Stable insulin-secreting ducts formed by reprogramming of cells in the liver using a three-gene cocktail and a PPAR agonist

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With the long-term aim of developing a new type of therapy for diabetes, we have investigated the reprogramming of liver cells in normal mice toward a pancreatic phenotype using the gene combination Pdx1, Ngn3, MafA. CD1 mice were rendered diabetic with streptozotocin and given a single dose of Ad-PNM, an adenoviral vector containing all three genes. Ad-PNM induced hepatocytes of the liver to produce insulin, and the blood glucose became normalized. But over several weeks, the insulin-positive cells were lost and the blood glucose rose back to diabetic levels. Simultaneous administration of a peroxisome proliferator-activated receptor agonist, WY14643, caused remission of diabetes at a lower dose of Ad-PNM and also caused the appearance of a population of insulin-secreting ductal structures in the liver. The insulin-positive ducts were stable and were able to relieve diabetes in the long term. We show that the effect of WY14643 is associated with the promotion of cell division of the ductal cells, which may increase their susceptibility to being reprogrammed toward a beta cell fate.

INTRODUCTION

During the last 10 years, several laboratories have reported procedures whereby cells of the liver can be induced to produce insulin by the overexpression of genes normally involved in pancreatic endocrine development. However, these procedures require high virus doses and can sometimes be difficult to replicate. Wang et al. have reported that the effect of viral infection per se is necessary for the effect, in addition to the genes carried by the viral vector.

Recently, we have shown that the three-gene combination of Pdx1, Ngn3 and MafA was able to induce the formation of insulin-secreting, glucose-sensitive ductal structures in the livers of immunodeficient mice. This three-gene combination was first introduced by Zhou et al. and represents a logical choice for stimulating the pancreatic endocrine development. In a normal embryo, Pdx1 is required for pancreatic bud outgrowth; Ngn3 is required for endocrine precursor cell formation; and MafA (and Pdk1 again) is required for β-cell maturation. In our study, we showed that the insulin-producing ductal structures were able to relieve experimentally induced diabetes in the long term and that the cell of origin was a SOX9-positive progenitor. For that work, we used immunodeficient (NOD-SCID) mice because of a perception that adenovirus-transduced cells are attacked by the immune system of immunocompetent animals. With immunodeficient animals, the procedure worked reliably, using a dose of viral vector that gave no significant liver damage.

However, from a therapeutic point of view, a procedure that only works in immunodeficient animals is of limited interest. Here, we show that the same therapeutic effect can be obtained in normal mice if they are also given the peroxisome proliferator WY14643. This compound, also known as pirinixic acid, is an agonist of both peroxisome proliferator-activated receptors α and γ and is known to cause liver hyperplasia. We show that when normal mice are made diabetic and are fed WY14643 around the time of administration of Ad-PNM, a long-term remission of the diabetes can be achieved. As in immunodeficient mice, this is owing to the appearance of insulin-producing duct-like structures. The WY14643 treatment also permits use of the virus at a dose that does not cause liver damage. The fact that normal mice can now be cured of diabetes using the combination of Ad-PNM and WY14346 opens the road to future clinical development of this type of approach for treatment of diabetes in humans.

RESULTS

Diabetes of normal mice can be relieved by the administration of Ad-PNM

Normal CD1 mice were injected with streptozotocin to induce diabetes. The adenoviral construct Ad-PNM, containing the three genes Pdx1, Ngn3 and MafA was given by a single injection to the tail vein. This resulted in a lowering of blood glucose levels back to normal levels by about a week after virus delivery. However, very high doses of virus (2 × 10¹⁰ p.f.u. per mouse) were required to achieve remission of diabetes. Moreover, the effect was not permanent. The blood glucose persisted at a low level for 6–7 weeks, and then it rose again, the time course varying somewhat between individual mice (Figure 1).

