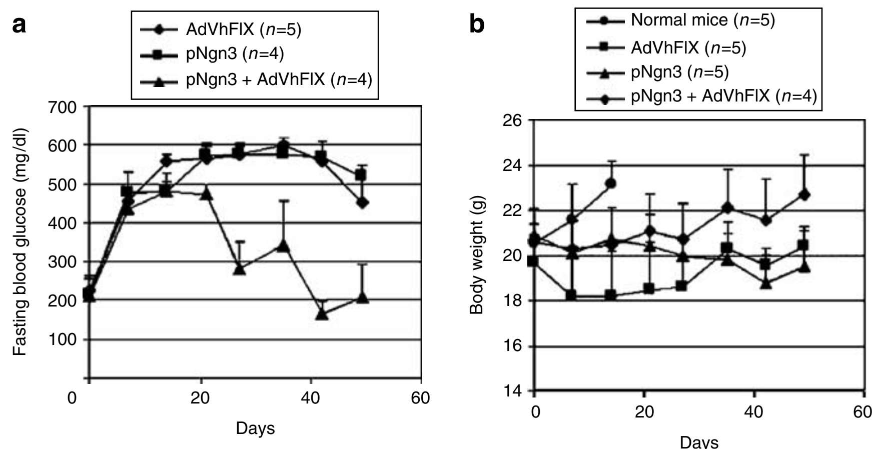

Figure 4 Blood glucose and growth parameters in STZ-diabetic mice undergoing various gene transfer protocols. (**a**) Fasting blood glucose levels of STZ-treated diabetic mice treated with pNgn3/AdVhFIX. Male C57/BL6 mice were injected with 150 mg/kg STZ and injected 2 weeks later with pPdx-1 via hydrodynamic transfection. After 24 h, AdVhFIX was injected through the tail vein. Glucose levels were determined after a 6 h fast from blood drawn from the retroorbital plexus. (**b**) Body weights of STZ-treated diabetic mice injected with pNgn3/AdVhFIX.

alone. The livers from mice treated with pNgn3 and AdVhFIX exhibited cystic lesions and enlarged nuclei (not shown).

Investigation of the role of the immune response to adenovirus in mediating Pdx-1/Ngn3-induced correction of diabetes

Adenoviral vectors are known to elicit a potent immune response *in vivo* when administered at high doses as compared with adeno-associated viral vectors,[23,24] a key difference that could explain the different results we observed in our studies. We therefore tested the hypothesis that adenoviral gene expression or a host inflammatory response to an adenoviral protein was needed to correct hyperglycemia in diabetic mice with Pdx-1 and Ngn3. To distinguish between these possibilities, we co-delivered plasmids expressing different portions of the adenoviral genome (plAd5—E2, E4; pAdHM4—entire AdV genome minus E1 and E3) along with pPdx-1 and Ngn3 by hydrodynamic transfection to STZ-treated diabetic mice. The mice treated with this combination did not show any improvement in their diabetic condition, suggesting that the expression of adenoviral gene(s) was not responsible for the cellular conversion allowing for glucose-regulated insulin secretion (**Figure 6a**).

We also co-delivered pPdx-1 or pNgn3 with 5×10^9 transducing units of an irrelevant helper-dependent adenoviral vector expressing canine coagulation factor IX (HDADcFIX) to STZ-treated mice. Helper-dependent adenoviral vectors have similar transduction efficiencies as first-generation adenoviral vectors, but lack all adenoviral genes.[25–27] HDADcFIX in combination with Pdx-1 or Ngn3 was also able to mediate the correction of hyperglycemia in these diabetic mice (**Figure 6b**). This further suggested that the adenoviral capsid and not adenoviral gene expression was responsible in combination with Pdx-1 and Ngn3 for the correction of STZ-induced hyperglycemia.

