**Abstract**

**Background:** Pancreatic duodenal homeobox-1 (Pdx-1) or Pdx-1-VP16 gene transfer has been shown to induce in vitro rat liver-stem WB cell conversion into pancreatic endocrine precursor cells. High glucose conditions were necessary for further differentiation into functional insulin-producing cells. Pdx-1 has the ability to permeate different cell types due to an inherent protein transduction domain (PTD). In this study, we evaluated liver-to-pancreas conversion of WB cells following Pdx-1 or Pdx-1-VP16 protein transduction.

**Findings:** WB cells were grown in high glucose medium containing Pdx-1 or Pdx-1-VP16 recombinant proteins for two weeks. β-like cell commitment was analysed by RT-PCR of pancreatic endocrine genes. We found that WB cells in high glucose culture spontaneously express pancreatic endocrine genes (Pdx-1, Ngn3, Nkx2.2, Kir6.2). Their further differentiation into β-like cells expressing genes related to endocrine pancreas development (Ngn3, NeuroD, Pax4, Nkx2.2, Nkx6.1, Pdx-1) and β-cell function (Glut-2, Kir6.2, insulin) was achieved only in the presence of Pdx-1(-VP16) protein.

**Conclusion:** These results demonstrate that Pdx-1(-VP16) protein transduction is instrumental for in vitro liver-to-pancreas conversion and is an alternative to gene therapy for β-cell engineering for diabetes cell therapy.

**Background**

The difficulties encountered in obtaining sufficient supply of transplantable β-cells is a major problem in cell therapy of type I diabetes. Liver may be a potential source of cells for β-cell engineering. Indeed, liver and pancreas derive from the same endodermal region during embryogenesis [1] and hepatocytes and β-cells share similar built-in glucose-sensing systems.

Among transcription factors involved in pancreatic β-cell specification, Pdx-1 plays a central role. All progenitors of the endocrine as well as the exocrine pancreas express...
Pdx-1 [2,3]. In the adult, Pdx-1 expression is mainly repressed to β-cells where it regulates important β-cell functions like insulin transcription. Several in vitro studies, using viral or stable plasmid gene transfer, show that Pdx-1 expression in hepatic cells results in reprogramming into insulin producing cells [4-7]. Fusion of Pdx-1 to the VP16 activation domain from Herpes simplex virus (Pdx-1-VP16) leads to more efficient liver-to-pancreas conversion than Pdx-1 alone [8-12]. Stable mouse VP16) leads to more efficient liver-to-pancreas conversion [17]. Up to now, no study using protein transduction has achieved liver-to-endocrine pancreas conversion [17].

Safety of gene therapy remaining a prime concern, protein transduction offers a more secure alternative to induce stem cell differentiation. Indeed, protein transduction domains (PTD) allow proteins to translocate across the cytoplasmic membrane. Due to an Antennapedia-like PTD in its structure, Pdx-1 protein can permeate different cell types and induces insulin expression in pancreatic ducts [15,16]. However, in human embryonic stem cells, the adjunction of a PTD domain derived from the HIV Tat protein (TAT) is necessary for Pdx-1 cell transduction [17]. Up to now, no study using protein transduction has achieved liver-to-endocrine pancreas conversion in vitro.

Here, we evaluate if Pdx-1(-VP16) proteins have the capacity to induce a pancreatic endocrine shift in WB cells. To achieve this, we treat WB cells with Pdx-1 or Pdx-1-VP16 proteins, containing their own PTD, or fused to the PTD of TAT.

**Methods**

**Recombinant proteins synthesis**

Full-length mouse Pdx-1 and Pdx-1-VP16 open reading frames were cloned into pET28b-TAT-v2-1 expression plasmid containing the HIV Tat protein PTD (kind gift from S. Dowdy), in order to construct Pdx-1, Pdx-1-VP16, TAT-Pdx-1 and TAT-Pdx-1-VP16. PTD<sub>Pdx-1</sub>-eGFP was constructed by fusing the PTD of Pdx1 (RHKWFQNNRMK-WKK) to eGFP and subsequent cloning into pET28b-TAT-v2-1. Tat-eGFP was constructed by insertion of eGFP into pET28b-TAT-v2-1. eGFP was cloned into pET21a(+) expression vector (Novagen, WI, USA). See Additional data 1 for more details.

Recombinant proteins were produced according to Studier's method of auto-induction [18]. Proteins were purified by Ni<sup>2+</sup> affinity chromatography on Proteino Ni-TED resin (Macherey-Nagel, France), dialyzed against Centricron-Plus-20 centrifugal filter devices (Millipore, France), and stored at -20°C in PBS/pH8.0/25%glycerol.

**Luciferase assay**

The RIP2-reporter gene was constructed by cloning the [-683 bp, +11 bp] 5' flanking region of rat insulin-II gene into the EcoRV-site of pGL4.10 [luc2] (firefly luciferase) (Promega, France).

