TAT-Mediated Neurogenin 3 Protein Transduction Stimulates Pancreatic Endocrine Differentiation In Vitro

Juan Domínguez-Bendala,[1] Dagmar Klein,[1] Melina Ribeiro,[1] Camillo Ricordi,[1] Luca Inverardi,[1] Ricardo Pastori,[1] and Helena Edlund[1,2]

Stem cell technologies hold great potential for the treatment of type 1 diabetes, provided that functional transplantable β-cells can be selectively generated in an efficient manner. Such a process should recapitulate, at least to a certain extent, the embryonic development of β-cells in vitro. However, progress at identifying the transcription factors involved in β-cell development has not been accompanied by a parallel success at unraveling the pattern of their instructive extracellular signals. Here we present proof of principle of a novel approach to circumvent this problem, based on the use of the HIV/TAT protein transduction domain. Neurogenin 3 (ngn3), a factor whose expression is essential for pancreatic endocrine differentiation, was fused to the TAT domain. Administration of TAT/ngn3 to cultured pancreatic explants results in efficient uptake, nuclear translocation, and stimulation of downstream reporter and endogenous genes. Consistent with the predicted activity of the protein, e9.5 and e13.5 mouse pancreatic explants cultured in the presence of TAT/ngn3 show an increased level of endocrine differentiation compared with control samples. Our results raise the possibility of sequentially specifying stem/progenitor cells toward the β-cell lineage, by using the appropriate sequence and combination of TAT-fused transcription factors.
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Islet transplantation has proven successful for the treatment of type 1 diabetes (1,2), but the shortage of donor pancreata has hindered the widespread clinical implementation of this therapy. Therefore, it is essential to find additional sources of islets. Human embryonic stem cells may present one promising alternative for the in vitro generation of islet cells. For this prospect to be realistic, however, we need to identify the appropriate conditions that will favor differentiation of islet cell types. Ideally, such conditions should reproduce as accurately as possible the sequence of events that results in islet formation during embryogenesis. Although little is known about the first of such events (endodermal specification), subsequent steps in pancreatic development have been associated with the timed expression of key transcriptional factors, such as insulin promoter factor-1 (Ipf1)/pancreatic and duodenal homeobox factor-1 (pdx1), Ptf1a, neurogenin 3 (ngn3), Pax4, Pax6, and Isl1 (3–8). During murine pancreatic development, endocrine differentiation occurs through a lateral inhibition process, mediated by Notch signaling. Cells in which Notch is activated by the ligands delta or serrate express high levels of *HES-1*, which in turn represses the proendocrine gene *ngn3*. However, in ligand-expressing cells, *HES-1* expression is not upregulated, thus allowing robust *ngn3* expression and differentiation toward the endocrine lineage (5–8).

ngn3 encodes a class B basic helix-loop-helix factor, which has been shown by loss-of-function studies to be required for the development of the four endocrine cell lineages of the pancreas (5). The pro-endocrine role of *ngn3* has also been demonstrated in gain-of-function studies. Ectopic *ngn3* expression (6–9), as well as lineage tracing experiments (10), indicates that ngn3 is a cell-autonomous determinant and true marker of endocrine progenitor cells. The adoption of each endocrine fate within the islet (α-, β-, δ-, and PP cells) occurs preferentially at specific time points during embryogenesis, suggesting that *ngn3*-positive cells adapt their responses to an evolving milieu of signals. Premature expression of the *ngn3* gene in early pancreatic progenitor cells (e8.5–e9) results in their differentiation into glucagon-producing cells (6). Adenovirus-mediated expression of *ngn3* in adult human duct cells induces neuroendocrine differentiation (11). Finally, ectopic expression of *ngn3* in the chick gut leads to the differentiation of endodermal cells into endocrine cell types that form clusters in the mesenchyme (12). Taken together, these studies suggest that ngn3 could be used as a molecular agent to induce endocrine differentiation in islet neogenesis protocols.

Although gain-of-function studies are essential for the elucidation of gene function and regulation, genetic manipulation is not desirable for clinically oriented differentiation approaches. The unpredictability of both the site of integration and the number of gene copies, as well as the

From the [1]Diabetes Research Institute, University of Miami School of Medicine, Miami, Florida; and the [2]Umeå Center for Molecular Medicine, Umeå University, Umeå, Sweden.

Address correspondence and reprint requests to Helena Edlund, Diabetes Research Institute, University of Miami School of Medicine, 1450 NW 10th Ave., Miami, FL 33136. E-mail: helena.edlund@med.miami.edu or helena.edlund@ucmm.umu.se.

