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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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Abstract

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 towards 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 PPAR 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 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 for reprogramming towards a beta cell fate.

Keywords

Pdx1; Neurogenin3; MafA; WY14643; insulin; diabetes

Introduction

In the last ten 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 [1–6]. However, these procedures require high virus doses and can sometimes be difficult to replicate. Wang et al. have reported that the effect of virus infection per se is necessary for the effect, in addition to the genes carried by the viral vector [7].

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Conflict of Interest Statement

AB, LVG, JRD, JMWS have no commercial associations that might create a conflict of interest in connection with this manuscript.

Supplementary Information

Supplementary information is available at Gene Therapy's website.

Recently we showed that the three gene combination *Pdx1, Ngn3* and *MafA* was able to induce the formation of insulin-secreting, glucose-sensitive ductal structures in the livers of immunodeficient mice [8]. This three gene combination was first introduced by Zhou et al. [9] and represents a logical choice for stimulating pancreatic endocrine development. In the normal embryo *Pdx1* is required for pancreatic bud outgrowth, *Ngn3* for endocrine precursor cell formation, and *MafA* (and *Pdx1* again) for β-cell maturation [10]. In our study we showed that the insulin-producing ductal structures were able to relieve experimentally induced diabetes long term and that the cell of origin was a SOX9-positive progenitor [8]. 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 [11]. 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 receptor (PPAR) α and γ, and is known to cause liver hyperplasia [12, 13, 14, 15]. 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 due to the appearance of insulin-producing duct-like structures. The WY14643 treatment also permits use of the virus at a dose which 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 administration of *Ad-PNM*

Normal CD1 mice were injected with streptozotocin (STZ) 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 in blood glucose levels back to normal levels by about a week after virus delivery. However, very high doses of virus ($2x10^{10}$ pfu/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 (Fig 1).

Examination of the livers of the rescued mice one week after *Ad-PNM* administration showed the presence of the vector-delivered gene products, PDX1, 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 sensitivity of the antibodies used. (Fig 2A–C, J–L). 12±2.8 % of cells 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 (Fig 2G). However, they retained the overall shape of hepatocytes, and, like the normal hepatocytes around them, they also contained

albumin (Fig. 2H). After 6 weeks, the number of these cells was much reduced and they were no longer albumin-positive (Fig. 2I). Although all cells expressing insulin also expressed the vector-delivered proteins, 64% of cells PDX1-positive at one week were not positive for insulin. In previous work with immunodeficient mice we described insulin-positive ductal structures induced by *Ad-PNM* [8], 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 *Ad-GFP* vector (Suppl. Fig. 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 (Fig 2D–F). We consider this to be endogenous not virus-encoded, as endogenous expression was apparent on RT-PCR for the 3′UTR of *Pdx1* and outlasted the drop in expression of the vector-encoded *Pdx1* (Suppl. Fig. 2). The RT-PCR 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 is rising (Suppl. Fig. 2 and Fig. 1).

WY14643 modifies the response to *Ad-PNM* by inducing SOX9-positive cell proliferation and formation of insulin-positive ductal structures

WY14643 has been described previously as causing liver hyperplasia [14, 15]. We confirmed that feeding for 4–6 days did cause an increase in the size of the liver, and an increase in the proportion of cells labeled one day after an injection of EdU (Fig. 3A,B,C,G). The mean increase of liver wet weight was 26.6±3.3%. The overall histology of the liver was not affected (Fig. 3D), and there was little increase of liver enzymes in the serum (Suppl. Fig. 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 EdU-labeled comprised some hepatocytes and also cells lining bile ducts and some other cells in the periportal regions (Fig. 3E). This mitogenic effect was short lived. Six weeks after the *Ad-PNM*/WY14643 treatment, very little DNA labeling was seen following an EdU injection (Fig. 3F, H).