To test whether the immune response elicited by adenoviral transduction in conjunction with Pdx-1 and Ngn3 was necessary

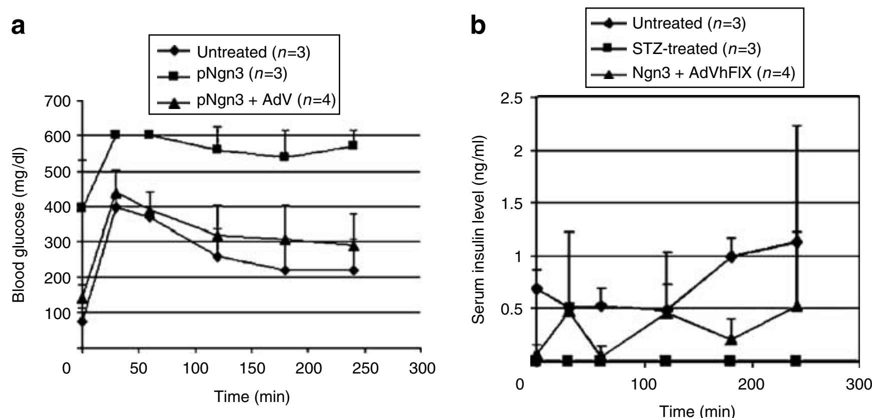

Figure 5 Glucose tolerance tests in STZ-diabetic mice undergoing various gene transfer protocols. (**a**) Glucose tolerance test performed on mice treated with pNgn3/AdVhFIX. Mice were fasted overnight and then injected with 1.5 g/kg glucose intraperitoneally. Blood glucose levels were determined from blood drawn from the retroorbital plexus. (**b**) Serum insulin levels during the glucose tolerance test from mice treated with pNgn3/AdVhFIX.

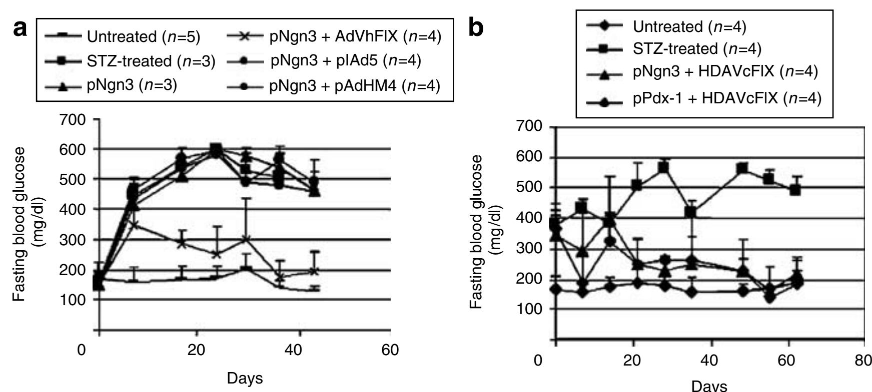

Figure 6 Glucose tolerance tests in STZ-diabetic mice undergoing various gene transfer protocols. (**a**) Fasting blood glucose measurements on STZ-treated mice treated with pPdx-1 or pNgn3 and various adenoviral plasmids. Male C57/BL6 mice were injected with 150 mg/kg STZ and injected 2 weeks later with pPdx-1 or pNgn3 in combination with the various adenoviral plasmids (plAd5, pAdHM4) via hydrodynamic transfection. (**b**) Fasting blood glucose measurements of STZ-treated mice treated with pPdx-1 or pNgn3/HDAdVcFIX. Male C57/BL6 mice were injected with 150 mg/kg STZ and injected 2 weeks later with pPdx-1 or pNgn3 via hydrodynamic transfection. After 24 h, 5×10^9 transducing units of the gene-deleted adenoviral vector HDAdVcFIX were injected through the tail vein. Glucose levels were determined after a 6 h fast from blood drawn from the retroorbital plexus.

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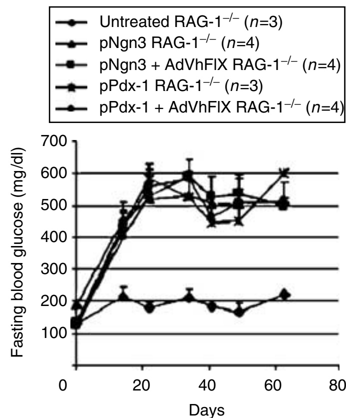

Figure 7 Blood glucose levels in immunodeficient STZ-diabetic mice. Fasting blood glucose measurements of STZ-treated RAG-1$^{-/-}$ mice treated with pPdx-1 or pNgn3 and AdVhFIX. Male RAG-1$^{-/-}$ mice were injected with 150 mg/kg STZ and injected 2 weeks later with pPdx-1 or pNgn3 via hydrodynamic transfection. After 24 h, AdVhFIX was injected through the tail vein. Glucose levels were determined after a 6 h fast from blood drawn from the retroorbital plexus.