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β-gal, β-galactosidase; Ipf1, insulin promoter factor-1; ngn3, neurogenin 3; pdx1, pancreatic and duodenal homeobox factor-1.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

side effects often observed when using viral vehicles (13,14), are just a few of the drawbacks of conventional gene transfer strategies. Furthermore, terminal endocrine differentiation is invariably associated with *ngn3* silencing, which is only transiently expressed in cells that are about to exit the cell cycle (8). Therefore, any possible use of ngn3 as an in vitro pro-endocrine agent should ideally be transient and not involve the transfer of the gene itself. The use of protein transduction domains would circumvent such restrictions by providing a versatile transduction system, where the protein of interest could be added to the culture medium at the appropriate concentration and only for the time its function is required. The protein transduction domain of the HIV/TAT protein has been extensively used because of its effectiveness and small size (11 amino acids) (15). Many TAT-fused full-length functional proteins have been transduced into cells and tissues (16–24,25). When systemically administered to rodents in vivo, TAT-protein hybrids have been shown to freely diffuse across all tissues, crossing the hemato-encephalic barrier (26) and even the placenta (27). Recently, TAT has been used to deliver the homeobox HOXB4 protein to human hematopoietic stem cells, which resulted in rapid expansion without loss of normal in vivo potential for differentiation or long-term repopulation (28). Intriguingly, many homeobox proteins already have protein transduction domains embedded in their amino acid sequence. In fact, IPF1/PDX1 has its own antennapedia-like protein transduction domain, which has been used to successfully deliver native IPF1/PDX1 protein to islets and cultured duct cells, where it enhanced insulin expression (29). However, ngn3 lacks such intrinsic domain.

Here we report that a TAT/ngn3 fusion protein is effectively taken up by cells and functions in vitro in a manner consistent with the reported activity of native ngn3. These results raise the possibility of using protein transduction domain technology to sequentially introduce critical transcription factors to stem and progenitor cells in vitro as a way of promoting their differentiation into functional cell types in a controlled and reproducible manner.

RESEARCH DESIGN AND METHODS

Vector construction and protein purification. The TAT/ngn3 construct (online appendix available at http://diabetes.diabetesjournals.org) was generated by inserting the coding region of the mouse *ngn3* in the *Nco*I/*Age*I sites of a pTAT expression vector (provided by Stephen Dowdy, University of California San Diego, San Diego, CA) in frame with the TAT/protein transduction domain peptide (YGRKKRRQRRR). The *ngn3* cDNA inserted into the pTAT vector was generated by PCR amplification of the *ngn3* cDNA (6). The oligonucleotides CCATGGCGCCTCATCCCTTGG and ACCGGTTCACAAGAA GTCTGAGAAC were used as forward and reverse primers, respectively. The ngn3 bacterial expression vector was generated by removing the TAT domain from the TAT/ngn3 construct. The TAT/β-galactosidase (β-gal) expression vector was also generously provided by Stephen Dowdy. TAT expression vectors feature a 6(x)His-affinity tag, which allows the purification of the fusion proteins by affinity chromatography using the nickel/nitryloacetic acid system (Qiagen, Valencia, CA). Then, 100-ml LB/Amp overnight culture of BL21(DE3)LysS bacteria expressing the protein of interest were inoculated into 1 l of LB/Amp and grown overnight at 37°C. Next, 0.4 mmol/l IPTG (isopropyl β-D-1 thiogalactopyranoside) was added 2 h before harvesting. Cells were centrifuged and washed with 50 ml PBS. Pellets were resuspended and combined in 10 ml of buffer Z (8 mol/l urea, 100 mmol/l NaCl, 20 mmol/l HEPES, pH 8.0) and 20 mmol/l imidazole. Cells were sonicated on ice and centrifuged at 12,000 rpm for 25 min. The supernatant was applied to a 5-ml nickel/nitryloacetic acid column pre-equilibrated with 20 mmol/l imidazole. The column was washed with 50 ml of imidazole (20 mmol/l) in buffer Z, and the protein was eluted with 250 mmol/l imidazole in buffer Z. Fractions were monitored by colorimetric determinations using a protein assay kit (Bio-Rad). The protein was desalted on a PD-10 column (Amersham), and final protein concentration was determined spectrophotometrically using the Bio-Rad protein assay kit. The TAT peptide was custom made by Sigma.

Western blot. Protein aliquots (15 μl) were diluted in 2× protein loading buffer (National Diagnostics) and run in a 15% polyacrylamide gel (Bio-Rad). For in situ staining, GelCode blue stain reagent (Pierce) was used. Transfer to Amersham enhanced chemiluminescence membranes was performed using the semidry method. Membranes were probed with rabbit anti-ngn3 antibodies (30) at 1:500 dilution.