If mice were injected with *Ad-PNM* during a 4 day period of feeding on a diet containing WY14643, a twentyfold lower virus dose ($1x10^9$ pfu/mouse) produced a similar fall in blood glucose to that seen with $2x10^{10}$ pfu/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) (Fig. 1). In these animals, in addition to the scattered insulin-positive hepatocyte-like cells described above, were seen many insulin-positive duct-like structures (Fig. 4), very similar to those previously seen when immunodeficient mice were treated with *Ad-PNM* [8]. Incorporation of EdU into the insulin-positive ducts was apparent at 1 week after the *Ad-PNM* dose. At this stage the ducts still expressed SOX9 as well as insulin (Fig. 4A,B). Unlike the scattered insulin-positive cells, they do not contain any albumin and are obviously distinct from hepatocytes (Fig. 4R). They contain C-peptide, indicative of endogenous insulin synthesis, but also contain glucagon, somatostatin, and PYY, hormones normally produced by other types of islet endocrine cell

(Fig. 4I–L). In general the phenotype has features both of endocrine cells and of ducts. They are positive for ECAD, EpCAM, CD133, CK19 but not for OV6 or albumin (Fig. 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 (Fig. 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 (Fig. 4F–H), and the labeling following EdU injection was mostly negative indicating a cessation of expansion (Fig. 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 control for 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 (Suppl. Fig. 3).

To determine if 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 glucose disposal better than the diabetic controls (Fig. 5A,B). In both experimental groups the restoration of normoglycemia was slower at 6w post-*Ad-PNM* than at 1 week. This may be due to the loss of the scattered insulin-positive population over this period. The glucose clearance effect correlated with an elevation in serum insulin level (Fig. 5C,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 normal controls, 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 disappear. To examine whether this was due to an immune process, sections were immunostained for F4/80, a marker of macrophages/Kupffer cells [16]. Normal liver contains many of these cells (Fig. 6E) and, following *Ad-PNM* treatment, their numbers increased in the vicinity of the insulin-positive hepatocytes (Fig. 6A,J). A similar increase was seen following treatment with *Ad-GFP* (i.e adenovirus expressing just GFP, without the transcription factors) (Fig. 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 (Fig. 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 attack in the intestine [17] (Fig. 6C,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 (Fig. 6F). In these experiments the tacrolimus did prolong the fall of blood glucose caused by the *Ad-PNM* and maintained a near normal glycemia long term (Fig. 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 cells [21] (Fig. 6L–M).

Discussion

Our most important new result is the discovery that the PPAR agonist WY14643 modifies the response of normal mice to *Ad-PNM* such 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, which resemble hepatocytes and which gradually disappear concurrent 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 which 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 did not see any at all in our STZ-treated controls. We cannot however exclude some role in the overall process for the STZ treatment or the diabetic condition itself.

The scattered insulin-positive cells initially expressed albumin as well as insulin (Fig. 2H) indicating that they retain 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 the hepatocyte. In vitro studies have shown a higher degree of reprogramming than this [23] but this is probably due to 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 liver [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 one 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 MAFA to represent endogenous expression for several reasons. First, after 6 weeks the vector has mostly disappeared based on observation of *Ad-GFP* treated animals (Suppl. Fig. 1 and Fig. 6I), secondly, some endogenous *Pdx1* expression is seen by RT-PCR (Suppl. Fig. 2), and thirdly, normal mature β-cells would be expected to express PDX1 and MAFA but not NGN3 [10]. Since 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 and some other cells in the periportal region which may possibly include progenitor cells capable of forming both hepatocytes and bile ducts [27, 28] (Fig. 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. Reprogramming of cell type by overexpression of transcription factors has been studied most intensively in the generation of induced pluripotent stem cells (iPS cells) [29–31]. In this procedure only a small fraction of cells expressing the four transcription factors become iPS 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 chromatin can be produced by small molecules [32], macromolecular agents [33], or the use of modified transcription factors with chromatin penetration or “pioneer” qualities [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 (Fig. 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 β-cells in the pancreas over the time course of our experiments [8], and as the expression of *Ad-PNM* is overwhelmingly in the liver, it is 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 relieved 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 that the induced insulin-positive ducts are glucose-responsive because of the increase of serum insulin following a glucose challenge (Fig. 5C,D). Moreover in our previous work we showed directly that cells isolated from the livers demonstrated glucose-sensitive insulin secretion *in vitro* [8].