Examination of the livers of the rescued mice 1 week after Ad-PNM administration showed the presence of the vector-delivered gene products, pancreatic and duodenal homeobox 1 (PDX1), neurogenin 3 (NGN3) and MAFA, in many cells. The percentage of cells immunopositive for PDX1 was 32 ± 7.1%. PDX1 protein is detected in more cells than NGN3 or MAFA, but we believe this is due to the differing sensitivities of the antibodies used (Figures 2a–c, j–l). Cells (12 ± 2.8%) expressed insulin, and these were all also positive for the vector-encoded proteins.
Insulin-positive cells also contained C-peptide, indicating that they could synthesize and process insulin and are not simply concentrating it from the bloodstream (Figure 2g). However, they retained the overall shape of hepatocytes, and, like the normal hepatocytes around them, they also contained albumin (Figure 2h). After 6 weeks, the number of these cells was much reduced and they were no longer albumin-positive (Figure 2i). Although all cells expressing insulin also expressed the vector-delivered proteins, 64% of cells that were PDX1-positive at 1 week were not positive for insulin. In our previous work with immunodeficient mice, we described insulin-positive ductal structures induced by Ad-PNM, but in the present work, using Ad-PNM alone on normal mice, very few of these structures were seen. By 6–8 weeks, most of the vector-expressed proteins had been lost. This was directly observed by green fluorescence using the Ad-GFP vector (Supplementary Figure 1). In mice injected with Ad-PNM, after 6 weeks, NGN3 was no longer visible at all and PDX1 and MAFA expression was confined to the remaining insulin-positive cells (Figures 2d–f). We consider this to be endogenous and not virus-encoded, as endogenous expression was apparent on reverse transcription-polymerase chain reaction for the untranslated region of Pdx1 and outlasted the drop in expression of the vector-encoded Pdx1 (Supplementary Figure 2). The reverse transcription-polymerase chain reaction analysis of the livers of responding mice also revealed high levels of endogenous expression of several gene products characteristic of β-cell development or function. By 8 weeks, this new gene expression was much reduced, correlating with the time when the fasting blood glucose level was rising (Supplementary Figure 2 and Figure 1).

**Figure 1.** Feeding with peroxisome-proliferator-activated receptor agonist WY14643 improves the Ad-PNM effect on CD1 mice and rescues diabetes in the long term: (a) Time line of the different treatments done before and after Ad-PNM delivery. (b) CD1 mice are made diabetic with streptozotocin and treated with Ad-PNM. This causes a lowering of blood glucose, which is gradually lost. If WY14643 feeding is carried out around the time of the Ad-PNM dose, then the blood glucose remains normal for at least 12 weeks. The graph is a measure of the blood glucose of five animals in each group.

The proportion of cells labeled 1 day after an injection of EdU (Figures 3a–c and g). The mean increase of liver wet weight was 26.6 ± 3.3%. The overall histology of the liver was not affected (Figure 3d), and there was little increase of liver enzymes in the serum (Supplementary Figure 3), showing that damage to the liver was minimal. No hepatic tumors were seen in this work, and there is no additional increase of liver size due to Ad-PNM. In animals given Ad-PNM and WY14643, the cells becoming Ad-PNM-labeled comprised some hepatocytes and also cells lining bile ducts and some other cells in the periportal regions (Figure 3e). This mitogenic effect was short-lived. At 6 weeks after the Ad-PNM/WY14643 treatment, very little DNA labeling was seen following an EdU injection (Figures 3f and h).

If mice were injected with Ad-PNM during a 4-day period of feeding on a diet containing WY14643, a 20-fold lower virus dose (1 × 10^6 p.f.u. per mouse) produced a similar fall in blood glucose to that seen with 2 × 10^7 p.f.u. per mouse in its absence. Furthermore, the remission of diabetes was long-term, and the mice did not revert to hyperglycemia within the course of the study (12 weeks from the Ad-PNM dose) (Figure 1). In these animals, in addition to the scattered insulin-positive hepatocyte-like cells described above, were seen many insulin-positive duct-like structures (Figure 4), very similar to those previously seen when immunodeficient mice were treated with Ad-PNM. Incorporation of EdU into the insulin-positive ducts was apparent by 1 week after the Ad-PNM dose. At this stage, the ducts still expressed SOX9 as well as insulin (Figures 4a and b). Unlike the scattered insulin-positive cells, they do not contain any albumin and are obviously distinct from hepatocytes (Figure 4r). They contain C-peptide, indicative of endogenous insulin synthesis, but they also contain glucagon, somatostatin and PYY, hormones normally produced by other types of islet endocrine cell (Figures 4i–l). In general, the phenotype has features both of endocrine cells and of ducts. They are positive for ECAD, EpCAM, CD133 and CK19 but not for OV6 or albumin (Figures 4m–r).