18 × 10<sup>3</sup> HepG2 cells/well were seeded onto a 96-well plate and grown 24 h in 10% FCS DMEM (5% CO<sub>2</sub>, 37°C). 0.12 μg of RIP2-reporter and 0.13 μg of HSV-TK-hRluc control vector (renilla luciferase, pGL4-74, Promega) were co-transfected in these cells using JetPEL reagent (Polyplus-Transfection, France). 12 h after transfection, the medium was replaced with medium containing 5 μM protein or storage buffer for negative controls. 36 h later, cells were assayed for luciferase activities using Dual-Glo-System (Promega) and a scintillation counter (MicroBeta-Trilux, Wallac/Perkin-Elmer, France).

**WB cell culture and protein treatment**

WB-F344 cells, kindly provided by N. Malouf [19], were grown in low glucose (LG) medium: 10% FCS RPMI-1640 (11 mM glucose, Invitrogen, France) (5% CO<sub>2</sub>, 37°C).

To evaluate transduction efficiency, 50 × 10<sup>3</sup> WB cells/well were seeded onto a 24-well plate. 24 h later, the medium was replaced with fresh medium containing 15 μM eGFP, PTD<sub>Pdx-1</sub>-eGFP, or Tat-eGFP proteins and incubated for another 24 h before confocal microscopy analysis.

To evaluate effects of Pdx-1(-VP16) proteins, 25 × 10<sup>3</sup> WB cells/well (passage 15) were seeded onto a 96-well plate with medium adjusted to HG concentration (25 mM D-glucose, Sigma-Aldrich). 12 h later, the medium was replaced by HG medium containing 1 μM protein (Pdx-1 (n = 4), Pdx-1-VP16 (n = 5), or TAT-Pdx-1-VP16 (n = 5)) or storage buffer for HG control cells (n = 5). The medium was replaced every 3–4 days. Control cells in LG medium (n = 5) were also grown. To favour differentiation rather than proliferation, WB cells were treated for 2 weeks without being trypsined. Then mRNAs were collected and analysed by RT-PCR. Each sample (n) represents one culture well.

**Laser scanning confocal microscopy**

Cells were treated 10 minutes with trypsin/EDTA, washed with PBS, stained with Vybrant-CM-Dil (1:200 dilution in PBS, Molecular Probes), and washed twice. eGFP signal (λ<sub>exc</sub> 488 nm, λ<sub>em</sub> 507 nm) and CM-Dil staining (λ<sub>exc</sub> 553 nm, λ<sub>em</sub> 570 nm) were examined by laser scanning confocal microscopy (Nikon TE-2000, France).

**RT-PCR analysis**

mRNAs were isolated with the Dynabeads-mRNA-Direct Kit (Invitrogen). First-strand cDNA was synthesized using M-MuLV reverse transcriptase (Promega) and random
proteins into cells.

Whereas LG control cells expressed proteins induced the expression of insulin and pancreatic-Pdx-1, Pdx-1-VP16, TAT-Pdx-1 and TAT-Pdx-1-VP16 activity in an equal manner as PTDPdx-1 alone. Pdx-1 fusion to PTDs in their structure, increased insulin promoter activity consistent with previous findings [10]. VP16 activation domain does not further enhance insulin transduction into hepatic WB and HepG2 cell lines. Intra-cellular localization of fusion proteins revealed by confocal microscopy analysis indicate a true uptake of proteins and not mere adherence to the cell surface. Furthermore, transduced recombinant Pdx-1, TAT-Pdx-1, Pdx-1-VP16 and TAT-Pdx-1-VP16 proteins exert biological activity on an insulin promoter reporter system. Pdx-1 fusion to VP16 activation domain does not further enhance insulin promoter activity consistent with previous findings [10]. Our results confirm reports of Pdx-1 transduction [15,20] and are the first demonstration of Pdx-1-VP16 protein transduction.