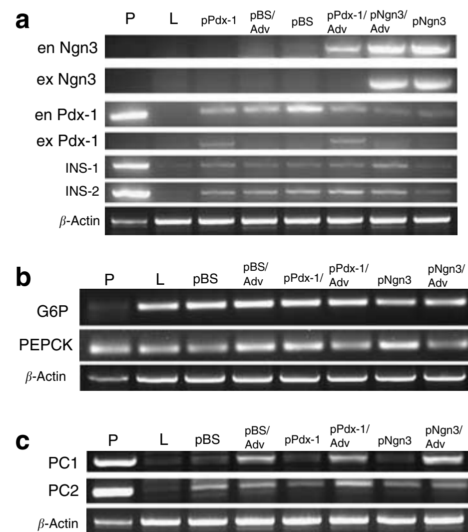

Figure 8 Gene expression profiles in liver of treated animals. (**a**) RT-PCR analyses of total RNA from untreated mouse pancreas (P), untreated mouse liver (L), or mice treated with pBS, pPdx-1, pNgn3, pBS/AdV, pPdx-1/AdV, pNgn3/AdV. INS-1 = insulin-1, INS-2 = insulin-2. (**b**) Glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression in untreated mouse pancreas (P), untreated mouse liver (L), or mice treated with pBS, pPdx-1, pNgn3, pBS/AdV, pPdx-1/AdV, pNgn3/AdV. (**c**) PC1/3 and PC2 mRNA expression in untreated mouse pancreas (P), untreated mouse liver (L), or mice treated with pBS, pPdx-1, pNgn3, pBS/AdV, pPdx-1/AdV, pNgn3/AdV.

for the correction of hyperglycemia in STZ-treated mice, we repeated the co-delivery of pPdx1 or pNgn3 with AdVhFIX in immunodeficient RAG-1$^{-/-}$ mice made diabetic with STZ.[28] Interestingly, these mature B- and T-cell-functionally deficient mice did not show any improvement in hyperglycemia in response to co-administration of either pPdx-1 and AdVhFIX or pNgn3 and AdVhFIX (**Figure 7**). This suggested that the immune response elicited by the adenoviral capsid played a critical role in combination with Pdx-1 and Ngn3 in ameliorating the hyperglycemic state of the diabetic mice.

Gene expression in transduced livers

The livers of mice treated with pPdx-1, pNgn3, pPdx-1/AdV, and pNgn3/AdV were harvested, and total liver RNA was extracted for RT-PCR analyses to confirm transcription of the pancreatic transcription factors. As expected, exogenous Pdx-1 messenger RNA (mRNA) (ex Pdx-1) was detected in mice treated with pPdx-1, and exogenous Ngn3 mRNA (ex Ngn3) was detected in mice treated with pNgn3 (**Figure 8a**). Interestingly, endogenous Pdx-1 (en Pdx-1) expression was detected in all the liver samples taken from diabetic mice, but was not detected in the livers of untreated normoglycemic mice. Endogenous Ngn3 (en Ngn3) expression was only detected in mice treated with pNgn3, and only in mice treated with pPdx-1 and the irrelevant adenovirus, but not in mice treated only with pPdx-1, suggesting that the adenoviral cofactor was necessary for the activation of the transcription factors like Ngn3, which are postulated to be induced by Pdx-1 in the embryonic pancreas.[29]

RT-PCR analyses were also performed on the livers of mice treated with pPdx-1 or pNgn3 and the irrelevant adenovirus to determine whether expression of these genes in the liver induced insulin mRNA expression. Interestingly, insulin-1 and insulin-2 mRNA was detected in all the liver samples taken from STZ-treated diabetic mice (including mice not treated with Pdx-1 or Ngn3), but not in non-diabetic control mice (**Figure 8a**). This observation is consistent with studies by Kojima *et al.*[30] who reported that extra-pancreatic insulin mRNA expression was detectable in multiple organs, including the liver, in hyperglycemic mice and rats.