Shortly after the Ad-PNM treatment, the ductal structures, as well as the scattered insulin-positive cells, were found to express the three vector-encoded proteins PDX1, NGN3 and MAFA (Figures 4c–e). By 6 weeks, the ductal structures had become larger and more evident, and the scattered insulin-positive hepatocyte-like cells had largely disappeared. At this stage, the insulin-positive ductal structures were still found to express PDX1 and MAFA but not NGN3 (Figures 4f–h), and the labeling following EdU injection was mostly negative, indicating a cessation of expansion (Figure 3f). Although WY14643 on its own is not particularly toxic, we found that when it was combined with Ad-PNM, a 6-day period of feeding often led to death, to which hypoglycemia was probably a contributing factor. So a 4-day feed was adopted as standard. In order to examine the occurrence of liver damage, we measured total bilirubin, alanine aminotransferase and aspartate transaminase in serum of treated mice of each treatment group. The levels were slightly elevated but not to a level indicating significant liver damage (Supplementary Figure 3).

To determine whether the mice were capable of glucose-sensitive insulin secretion, intraperitoneal glucose tolerance tests were performed, along with measurement of serum insulin. The glucose challenge showed that the Ad-PNM-treated mice without or with WY14643 had better glucose disposal than the diabetic controls (Figures 5a and b). In both experimental groups, the restoration of normoglycemia was slower at 6 weeks post-Ad-PNM than at 1 week. This may be owing to the loss of the scattered insulin-positive population over this period. The glucose clearance effect correlated with an elevation in the serum insulin level (Figures 5c and d), which was highest in the WY14643-treated group. However, neither glucose tolerance nor insulin secretion of the WY-14643 group was as good as that of the normal controls, which is consistent with the incomplete nature of the transformation described above.
Effects of immunosuppression

It is notable that the insulin-positive ducts persisted in the treated animals, whereas the scattered insulin-positive cells disappeared. To examine whether this was due to an immune process, sections were immunostained using F4/80, an antibody recognizing macrophages/Kupffer cells.16 Normal liver contains many of these cells (Figure 6e) and, following Ad-PNM treatment, their numbers increased in the vicinity of the insulin-positive hepatocytes (Figures 6a and j). A similar increase was seen following treatment with Ad-GFP (that is, adenovirus expressing just GFP, without the transcription factors) (Figures 6g–i, j). This indicates that the macrophage response is to the virus rather than the specific transcription factors used. Interestingly, the insulin-positive ducts were not invested by macrophages (Figure 6b). We found that the insulin-positive ducts produced the mucin MUC2, which has been shown in other studies to function as a barrier against immune attacks in the intestine17 (Figures 6c and d).

Because WY14643 makes the CD1 mice behave like the NOD-SCID mice in response to the effects of Ad-PNM, we considered that this might be due to an immunosuppressive effect of WY14643. To test this possibility, we examined Ad-PNM-treated mice that were given a daily dose of the immunosuppressant drug tacrolimus. CD1 mice were made diabetic with streptozotocin; then a daily dose of tacrolimus was commenced before the Ad-PNM injection and continued thereafter. Animals receiving tacrolimus contained few, if any, macrophages (Figure 6f). In these experiments, the tacrolimus did prolong the fall of blood glucose caused by the Ad-PNM and maintained a near-normal glycemia in the long term (Figure 6k). However, the mechanism appeared quite distinct from that of WY14346. Analysis of the tacrolimus-treated livers revealed very few insulin-positive ducts. Most of the insulin-positive cells visible after 6 weeks were, instead, small round cells. These resemble oval cells, which appear following treatment of rodents with carcinogens and which are capable of forming either hepatocytes or biliary epithelial cells.18–20 Unlike the insulin-positive ducts seen with WY14643, these cells did stain positive with the OV6 antibody, which is a marker for oval cells21 (Figures 6l–m).
DISCUSSION

Our most important new result is the discovery that the peroxisome-proliferator-activated receptor agonist WY14643 modifies the response of normal mice to Ad-PNM so that insulin-positive ducts arise in significant numbers in the liver. These are stable to immune attack, perhaps because of their production of MUC2. This means that reliable long-term control of STZ-induced diabetes can be achieved for normal animals.