Cell and tissue culture. Mouse ES cells and fibroblasts were cultured as previously described (31). β-TC3 cells were cultured at 37°C (5% CO_2) on opaque 96-well plates (Nunclon) and fed daily with Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 0.1 mmol/l minimum essential medium nonessential amino acids (Invitrogen), sodium pyruvate, 5% (vol/vol) newborn bovine serum, 5% (vol/vol) fetal calf serum, 0.1 mmol/l 2-mercaptoethanol, penicillin (100 units/ml)/streptomycin (100 μg/ml), and L-glutamine (250 μmol/l) from Invitrogen. Pancreata from e12.5–e13.5 embryos resulting from CBA × B6 crosses (where 12:00 P.M. of the day a vaginal plug is found is considered 0.5 days of gestation) were isolated, microdissected in ice-cold L-15 medium (Invitrogen), and cultured in explant medium (199 minimum essential medium, 10% calf serum, penicillin/streptomycin, and Fungizone) on top of 12-mm Millicell culture plate inserts. Whole guts of e9.5 embryos were dissected and cultured as above. Purified protein was added freshly every day to the culture medium.

Immunostaining and image analysis. Explants were grown as above for 48 h and then fixed with 4% paraformaldehyde (30 min.), washed with PBS (30 min), and frozen in optimal cutting temperature compound (Sakura). Pancreatic rudiments were sectioned in their entirety (5 μm) and mounted with DAPI (4,6-diamidino-2-phenylindole)-Vectashield (Vector). Guinea pig anti-insulin and rabbit anti-glucagon antibodies (ready-to-use solution; BioGenex) were used for double staining. Rabbit anti-ngn3 antibodies (30) were used at a 1:500 dilution. Metamorph imaging was used to quantify relative amounts of insulin and glucagon staining in each section. This software allows the detection and precise quantification of any given fluorescent signal in biological samples. Positive areas were calculated as a percentage relative to the total area of individual histological or confocal sections and then averaged for the entire sample.

TAT/ngn3 in vitro reporter system. β-TC3 cells were transiently transfected with the vector pBETA2(1.0)-Luc (kindly provided by Ming-Jer Tsai, Baylor College of Medicine, Houston, Texas) using an Effectene transfection kit (Qiagen). Protein was added to the medium 24 h later and maintained for another day. Luciferase measurements were performed with a Promega Bright-Glo luciferase reporter kit and a Molecular Diagnostics luminometer.

RT-PCR. β-Actin primers were: ATGGATGACGATATCGCT (forward) and ACCTGACAGACTACCTCAT (reverse), with 568 bp. beta2/neuroD primers were: CTTGGCCAAGAACTACATCTGG (forward) and TTCCCGGTGCATC CCTACTCC (reverse), with 228 bp. A Lightcycler instrument (Roche) was used for real-time RT-PCR analyses (primers as above).

RESULTS

Transduction of TAT/ngn3 into mammalian cells and pancreatic buds. TAT-fusion proteins are known to effectively transduce mammalian cells (15). Using a TAT/β-gal reporter protein, we observed that the uptake efficiency of TAT-fused proteins by ES cells is concentration dependent (Fig. 1*A–D*). This was further confirmed by ngn3 immunostaining of TAT/ngn3-transduced ES cells (data not shown). Neither protein was toxic to the cells within the concentration range used in our experiments (100 nmol/l to 5 μmol/l).

After 24-h incubation with 1 μmol/l TAT/ngn3, UV microscopic examination of mouse ES cells shows that virtually 100% of the cells stain positively for ngn3. Immunostaining is evident throughout the cell, although it appears to concentrate in granular structures. This is consistent with the prevailing view that TAT promotes cellular uptake via endocytosis (18,32,33). Confocal analysis confirmed the presence of ngn3-positive vesicles in the cytoplasm of the transduced cells, as well as diffuse nuclear staining (Fig. 1*E*). Incubation with ngn3 alone did not result in cellular uptake, as detected by immunohistochemistry (Fig. 1*F*).