In earlier work using the gene combination *Ngn3* and *β-cellulin*, Yechoor et al. showed two phases of insulin secretion in the liver, the first due to expression of the *insulin* gene in hepatocytes and the second due to permanent reprogramming of progenitor cells [6]. Although with their gene combination Yechoor 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 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 due to loss of the virus DNA (adenovirus is non-integrating) or to immune destruction of the cells, or both. The present results indicate that both effects are occurring. 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 due 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 (Fig 4I–R). This is consistent with the maintenance in blood glucose levels over a longer period of time compared to the mice given *Ad-PNM* alone (Fig 1). The absence of macrophage infiltration of the insulin-positive ducts (Fig. 6B) indicates that they are more resistant to immune attack than the scattered cells. This may be due to 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 [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.

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 such that acceptable doses of viral vector could be employed. Under these circumstances we can expect reprogramming of progenitor cells in the liver eventually to 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 was described by Akinci et al. [38]. *Ad-GFP* was prepared as described by Dutton et al. [39].

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 (STZ) (Sigma or Teva Pharmaceutical Industries Ltd.) This drug works by destroying the beta cells of the pancreas [40]. The dose was 180mg/kg body weight and blood glucose was monitored every four days with an Accu-Chek glucose meter (Roche). Mice showing blood glucose levels in the range from 400–600mg/dl over at least 10 days were considered as diabetic and were used for experiments.

For treatment with *Ad-PNM*, a volume of 200μl of the 10^{11} pfu/ml stock (i.e $2x10^{10}$ pfu/mouse) was injected into each of the diabetic mice by tail vein injection. A lower dose of 100μl of the 10^{10} pfu/ml stock (i.e $1x10^{9}$ pfu/mouse) was injected in WY14643-treated or tacrolimus-treated mice. So long as the injection was successful, all treated mice responded with a fall in blood glucose. *Ad-GFP* was also administered at $1x10^{9}$ pfu/mouse.

For WY14643 treatment, diabetic mice were fed with WY14643 (Cayman Laboratories) for 4–6 days. WY14643 was dissolved in acetone (Sigma) and mixed into the normal powdered chow diet at a dose of 10mg/kg 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 two more days after delivery.

Tacrolimus is a macrolide 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 US) at a dosage of 2.5 – 5.0mg/kg, beginning three days prior to *Ad-PNM* administration and continuing each day thereafter for the duration of the experiment.

For cell division studies, mice were injected intraperitoneally with 40μl of 100mM 5-ethynyl-2′-deoxyuridine (EdU, Invitrogen) one day prior to sacrifice. The liver tissue was fixed in 10% formalin buffered with PBS (Fischer) washed, soaked in 15% sucrose solutions overnight and frozen in optimal cutting temperature compound (OCT). Sections of 8–10μm were stained to visualize the number of EdU labeled cells using a Click-iT EdU Assay kit (Invitrogen) according to the manufacturer's instructions.

For analysis of liver enzymes, blood was collected from the optic vein of differently treated mice groups. The blood was then analyzed by the University of Minnesota College of

Veterinary Medicine Pathology Laboratories for aspartate transaminase, alanine aminotransferase and bilirubin concentrations.

The blood glucose levels in the *Ad-PNM* injected mice were monitored every four days or once every week as needed, along with measurements of body weight. A glucose tolerance test was perfomed after 4 weeks for the different groups of mice. They were made to fast for 16hrs followed by an intraperitoneal injection of a glucose solution (2gm/kg body weight). Blood glucose levels were measured at 0, 30, 60 and 120 mins after glucose administration. Also serum was collected from the mice at the indicated time points. The blood was allowed to clot and was spun at 3K for 20 mins. The serum was separated from the clots and frozen at −80C. Serum insulin levels were then measured using an Ultrasensitive ELISA kit for mouse (Alpco) according to the manufacturer's instructions.