We examined the expression of the two key rate-limiting enzymes involved in gluconeogenesis, glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK), in the livers of mice treated with the various vectors, as a disruption of gluconeogenesis in the liver can also ameliorate hyperglycemia. RT-PCR analyses on the livers of mice treated with Pdx-1 or Ngn3 and the irrelevant adenovirus revealed similar amounts of both G6P and PEPCK compared with samples from control mice, suggesting that decreased gluconeogenesis was not a contributing factor in the reversal of hyperglycemia in mice treated with Pdx-1 or Ngn3 and the irrelevant adenovirus (**Figure 8b**).

To further investigate the role of adenovirus transduction in mediating increased insulin production in mice treated with pNgn3 or pPdx-1, we investigated whether expression of the prohormone convertases (PCs) was altered in response to adenovirus transduction in the liver. The PCs, PC1/3 and PC2, are normally expressed in pancreatic beta cells and are implicated in the processing of the weakly active proinsulin into its active mature form.[31,32] PC2 mRNA was detected by RT-PCR in the livers of all the diabetic mice. Interestingly, PC1/3 mRNA was also detectable in the livers of all the diabetic mice, but mRNA levels were elevated in the mice treated with adenovirus, suggesting that adenovirus transduction may be upregulating PC1/3 expression in the livers of diabetic mice (**Figure 8c**).

DISCUSSION

It was somewhat surprising that AAV8-mediated delivery of Pdx-1 and Ngn3 to the liver did not result in a correction of STZ-induced hyperglycemia in mice, given that previous studies using adenoviral vectors expressing Pdx-1 or NeuroD, a downstream factor of Pdx-1, were able to correct STZ-induced diabetes in mice by perhaps inducing liver cells to transdifferentiate into human beta-like cells.[12,14] Both adenoviral and AAV8 vectors are extremely efficient at transducing hepatocytes in mouse liver. However, the first-generation adenoviral vectors have also been shown to elicit a strong inflammatory response and even liver toxicity[33–35] whereas the helper-dependent adenoviral vectors have reduced immunogenicity owing to the removal of the adenoviral genes present in first-generation vectors, but the adenoviral capsid alone is still immunogenic.[27]

The initial studies carried out with Pdx-1 to the liver using first-generation adenoviral vectors by Ferber *et al.*[12] demonstrated a correction of STZ-induced hyperglycemia. In contrast, a later study performed by Kojima *et al.*[14] using a helper-dependent adenoviral vector to deliver Pdx-1 demonstrated severe liver toxicity and only a very transient correction of hyperglycemia, whereas delivery of NeuroD, a downstream factor of Pdx-1, along with betacellulin, a beta cell growth factor, were able to correct STZ-induced hyperglycemia without any toxicity. These differences were explained by the transient expression from first-generation adenoviruses, where the duration expression from helper-dependent adenoviruses was significantly prolonged owing to the lack of viral proteins, although our laboratory has shown that when the transgene sequences are the same there was no difference in the duration of expression between first-generation and helper-dependent adenoviral vectors.[26] Another study investigating the long-term effect of expressing Pdx-1 in the livers using transgenic mice demonstrated that Pdx-1 was able to initiate but not complete the conversion process, resulting in abnormal liver lobe structures and dysmorphogenesis.[15]

The duration of transgene expression from AAV vectors is indefinite; therefore, it was not entirely unexpected that AAV8-Ngn3 also resulted in hepatotoxicity and did not result in a correction of STZ-induced hyperglycemia. It appears likely that the duration of expression of Pdx-1 is critical in determining its effect in the adult liver, with extended expression being detrimental to liver function. In addition, the kinetics of expression from AAV8 vectors is typified by a slow rise in expression followed by stable expression after about 2 weeks, whereas expression from first-generation adenoviruses is typified by a rapid rise in expression within a few days, and a significant drop in expression within a week owing to the immune response.[36] Therefore, it may also be possible that the differing kinetics between adenoviral and AAV vector mediated transduction may also help explain the differences observed between adenoviral- and AAV-mediated delivery of Pdx-1. In contrast, delivery of the Pdx-1 gene by hydrodynamic transfection was also unable to correct hyperglycemia in diabetic mice suggesting the kinetics of transgene expression cannot explain these results because transgene expression from hydrodynamic transfection is similar to that of adenoviral transduction.