Pancreatic differentiation experiments were conducted in an HG environment. In fact, long-term HG culture further liver cell commitment towards a pancreatic fate [4,6,13,14,21]. Previous studies of Pdx-1(-VP16) expression in WB cells do not distinguish between respective contributions of transgene expression and HG culture on differentiation. Here, we show that HG culture alone converts WB cells into pancreatic endocrine precursor cells. In contrast to LG cultures, HG cultures express four pancreatic endocrine genes: Ngn3, Nlx2.2, Pdx-1 and Kir6.2. These results concur with Yang et al. study, where confluent culture of hepatic oval stem cells for 2 months in HG medium induces conversion into insulin-producing cells [21]. Kir6.2 expression is at odds with previous reports where Kir6.2 was detected in WB cells overexpressing Pdx-1 or Pdx-1-VP16 genes only (i) after 2–3 months in HG culture [14], (ii) 40 days post-transplantation into diabetic mouse [13], or (iii) after Pax4 co-expression [22].
Figure 1
**PTDPdx-1 and TAT fusion protein synthesis and their ability to transduce HepG2 and WB cells.** (a) Schematic structure (top panel) and purity (bottom panel) of fusion proteins. PTDPdx-1 and TAT represent protein transduction domains of the Pdx-1 protein and the HIV TAT protein, respectively. His represents hexahistidine tag used to purify proteins by His-tag affinity chromatography. Purified proteins were run on a SDS-PAGE gel (8%) stained with Coomassie blue. Molecular weights are 28 kDa for eGFP, 31 kDa for PTDPdx-1-eGFP and TAT-eGFP. (b) Observation by confocal microscopy of eGFP fluorescence in HepG2 and WB cells treated for 24 hours with 15 μM PTDPdx-1-eGFP, TAT-eGFP, or eGFP protein lacking PTD. Treated cells were observed by confocal microscopy without being fixed in order to exclude artifactual protein uptake [24]. CM-Dil was used to visualize cytoplasmic membrane (red staining). Scale bars = 50 μm.
Pdx-1, TAT-Pdx-1, Pdx-1-VP16, or TAT-Pdx-1-VP16 proteins further induce expression of NeuroD, Pax4, Nkx6.1, insulin 1 and Glut-2 after two weeks of treatment. Some samples express just a part of these markers or display RT-PCR expression patterns similar to HG controls. Quantitative analysis would help to further nuance pancreatic gene expression in-between these samples, in particular up-regulation of the endogenous Pdx-1 gene. The heterogeneity between samples may be a consequence of: (i) infrequent liver to endocrine pancreas conversion, leading to few pancreatic gene positive cells which may be difficult to detect, (ii) different kinetics of gene expression between wells. According to Tang et al. study, our experiments do not reveal more efficient differentiation following Pdx-1-VP16 protein treatment compared to Pdx-1 protein treatment [14]. TAT-mediated transduction does not lead to more advanced differentiation suggesting that containing two PTDs (PTD<sub>pdx-1</sub> and TAT) does not increase transduction efficiency. Surprisingly, insulin 1 and Pax4, two of the pancreatic genes expressed after our protein treatments, are not detected in a previous in vitro study on WB cells transduced with Pdx-1 or Pdx-1-VP16 genes, even after 3 months of HG culture [14]. Here, despite insulin 1 expression, neither insulin 2 nor glucokinase are detected pointing at the possible need for long-term culture in HG medium to obtain mature β-like cells [14]. Moreover, Ngn3 expression in wells scoring positive for all other pancreatic genes, including insulin 1, suggests the presence of remaining subpopulations of immature pancreatic precursor cells.

Overexpression of Pdx-1 or Pdx-1-VP16 genes in hepatic cells leads to exocrine as well as a range of endocrine cell types [4,7,8,13,14]. In our study, Pdx-1(-VP16) or TAT-Pdx-1(-VP16) protein treatments result in expression of PP, but not of glucagon suggesting that Pdx-1(-VP16) transduction in hepatic stem-like cells may lead to endocrine β-cell and non-β-cell phenocopies. Amylase,
Gene expression profile of WB-cells treated with Pdx-1(-VP16) or TAT-Pdx-1(-VP16) proteins in high glucose culture. RT-PCR analysis was performed after two weeks of protein treatment. cDNA from rat pancreas served as control for pancreatic gene expression, and cDNA from rat liver served as positive control for albumin expression (*). Each sample is represented by a single column and corresponds to an independent biological repeat of the experiment (LG (n = 5), HG (n = 5), Pdx-1 (n = 4), TAT-Pdx-1 (n = 4), Pdx-1-VP16 (n = 5), TAT-Pdx-1-VP16 (n = 5)). Each gene is represented by a single row of coloured boxes. Black colouring represents RT-PCR positive samples, whereas white colouring represents negative samples. LG: Low Glucose control WB cells; HG: High Glucose control WB cells. (See additional data 3 for original electrophoresis gel images).
expressed in rat liver or exocrine pancreatic cells, is detected in all conditions tested here [23].

Recently, Koya et al. demonstrated that Pdx-1 protein delivery into diabetic mice restores euglycemia mainly through pancreatic β-cell regeneration. The authors observed β-cell gene expression and insulin synthesis in the pancreas and in the liver of treated mice suggesting hepatic insulin contribution to euglycemia [20]. In complement, our findings provide the first direct evidence that Pdx-1(-VP16) protein transduction in conjunction with HG culture reprograms hepatic stem-like cells into cells displaying similarities with β-cells in vitro. At a point where strategies for targeted β-cell differentiation begin to surface, our study illustrates how simple exposition to Pdx-1(-VP16) protein in the surrounding medium triggers short-term pancreatic endocrine conversion. This study may contribute to the development of protein transduction therapy, a new concept to induce β-cell differentiation.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JC, SB and VL contributed to protein synthesis. LD and SB carried out confocal microscopy imaging. JC and VL performed luciferase assay, cell culture, RT-PCR analysis and drafted the manuscript. All authors contributed to the design of the study and interpretation of data. All authors read and approved the final manuscript.

Additional material

Additional file 1

Supplementary materials and methods.

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Additional file 2

List of primer information for RT-PCR.

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Additional file 3

Electrophoresis gel of RT-PCR analysis of WB cells treated with Pdx-1(-VP16) proteins.

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