Immunohistochemistry

Livers were fixed in 10% formalin buffered with PBS (Fischer) for 2 hours or overnight at room temperature, washed and soaked in 15% sucrose solutions overnight and were either frozen in OCT embedding compound or embedded in paraffin. Sections of 5–7μm were permeabilized with 0.2% Triton-X 100 for 20 mins, blocked in 10% goat or sheep serum, and incubated with the appropriate primary antibody overnight at 4°C. Then they were washed in PBS and treated with secondary antibody for 1hr to visualize the required immunofluorescence staining. The primary antibodies used were insulin 1:200 (Abcam), PDX1 1:1000 (Millipore), SOX9 1:5000 (Millipore), NGN3 1:100 (Santa Cruz), MAFA 1:100 (Santa Cruz), Somatostatin and Glucagon 1:250 (Dako), PYY 1:100 (Acris Antibodies), F4/80 1:100 (e-Biosciences), OV6 1: 100 (R& D Systems), CD-133 1:100 (Millipore), EpCAM 1:100 (Santa Cruz), ECAD 1:250 (eBiosciences), CK19 1:100 (Santa Cruz), C-Peptide 1:100 (Cell Signaling Technologies), Albumin 1:100 (Bethyl Laboratories). Secondary antibodies used were: anti-rabbit Alexa 488, 633 and 594, anti-mouse Alexa 488, anti-goat Alexa 488, anti guinea-pig Alexa 594 at 1:500 (Molecular Probes).

For the cell counts, at least three equal areas were counted for each slide, each containing >100 cells. Results are expressed as mean ± standard error.

Reverse transcription–polymerase chain reaction (RT-PCR)

Mice that responded to *Ad-PNM*, as detected by monitoring of the blood glucose levels, were sacrificed, and the liver 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 minute, annealing at 55–60°C for 1 minute (depending on the primer), and elongation at 72°C for 1 minute (35 cycles). The PCR primers and the length of the amplified products are listed in Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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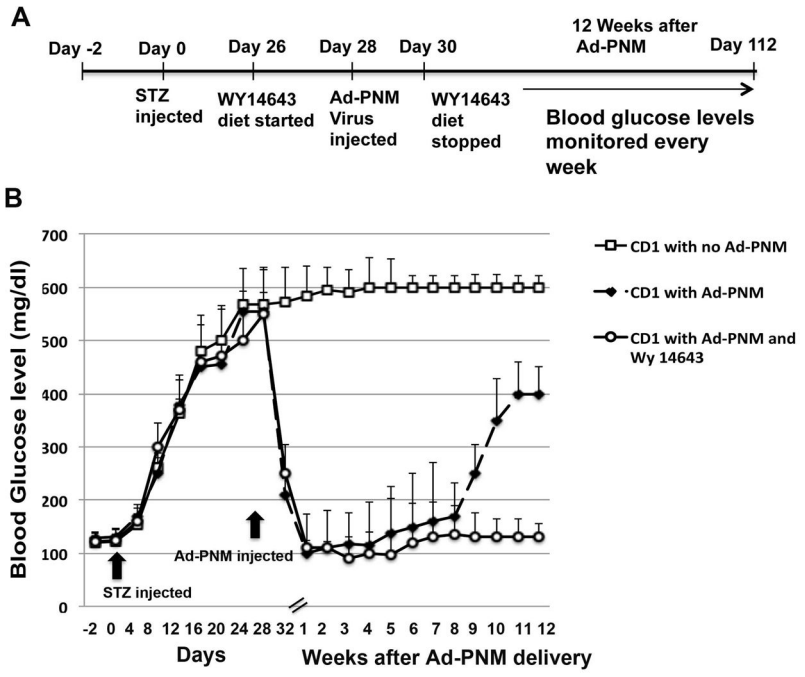

Figure 1. Feeding with PPAR agonist WY14643 improves the *Ad-PNM* effect on CD1 mice and rescues diabetes long term

A. Time line of the different treatments done before and after *AD-PNM* delivery. **B.** CD1 mice are made diabetic with streptozotocin (STZ) 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 blood glucose from five animals in each group.