Our results also show that there is a considerable difference in response to Ad-PNM between immunodeficient (NOD-SCID) and normal (CD1) mice. In the absence of WY14643, diabetic CD1 mice require a high virus dose to relieve the diabetes, and the response consists mostly of the appearance of scattered insulin-positive cells in the liver parenchyma that resemble hepatocytes and gradually disappear, concurrently with the gradual rise of blood glucose back to the diabetic level. By contrast, NOD-SCID mice require a lower virus dose, and, in addition to some scattered insulin-positive cells, the Ad-PNM generates the insulin-positive duct-like structures that persist long-term and display glucose-sensitive insulin secretion.8

Mechanism of effect

In preliminary experiments, separate viruses containing Pdx1, Ngn3 and MafA were used, but they were much less effective than the combined vector, Ad-PNM, so we believe that co-expression of the three genes is necessary. We attribute both types of insulin-positive cells induced in the liver to the effect of the Ad-PNM and not the streptozotocin. Although ectopic insulin production has previously been described in response to STZ treatment alone,22 we saw none at all in our STZ-treated controls. We cannot, however, exclude some role in the overall process of the STZ treatment or the diabetic condition itself.

The scattered insulin-positive cells initially expressed albumin as well as insulin (Figure 2h), indicating that they retained aspects of the hepatocyte phenotype. By 6 weeks, the few scattered insulin-positive cells remaining were not positive for albumin, indicating a shift in phenotype away from that of the hepatocyte. in vitro studies have shown a higher degree of reprogramming than this,23 but this is probably on account of the propensity of hepatocytes to de-differentiate in vitro even without any other treatment.24 A higher degree of reprogramming is also seen in experiments on immature livers,25 in which the liver transcription factors are less tightly coupled than in adult life.26

Expression of all three vector-encoded proteins is seen 1 week after treatment, but by 6 weeks, the expression was lost from most cells and only Pdx1 and MafA were retained in the insulin-positive cells. We consider this long-term retention of PDX1 and MAF to represent endogenous expression for several reasons. First, after 6 weeks, the vector had mostly disappeared, based on the observation of Ad-GFP-treated animals (Supplementary Figure 1 and Figure 6i); second, some endogenous Pdx1 expression is seen using reverse transcription-polymerase chain reaction (Supplementary Figure 2); and third, normal mature β-cells would be expected to express PDX1 and MAF, but not NGN3.19 As the three genes make up a single transcription unit in the vector, we would not expect to lose just one of the three proteins from vector-encoded synthesis.

The most obvious effect of WY14643 is to increase the cell division rate in the liver. In animals given both Ad-PNM and WY14643, the cells becoming EdU-labeled comprised some hepatocytes and also the SOX9-positive cells lining bile ducts

Figure 3. Peroxisome-proliferator-activated receptor agonist WY14643 causes transient cell proliferation of the liver. (a, b) Increase of EdU-labeled cells in livers of normal mice fed with WY14643. (c) The liver size increases upon WY14643 feeding. (d-f) shows mice treated with Ad-PNM and WY14643. (d, e and h) staining shows no obvious damage to the liver. (e) EdU labeling shows proliferation of cells, particularly in the bile ducts. (f) After 6 weeks, almost no cells in the ducts are labeled, following an injection of EdU. The arrow shows the presence of two EdU-positive cells. CV = central vein; D = duct. Scale bars 100 μm. The results are representative of at least three different samples. (g) Increase of cell division caused by WY14643. (h) EDU labeling index for ductal structures after 1 week and 6 weeks after WY14643 feeding. Error bars represent standard errors.