The principal finding of this study is that non-viral delivery of Pdx-1 or Ngn3 in combination with an irrelevant adenoviral vector expressing human factor IX could correct STZ-induced hyperglycemia in diabetic mice and amelioriate diabetes-induced weight loss. The mice treated with Pdx-1 or Ngn3 and AdVhFIX also exhibited elevated serum insulin levels, and improved glucose tolerance as compared with untreated diabetic mice. These findings suggest that adenoviral transduction is required for the correction of STZ-induced diabetes using the pancreatic transcription factors Pdx-1 and Ngn3 in the liver of mice.

Several possibilities exist to explain how adenovirus may be playing a role in the correction of STZ-induced diabetes using Pdx-1 and Ngn3 in the livers of mice. Based on our co-transfection studies with plasmids that express adenoviral genes and the inability to achieve correction of diabetes, we do not believe that an adenoviral gene is directly responsible. As we were able to achieve the same correction of diabetes by co-delivering Pdx-1 or Ngn3 with an irrelevant helper-dependent adenoviral vector, it lends further support that the adenoviral capsid works together with Pdx-1 or Ngn3 expression.

Adenovirus transduction is known to elicit a strong immune response and trigger a large cascade of immune molecules, one of which may be involved directly or indirectly in the presumed transdifferentiation event.[33,35] Indeed, STZ-treated immunodeficient RAG-1 null mice were not able to be corrected of diabetes using the Pdx-1 or Ngn3 and adenovirus combination, suggesting that an antigen-dependent immune response was required for the correction. Microarray studies performed on mice treated with adenovirus and AAV vectors show a dramatic difference in gene expression profiles, with adenovirus altering the expression of far more genes, many of which are involved in the immune response, as compared with AAV (not shown). In regard to mechanism, adenovirus was also shown to upregulate several transcription factors significantly, including Stat1 (signal transducer and activator of transcription 1) and the TATA-binding protein Abt1, either of which may be involved in upregulating a key cofactor involved in inducing islet neogenesis in the liver. The adenoviral co-activation requirement may represent a specific signaling pathway because we were unsuccessful in inducing normoglycemia in diabetic animals when carbon tetrachloride-induced inflammation was substituted for the adenoviral vector (not shown). More studies will be required to identify the pathway responsible.

Our studies also demonstrated the expression of insulin-1 and insulin-2 mRNA in the livers of STZ-treated diabetic mice, in agreement with a recent study by Kojima *et al.*[30] Even though low levels of insulin were detected in livers of these mice, only mice treated with pPdx-1 or pNgn3 and the irrelevant adenovirus were corrected of hyperglycemia, whereas the other STZ-treated diabetic mice remained severely hyperglycemic, suggesting that these low levels of insulin mRNA found in the livers of diabetic mice alone were not sufficient to correct STZ-induced hyperglycemia. We also demonstrated elevated expression of PC1/3 and PC2 in the liver by RT-PCR in the livers of diabetic mice and highly elevated PC1/3 expression in response to adenovirus transduction. This suggests that the role of adenovirus transduction was perhaps mediating the correction

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of hyperglycemia by increasing the conversion of proinsulin to insulin in the liver. Even though insulin is being produced in the livers of diabetic mice, the additional conversion of proinsulin into insulin by PC1/3 may be necessary to produce the biologically relevant levels of insulin necessary to restore normoglycemia in STZ-treated diabetic mice. Interestingly, Pdx-1 has also been proposed to regulate PC1/3 expression through FGFR1 signaling and expression of a dominant-negative form of Pdx-1 in INS-1 cells results in severely defective proinsulin conversion.[37] Therefore, expression of PC1/3 together with Pdx-1 or Ngn3 may be necessary to mediate normal proinsulin processing and insulin release from the liver.