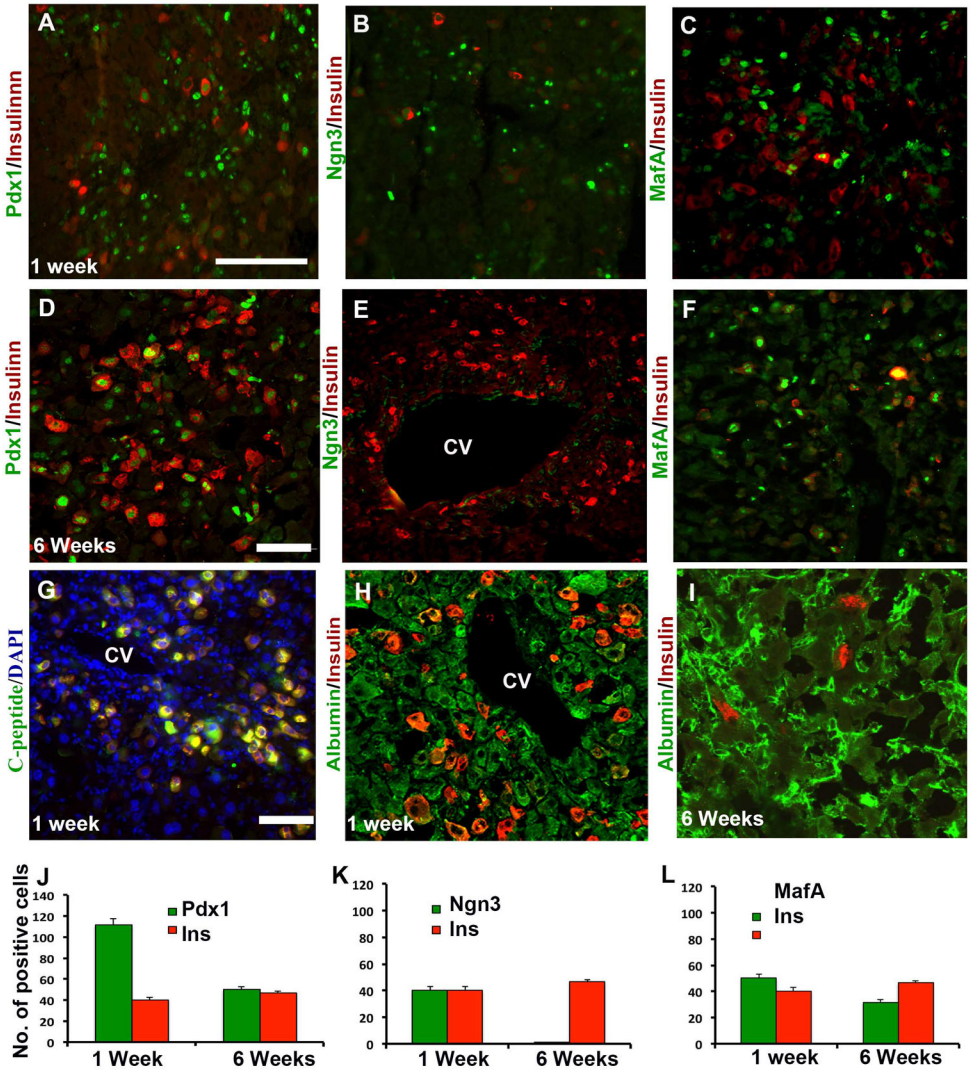

Figure 2. ***Ad-PNM*** **treatment of diabetic CD1 mice induces insulin expression by hepatocytes**

Immunostaining of livers from CD1 mice made diabetic and treated with *Ad-PNM*. **A,B,C.** After 1 week there are scattered insulin-positive cells which also express the adenoviral encoded PDX1, NGN3 and MAFA. **D, E, F.** 6 weeks after *Ad-PNM* infection NGN3 has been lost and there is persistent PDX1 and MAFA expression only in the insulin-expressing cells. **G, H.** The insulin-positive cells are also positive for C-peptide and albumin at 1 week. **I.** The insulin positive cells at 6 weeks are not positive for albumin. **J, K, L.** The histograms show the number of cells positive for insulin and the virus encoded PDX!, NGN3 and MAFA at 1 week and 6 weeks after *Ad-PNM* delivery. These represent counts on areas of the specimens equivalent to the fields shown. Error bars represent standard errors. CV=central vein. Scale bars 100μm.