Figure 4. Characterization of insulin-positive ducts in diabetic CD1 mice treated with Ad-PNM and WY14643. (a) EdU labeling (green) of SOX9-positive cells (lilac). (b) Transformed ductal structures are positive for insulin (red) and EdU at 1 week (green). (c-e) Transformed insulin-positive ductal structures express PDX1, NGN3 and MAF 1 week after Ad-PNM delivery (insulin: red; transcription factors: green). (f-h) The ductal structures still express PDX1 and MAF, but not NGN3, 6 weeks after Ad-PNM delivery. D = duct. Scale bars 100 μm, each horizontal set is of the same magnification. The results are representative of at least three different samples. (i-r) Characterization of the insulin-positive ductal structures at 6 weeks. In each case, insulin is red and the named product is green. (i) C-peptide. (j) Somatostatin. (k) Glucagon. (l) PY. (m) E-cadherin (ECAD). (n) Cytokeratin 19 (CK19). (o) EpCAM. (p) CD133. (q) OV6 (negative). (r) Albumin (negative). (s, t) Counts of insulin-positive cells in the liver parenchyma and in the ducts at 1 and 6 weeks after Ad-PNM delivery. At 1 week, more insulin-positive cells are found in the liver parenchyma than in ducts, but these cells are lost whereas those in the ducts persist. Error bars are standard errors.
and some other cells in the periportal region that may possibly include progenitor cells capable of forming both hepatocytes and bile ducts\textsuperscript{27,28} (Figure 3e). Proliferation involves DNA synthesis, which requires temporary disassembly of chromatin. Thus, genes that are normally in closed chromatin will become transiently accessible to the action of the three introduced transcription factors.
factors. Reprogramming of the cell type by overexpression of transcription factors has been studied most intensively in the generation of induced pluripotent stem cells.\textsuperscript{29–31} In this procedure, only a small fraction of cells expressing the four transcription factors become induced pluripotent stem cells. The fraction can be increased by strategies to open up closed chromatin, which enables the introduced transcription factors to find their targets in the DNA and to upregulate the genes that will lead to a new stable state of gene expression. This opening of the chromatin can be produced by small molecules,\textsuperscript{22} macromolecular agents\textsuperscript{33} or the use of modified transcription factors with chromatin penetration or ‘pioneer’ qualities.\textsuperscript{34}

We conclude that the WY14643 treatment renders the SOX9-positive cell population more susceptible to the effects of Ad-PNM by inducing proliferation and thereby making the target genes more accessible. EdU studies carried out at later stages in the protocol (6 weeks) showed that there was by then little labeling of the insulin-positive ductal structures (Figure 3f). This suggests that these structures grow for a while following the reprogramming process and then stop.

Although it is natural to suppose that the difference between NOD-SCID and CD1 mice arises from the immune deficiency of the former, we think it unlikely that WY14643 acts by immune suppression. We found that a daily dose of tacrolimus, sufficient to suppress the macrophage investment of transduced cells, does prolong the remission of diabetes but does not bring about the formation of the insulin-positive ducts.

Diabetes remission

We have previously shown that there is no regeneration of $\beta$-cells in the pancreas over the time course of our experiments,\textsuperscript{8} and as the expression of Ad-PNM is overwhelmingly in the liver, it may be concluded that the combined activity of the scattered insulin-positive cells and the insulin-positive ducts induced in the liver is responsible for secreting the insulin and thereby achieving the remission of diabetes. However, the fact that the diabetes was relived does not on its own prove that the transformed cells are necessarily glucose-responsive. A slow release insulin pellet, which releases insulin in an unregulated manner, can also relieve experimental diabetes. We consider the induced insulin-positive ducts to be glucose-responsive because of the increase of serum insulin following a glucose challenge (Figures 5c and d). Moreover, in our previous work, we directly showed that cells isolated from the livers demonstrated glucose-sensitive insulin secretion \textit{in vitro}.\textsuperscript{7}

In an earlier work, using the gene combination Ngn3 and $\beta$-cellulin, Yechoor \textit{et al.}\textsuperscript{6} showed two phases of insulin secretion in the liver—the first due to the expression of the insulin gene in hepatocytes and the second due to the permanent reprogramming of progenitor cells. Although with their gene combination, Yechoor \textit{et al.} did not obtain the characteristic insulin-positive ducts, we do concur with their overall interpretation. In CD1 mice, upon Ad-PNM delivery, adenovirus-encoded proteins are detectable in hepatocytes for a few weeks, along with the induction of insulin expression by the hepatocytes. However, by 6–8 weeks, the normalization of blood glucose levels has been lost, along with most of the insulin expression in the liver. We have previously been uncertain whether their loss is on account of the loss of the virus DNA (adenovirus is non-integrating) or the immune destruction of the cells, or both. The present results indicate that both effects occur. The loss of Ngn3 from the scattered insulin-positive cells indicates that the virus DNA has largely been lost after 6 weeks, but the loss of the scattered insulin-positive cells themselves indicates a process of cell removal, probably owing to the immune system.