Although our studies suggest that delivering Pdx-1 or Ngn3 to the livers of mice in combination with an irrelevant adenovirus leads to the correction of diabetes by inducing liver insulin production, we cannot entirely exclude that these mice may actually be recovering owing to insulin secretion from other tissues. It was recently demonstrated that several diabetic mouse models, including the STZ-induced diabetic mouse model, expressed insulin mRNA and protein in various tissues other than the pancreas.[30] It is possible that Pdx-1 and Ngn3 may also activate insulin gene expression from these extrapancreatic tissues, resulting in decreased blood glucose levels, although insulin secretion from these tissues would not be expected to be glucose dependent, as these organs do not possess the glucose-sensing mechanisms present in the pancreas and liver. Importantly, the distribution of AAV8 to non-hepatic tissues does occur but is similar to that achieved with adenoviral vectors.[18,38] However, plasmid transfection is more limited to hepatocytes in its distribution after hydrodynamic infusion, making non-hepatic insulin secretion much less likely.

In conclusion, we have demonstrated that adenovirus transduction is a necessary component in the correction of STZ-induced diabetes in mice by expression of the pancreatic transcription factors Pdx-1 and Ngn3 in the liver. It appears likely that an immune response to the viral capsid is a key component in inducing insulin expression in the liver in conjunction with Pdx-1 or Ngn3 expression. Our hypothesis is that the expression of Pdx-1 or Ngn3 may act by increasing proinsulin production in the liver and by regulating the expression of PC1/3. The upregulation of PC1/3 expression by adenoviral transduction in combination with Pdx-1 or Ngn3 expression results in the processing of proinsulin into mature insulin, resulting in the correction of hyperglycemia in STZ-treated diabetic mice. It remains to be determined which specific aspect of the immune response to adenoviral transduction is responsible for the observed effect as well as the specific-cell type in the liver responsible for insulin secretion. We believe this information is important for developing a liver-directed therapy for type I diabetes.

MATERIALS AND METHODS

Plasmid purification. Pdx-1 cDNA was PCR amplified from the plasmid pZL1-Pdx-1 (gift of C Wright) using primers 5′-GCGCAAGCT TATGAACAGTGAGGAG-3′ and 5′-GCGCAAGCTTTCACCGGGGTT CCTG-3′, and cloned into the *Hin*dIII site of expression plasmid p4.1e, which contains the elongation factor 1-alpha promoter. Ngn3 cDNA was cloned by RT-PCR of mouse pancreas total RNA using the Qiagen One Step RT-PCR Kit and primers 5′-GCGCAAGCTTAT GGCGCCTCATCCC-3′ and 5′-GGCCAAGCTTTCACAAGAAGTCTGA GAACACC-3′, and cloned into the *Hin*dIII site of p4.1e. For AAV production, the Pdx-1 and Ngn3 expression cassettes were excised from the p4.1e plasmid using *Not*I, and cloned into the *Not*I site of the shuttle plasmid pAAV. All plasmids used for injection or virus production were purified using the Qiagen EndoFree Plasmid Maxi Kit.

Virus production. AAV8-Pdx-1 and AAV8-Ngn3 were produced using a standard triple transfection method and packaged into AAV serotype 8 capsids using the plasmid R2C8, which contains the AAV serotype 2 Rep gene and AAV serotype 8 Cap gene. The AAVs were subsequently purified using cesium chloride density centrifugation and titered by quantitative dot blot analysis.

The recombinant adenoviral vector AdVhFIX (also called fgAdhFIX) was described earlier.[26] AdVhFIX was amplified from frozen stocks in 293 cells and purified by cesium chloride centrifugation and dialysis. The virus was quantitated by UV spectroscopy. HDADcFIX, which contained a canine factor IX expression cassette,[39] was produced using a suspension culture method,[40] and subsequently purified by cesium chloride centrifugation and dialysis.