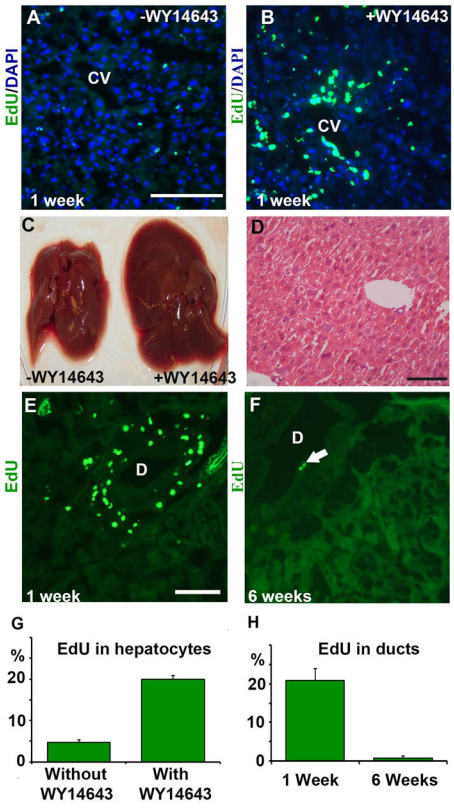

Figure 3. PPAR agonist WY14643 causes transient cell proliferation of the liver

A,B. Increase of EdU labeled cells in livers of normal mice fed with WY14643. **C.** The liver size is increased upon WY14643 feeding. **D–F** shows mice treated with *Ad-PNM* and WY14643. **D.** H & E staining shows no obvious damage in the liver. **E.** EdU labeling shows proliferation of cells particularly in the bile ducts. **F.** After 6 weeks almost no cells in the ducts label following an injection of EdU. The arrow shows the presence of two EdU positive cells. CV=central vein; D=duct. Scale bars 100μm. The results are representative of at least three different samples. **G.** Increase of cell division caused by W14643. **H.** EdU labeling index for ductal structures after 1 week and 6 weeks. Error bars represent standard errors.

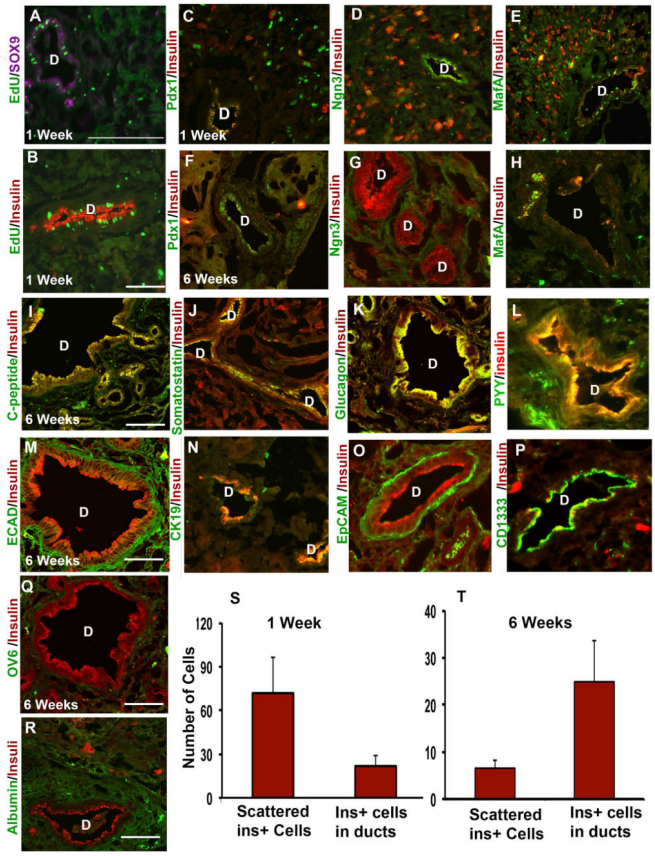

Figure 4. Characterization of Insulin-positive ducts in diabetic CD1 mice treated with *Ad-PNM* and WY14643

A. EdU labeling (green) of SOX9-positive cells (lilac). **B.** Transformed ductal structures are positive for insulin, and EdU at 1 week (green). **C–E.** Transformed insulin-positive ductal structures express PDX1, NGN3 and MAFA 1 week after *Ad-PNM* delivery (insulin red; transcription factors green). **F–H.** The ductal structures still express PDX1 and MAFA, but not NGN3, 6 weeks after *Ad-PNM* delivery. D=duct. Scale bars 100μm, each horizontal set is 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 green. **I.** C-peptide. **J.** Somatostatin. **K.** Glucagon. **L.** PYY. **M.** E-caherin (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 one and six 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 while those in the ducts persist.. Error bars are standard errors.