When the CD1 mice are given Ad-PNM along with WY14643 feeding, by 1 week, we see two types of insulin-positive cells—the scattered as well as the ductal. But by 6 weeks, we could observe that most of the scattered insulin-positive cells have been lost, and the ductal insulin-positive cells are left as the predominant population (Figures 4i–r). This is consistent with the maintenance of blood glucose levels over a longer period of time, compared with the mice given Ad-PNM alone (Figure 1). The absence of

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\caption{Diabetic CD1 mice treated with Ad-PNM/WY14643 show improved glucose tolerance and insulin secretion. (a, b) Blood glucose and serum insulin levels in response to glucose challenge, 1 week after Ad-PNM delivery. (c, d) Blood glucose and serum insulin levels in response to glucose challenge, 6 weeks after Ad-PNM delivery. The graphs show the response from five animals in each group, except for the PNM + tacrolimus groups, which had three animals. The control and diabetic groups in A and C are the same animals.}
\end{figure}
macrophage infiltration of the insulin-positive ducts (Figure 6b) indicates that they are more resistant to an immune attack than are the scattered cells. This may be on account of their expression of MUC2, which is a major mucin, also expressed in the normal adult intestine. In the intestine, the mucins are known to form a gel barrier and protect the body’s mucosal surfaces from pathogens and from immunological attack.\textsuperscript{17,35,36} Thus, the expression of MUC2 in the insulin-positive ducts may explain how they can persist long-term and continue to relieve diabetes in immunocompetent mice.

**Figure 6.** Macrophages in the livers of treated animals. (a) Presence of numerous macrophages, immunostained for F4/80 (green), in the vicinity of scattered insulin-positive cells (red). This mouse was made diabetic and treated with Ad-PNM alone. (b) Absence of macrophages from the immediate vicinity of insulin-positive ducts. (c, d) show the same section stained for insulin (red) and for MUC2 (green) and F4/80 (lilac). (d) These mice were made diabetic and treated with Ad-PNM + WY14643. (e, f) Treatment with tacrolimus eliminates macrophages from the liver. (e) Macrophages visualized using F4/80 (green) in normal CD1 mouse liver. (f) Absence of macrophages after tacrolimus treatment. (g–i). Macrophage number is elevated in Ad-GFP-treated mouse liver. (g, h) Ad-GFP-treated mouse liver after 1 week, GFP green, F4/80 red. (i) After 6 weeks, most green cells are lost. (j) Increase in the number of F4/80 cells following Ad-PNM or Ad-GFP administration. This shows F4/80 cells as a percentage of all cells in a standard area. Error bars are standard errors. (k) Tacrolimus prolongs the remission of diabetes caused by Ad-PNM. Arrows indicate the day of administration of STZ and Ad-PNM. (l, m) Appearance of insulin-positive cells in diabetic mice treated with Ad-PNM and tacrolimus, 8 weeks after Ad-PNM delivery. Insulin is red, and OV6, a marker of oval cells, is green. (n) OV6-positive cells are rare in a control liver. CV = central vein; D = duct. Scale bars 100 μm. The results are representative of at least three different samples.
Future implications

WY14643 is considered a somewhat toxic compound, and its use is restricted to laboratory experimentation. But a similar pharmacological effect is exerted by the fibrate drugs, widely used in human medicine.37 The present results indicate that the method is now effective and reproducible, and it is possible that fibrates could be used to sensitize the patient so that acceptable doses of viral vector could be employed. Under these circumstances, we can expect the reprogramming of progenitor cells in the liver to eventually become an acceptable option for diabetes therapy in humans.

MATERIALS AND METHODS

Adenovirus vector

The polycistronic construct Ad-PNM is a first generation adenoviral vector containing coding regions for mouse Pdx1, Ngn3 and Mafa, separated by 2A sequences and driven by a CAGS promoter. Its construction and preparation were described by Akinci et al. Ad-GFP was prepared as described by Dutton et al.

Animal procedures

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Diabetes was induced in mice with an intraperitoneal injection of streptozotocin (Sigma, St Louis, MO, USA or Teva Pharmaceutical Industries Ltd., North Wales, PA, USA) This drug works by destroying the beta cells of the pancreas.40 The dose was of 180 mg kg\(^{-1}\) of body weight, and blood glucose was monitored every 4 days using an Accu-Chek glucose meter (Roche, Indianapolis, IN, USA). Mice showing blood glucose levels in the range of 400–600 mg dl\(^{-1}\) over at least 10 days were considered as diabetic and were used for experiments.