Animal experiments. Male 8- to 10-week-old C57/Bl6 mice were made diabetic by injection with a single intraperitoneal injection of 150 mg/kg STZ dissolved in 0.1 M citrate buffer (pH 4.5). The following week, the mice were fasted for 6 h, blood was taken from blood extracted from the retroorbital plexus, and blood glucose levels were measured using a OneTouch Ultra Blood Glucose Monitor and OneTouch Ultra Test Strips (Lifescan, Milpitas, CA). Mice with fasting blood glucose levels of 300–600 mg/dl were considered diabetic, and selected for the experiments. Subsequent fasting blood glucose measurements were performed after a 6 h fast.

The AAVs were injected into the diabetic mice through the tail vein, in a total injection volume of 200 μl diluted with saline. Hydrodynamic transfections were performed by injecting the various plasmids diluted in saline in a total volume of 1.8–2.0 ml in under 10 s into the tail vein. Adenovirus (AdVhFIX) or helper-dependent adenovirus (HDADcFIX) was subsequently injected through the tail vein 24 h later, in a total volume of 200 μl diluted in Dulbecco's phosphate-buffered saline.

Functional studies. Glucose tolerance tests were performed on the mice by fasting them overnight, with access to water. Baseline blood and serum samples were taken, and the mice were subsequently injected intraperitoneally with a single bolus of 1.5 g/kg D-glucose dissolved in sterile water. Blood and serum samples were taken periodically up to 4 h post-injection. Blood glucose levels were determined immediately following sampling as described above, and serum was separated and stored at $-20°C$ until insulin determination. Insulin levels were determined by enzyme-linked immunosorbent assay using a Rat Ultrasensitive Insulin enzyme-linked immunosorbent assay Kit (Crystal Chem, Downers Grove, IL) using Mouse Insulin Standards.

Immunohistochemistry. Freshly harvested mouse livers were embedded in Tissue-Tek optimal cutting temperature and frozen on dry ice. The livers were then sectioned at 10 μM using a cryostat and fixed onto glass slides using 1.25% glutaraldehyde. The slides were then blocked with 10% normal goat serum and incubated with antibodies against insulin (Linco, St Louis, MO) diluted 1:500 in phosphate-buffered saline/1% bovine serum albumin/10% goat serum for 2 h. Secondary antibody was applied at a dilution of 1:500 for 30 min, and the ABC reagent (Vector Laboratories, Burlingame, CA) was applied for 30 min. The slides were developed by incubating with 0.06%

3,3′-diaminobenzidine and 0.01% H_2O_2 for 5 min, and counterstained using hematoxylin.

Gene expression studies. Total RNA was isolated from mouse pancreas and liver samples using the Qiagen RNEasy Protect Kit with the optional DNAse I digestion step. RT-PCR was performed using the Qiagen One-Step RT-PCR Kit and 0.5 μl of total RNA. Thirty-five cycles were used for amplification. The following primers were used for amplification:

β-Actin: 5′-TCTGTCAGGTCCCGGCCA/3′-TCTGTCAGGTCCCGGCCA

enNgn3: 5′-GCGCAAGCTTATGGCGCCTCATCCC/3′-GCCAAGCTTTCACAAGAAGTCTGAG

exNgn3: 5′-GCGCAAGCTTATGGCGCCTCATCCC/3′-GTCACAGGGATGCCACC

enPdx-1: 5′-ATGAACAGTGAGGAGCAGTACTACG/3′-GGAGCCCAGGTTGTCTAAAT

exPdx-1: 5′-AACCGTCGCATGAAGTG/3′-GTCACAGGGATGCCACC

Ins-1: 5′-ATGGCCCTGTTGGTGCACTTC/3′-TTAGTTGCAGTAGTTCTCCAGCTG

Ins-2: 5′-ATGCGGCTGTGGATGCGCTT/3′-CTAGTTGCAGTAGTTCTCCAGCTG

PC1: 5′-GATGGCTACACAGACAGC/3′-GGATCAGCCAAATCCACCAG

PC2: 5′-GACTGGTTCAACAGCCATGG/3′-TAGCCGTCACAGTTGCAGTC

G6P: 5′-GAAGGCCAAGAGATGGTGTGA/3′-TGCAGCTCTTGCGGTACATG

PEPCK: 5′-ATGTTCGGGCGGATTGAAG/3′-TCAGGTTCAAGGCGTTTTCC

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