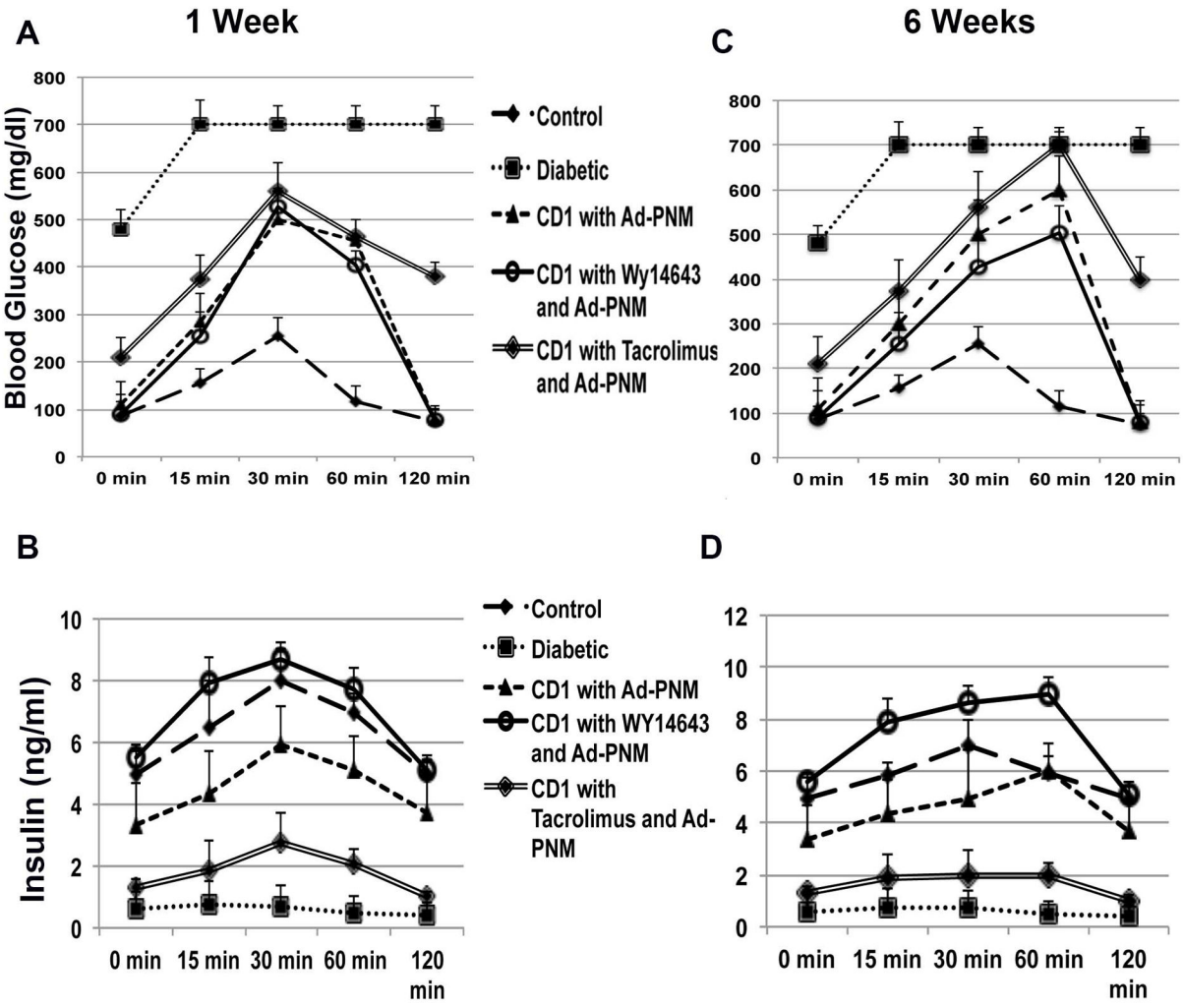

Figure 5. Diabetic CD1 mice treated with *Ad-PNM*/WY14643 show improved glucose tolerance and insulin secretion

A,B. Blood glucose and serum insulin levels in response to glucose challenge, one week after *Ad-PNM* delivery. **C,D**. Blood glucose and serum insulin levels in response to glucose challenge, six weeks after *Ad-PNM* delivery. The graphs show response from 5 animals in each group except the PNM+tacrolimus groups which had 3 animals. The control and diabetic groups in A and C are the same animals.