For treatment with Ad-PNM, a volume of the \(10^9\) p.f.u. ml\(^{-1}\) stock (that is, \(2 \times 10^{10}\) p.f.u. per mouse) was injected into each of the diabetic mice by tail vein injection. A lower dose of \(10^7\) of the \(10^9\) p.f.u/ml stock (that is, \(1 \times 10^9\) p.f.u. per mouse) was injected into WY14643-treated or tacrolimus-treated mice. As long as the injection was successful, all treated mice responded with a fall in blood glucose. Ad-GFP was also administered at \(1 \times 10^9\) p.f.u. per mouse.

For WY14643 treatment, diabetic mice were fed with WY14643 (Cyman Laboratories, Ann Arbor, MI, USA) for 4–6 days. WY14643 was dissolved in acetone (Sigma) and mixed into the normal powdered chow diet at a dose of 10 mg kg\(^{-1}\) of mouse food. The acetone was allowed to evaporate before use. Feeding was started 2–4 days prior to the delivery of Ad-PNM and continued for 2 more days after delivery.

Tacrilmus is a macroline immunosuppressant that acts by inhibiting dephosphorylation of the transcription factor NF-AT and thereby decreases the activation of various cytokines.41,42 For tacrolimus experiments, diabetic mice were given a daily intraperitoneal injection of tacrolimus (Fisher Scientific or Astellas Pharma USA) at a dosage of 2.5–5.0 mg kg\(^{-1}\) of body weight and blood glucose was monitored every 4 days using an Accu-Chek glucometer (Roche, Indianapolis, IN, USA). Mice showing blood glucose levels in the range of 400–600 mg dl\(^{-1}\) over at least 10 days were considered as diabetic and were used for experiments.

For an analysis of liver enzymes, blood was collected from the optic vein (that is, 100 ml of blood were collected from the liver) and centrifuged for 20 min. The serum was separated from the clots and spun at 700 \(\times\) 120 min after glucose administration. Also, serum was collected from the liver (that is, 100 ml of liver preparation were described by Akinci et al. Ad-GFP was prepared as described by Dutton et al.

For the cell counts, at least three equal areas were counted for each slide, each containing more than 100 cells. Results are expressed as mean \(\pm\) standard error.

Reverse transcription–polymerase chain reaction

Mice that responded to Ad-PNM, as detected by monitoring of the blood glucose levels, were killed, and the liver was removed. Total RNA was isolated from the liver using Trizol. DNase-treated total RNA was used for the first-strand cDNA synthesis. This reaction was performed using SuperScript II and Oligo-dT (Invitrogen), following the manufacturer's protocol. cDNA samples were subjected to PCR amplification with specific primers under linear conditions in order to reflect the original amount of the specific transcript. The cycling parameters were as follows: denaturation at 94 °C for 1 min, annealing at 55–60 °C for 1 min (depending on the primer) and elongation at 72 °C for 1 min (35 cycles). The PCR primers and the length of the amplified products are listed in Table S1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Ferber S, Hallkin A, Cohen H, Ber I, Einav Y, Goldberg I et al. Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 2000; 6: 568–572.
2 Ber I, Shenhall K, Perl S, Olanuna Z, Goldberg I, Barshack I et al. Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem 2003; 278: 31950–31957.
3 Miyaitsu T, Kaneto H, Kitomyo T, Hirota S, Arawaka Y, Fujitani Y et al. Ectopically expressed PD9-1 in liver initiates endocrine and exocrine pancreas differentiation but causes dysmophogenesis. Biochem Biophys Res Comm 2003; 310: 1017–1025.
4 Kojima H, Fujimya M, Matsumura K, Younan P, Imaeda H, Maeda M et al. NeuroD-beta-cellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 2003; 9: 596–603.
5 Kaneto H, Nakatani Y, Miyaitsu T, Imaeda H, Matsui A, Morii M et al. PDX-1/PV16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. Diabetes 2005; 54: 1009–1022.
6 Yechoor V, Liu V, Espiritu C, Paul A, Oka K, Kojima H et al. Neurogenin3 is sufficient for transdifferentiation of hepatic progenitor cells into neo-islets in vivo but not transdifferentiation of hepatocytes. Dev Cell 2009; 16: 358–373.
7 Wang AY, Ehrhardt A, Xu H, Kay MA. Adenovirus transduction is required for the correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. Mol Ther 2007; 15: 255–263.

8 Banza A, Akinci E, Greder LV, Dutton JR, Slack JMW. In vivo reprogramming of Sox9+ cells to the liver in insulin-secreting ducts. Proc Natl Acad Sci USA 2012; 109: 15336–15341.

9 Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008; 455: 627–632.