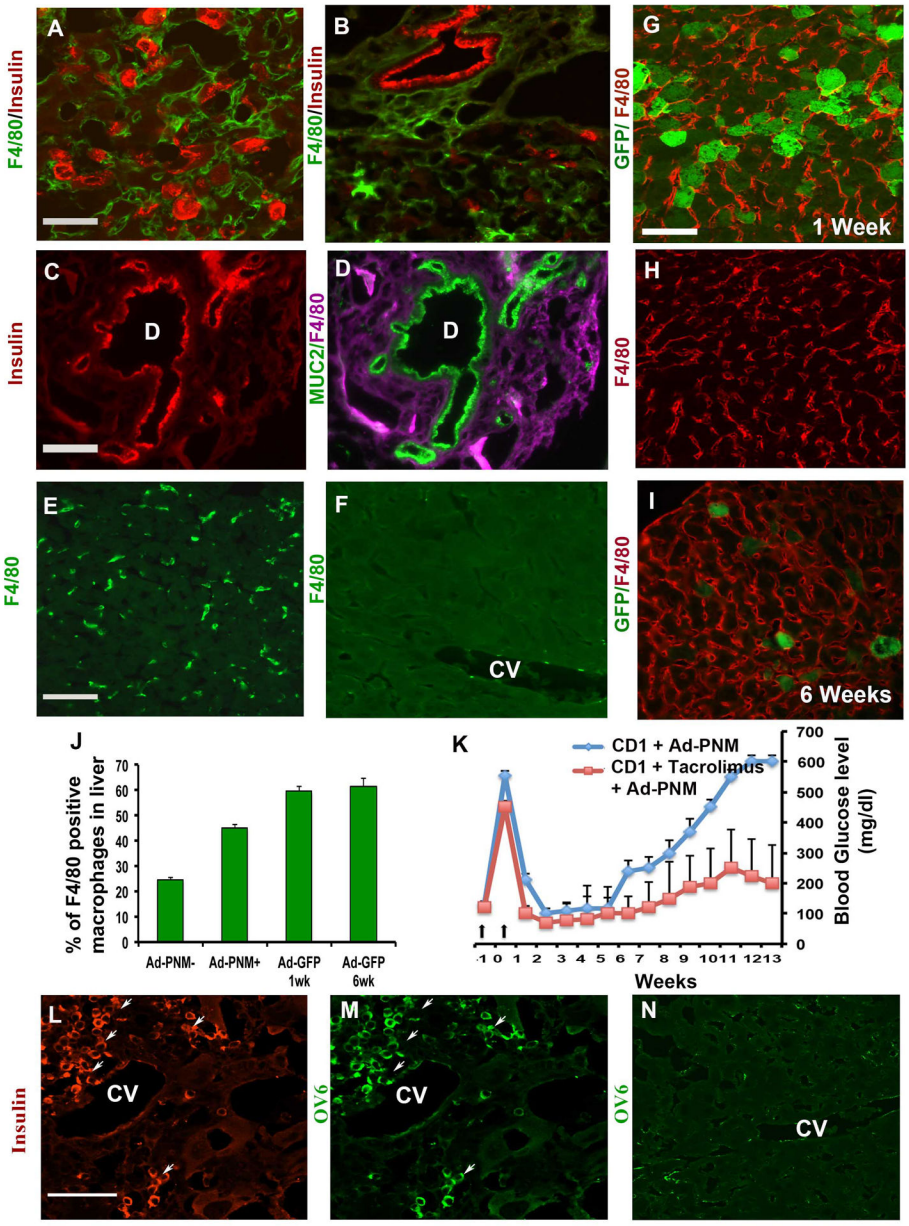

Figure 6. Macrophages in the livers of treated animals

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) (C) 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 with 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, 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 100μm. The results are representative of at least three different samples.