10 Gittes GK. Developmental biology of the pancreas: a comprehensive review. Dev Biol. 2009; 326: 4–35.

11 Yang Y, Nunes FA, Berenci K, Furth EE, Gonzelo E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci 1994; 91: 4407–4411.

12 Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W. The PPAR transactivation domain of MyoD.

13 Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliever SA. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J Biol Chem 1997; 272: 3406–3410.

14 Cohen AJ, Grasso P. Review of the hepatic response to hyperlipidaemic drugs in rodents and assessment of its toxicological significance to man. Food Cosmet Toxicol 1981; 19: 585–605.

15 Ledwith BJ, Johnson TE, Wagner LK, Pauley CJ, Manam S, Galloway SM et al. Correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. Cell Metab 2012; 15: 885–894.

16 Xin S, Walton G, Aoki R, Brondell K, Schug J, Fox A et al. Fox1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential. Genes Dev 2011; 25: 1193–1203.

17 McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. Nat Rev Microbiol 2011; 9: 265–278.

18 Shin S, Walton G, Aoki R, Brondell K, Schug J, Fox A et al. Fox1-Cre-marked adult hepatic progenitors have clonogenic and bilineage differentiation potential. Genes Dev 2011; 25: 1193–1203.

19 Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci USA 2003; 100: 11881–11888.

20 Duncan AW, Dorrill C, Grompe M. Stem cells and liver regeneration. Gastroenterology 2009; 137: 466–481.

21 Horb ME, Shen CN, Tosh D, Slack JMW. Experimental conversion of liver to pancreatic exocrine cells using Pdx1, Ngn3 and MafA. Cell Metab 2011; 238: 1349–1361.

22 Singhal N, Graumann J, Wu G, Arauzo-Bravo MJ, Han DW, Greber B et al. Chromatin-remodeling components of the BAF complex facilitate reprogramming. Cell 2010; 141: 943–955.

23 Shiyber D, Brosco P, Do JT, Ahn HS, Scholer HR, Ding S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds. Cell Stem Cell 2008; 25: 473–481.

24 McKeehan WL. Aspects of the hepatic response to hyperlipidaemic drugs in rodents and assessment of its toxicological significance to man. Food Cosmet Toxicol 1981; 19: 585–605.

25 Kubicek S, O'Sullivan R, August EM, Hickey ER, Zhang Q, Teodoru ML et al. Reversal of HSK99962 by a small-molecule inhibitor for the G9a histone methyltransferase. Mol Cell Biol 2007; 27: 255–263.

26 Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008; 455: 627–632.

27 Peters DH, Fitton A, Plosker GL, Faulds D. Tacrolimus—a review of its immunosuppressive activity. Drugs 1993; 46: 746–794.

28 Singhal N, Graumann J, Wu G, Arauzo-Bravo MJ, Han DW, Greber B et al. Chromatin-remodeling components of the BAF complex facilitate reprogramming. Cell 2010; 141: 943–955.

29 Andersson OS, Adams BA, Yoo D, Ellis GC, Gut P, Anderson RM et al. Adenosine signaling promotes regeneration of pancreatic β-cells in vivo. Cell Metab 2012; 15: 885–894.

30 Haumaitre C, Lenoir O, Kellner S, Karian P, Firpo M et al. Radical acceleration of nuclear reprogramming by chromatin remodeling with the transactivation domain of MyoD. Stem Cells 2011; 29: 1349–1361.

31 Horn P, Sollert C, Stuhr T, Ahn HS, Scholer HR, Ding S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds. Cell Stem Cell 2008; 25: 473–481.

32 Singhal N, Graumann J, Wu G, Arauzo-Bravo MJ, Han DW, Greber B et al. Chromatin-remodeling components of the BAF complex facilitate reprogramming. Cell 2010; 141: 943–955.

33 Accelrys SD. Reimaging of cells in the liver. A Banga et al.

34 Llacer J, Gudou D, Ribeiro A, Zhang Q, Teodoru ML et al. Reversal of HSK99962 by a small-molecule inhibitor for the G9a histone methyltransferase. Mol Cell Biol 2007; 27: 255–263.

35 Peters DH, Fitton A, Plosker GL, Faulds D. Tacrolimus—a review of its immunosuppressive activity. Drugs 1993; 46: 746–794.

36 Brazelton TR, Morris RE. Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: Tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr Opin Immunol 1996; 8: 710–720.

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