To test whether TAT/ngn3 would be able to evenly

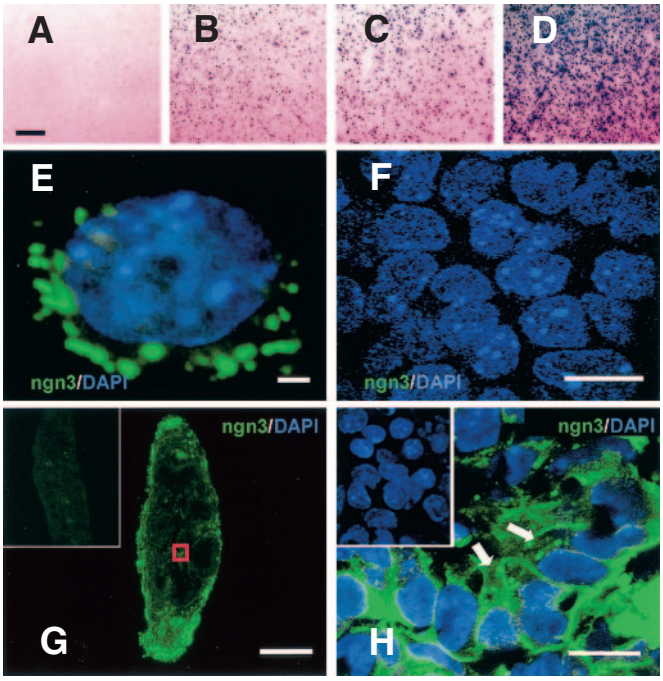


FIG. 1. Uptake of TAT/ngn3 by mammalian cells. TAT/β-gal uptake (X-gal staining) after incubation of ES cells (24 h) with the protein: nontransduced control (*A*), 100 nmol/l (*B*), 300 nmol/l (*C*), and 500 nmol/l (*D*). *E*: CARV confocal image of an ES cell incubated with TAT/ngn3 (2 μmol/l) for 24 h (ngn3 staining). *F*: Control. Cells were incubated with ngn3 protein (2 μmol/l). *G*: Ngn3 immunostaining of an e13.5 bud section cultured with 2 μmol/l TAT/ngn3 (12 h). *Inset*: TAT-treated bud stained for ngn3 (negative control). *H*: A region in the center of the bud (red square) is shown at a higher magnification (confocal). Ngn3$^+$ vesicles (white arrows) can be observed in virtually 100% of the cells. Nuclear staining (blue) of DAPI (4,6-diamidino-2-phenylindole) is shown. *Inset*: TAT-treated bud stained for ngn3 (negative control). Original magnification and scale bars: 100×, 150 μm (*A–D*); 400×, 1 μm (*E*); 400×, 10 μm (*F* and *H*); and 100×, 150 μm (*G*).

transduce structures thicker than a cell monolayer, e13.5 pancreatic buds were cultured with 2 μmol/l TAT/ngn3 for 12 h. Confocal analysis of ngn3-immunostained samples shows, as expected, an uptake gradient from the surface to the core of the bud (Fig. 1*G*). However, cells located in the center of the explant display the typical ngn3-positive vesicles observed in TAT/ngn3-transfected monolayers (Fig. 1*H*). This observation shows the ability of TAT/ngn3 to efficiently transduce cells in a three-dimensional structure.

Exogenously administered TAT/ngn3 activates a beta2/neuroD reporter system in vitro. To demonstrate that transduced TAT/ngn3 functions at the nuclear level in vitro, we used the reporter vector β-*luc*, where expression of luciferase is driven by a 1.0-kb fragment of the *beta2/neuroD* promoter. *beta2/neuroD* is a downstream target of ngn3 during endocrine differentiation (34). Therefore, nuclear import of active TAT/ngn3 would result in stimulation of the *beta2/neuroD* promoter and expression of luciferase in our system. First, we examined the inducibility of the reporter system upon ectopic expression of *ngn3*. β-TC3 cells, which do not express detectable levels of endogenous *ngn3* (Fig. 2*A*), were sequentially transfected with 250 ng of β-*luc* (day 1) and either 500 or 1,000 ng of a CMV (cytomegalovirus)-*ngn3* expression vector at day 2. We observed two- and threefold increases in luciferase activity, respectively, compared with mock controls (Fig. 2*B*).

Next, β-TC3 cells that had been transiently transfected with β-*luc* were incubated for 24 h in the presence of TAT/ngn3 (2 and 5 μmol/l). Two control groups were treated with PBS or native ngn3 protein. As shown in Fig. 2*C*, luciferase activity was increased 2.5- and 3.5-fold in cells that had been treated with TAT/ngn3 (2 and 5 μmol/l, respectively) compared with the basal level of luciferase expression observed in PBS and ngn3 controls.

Nonlinearized vectors tend to remain episomal after transfection (35). We reasoned that the effectiveness of TAT/ngn3 at inducing expression of an episomal promoter might not necessarily correlate with its ability to regulate the expression of endogenous genes. To test whether TAT/ngn3 was able to enhance the expression of the endogenous *beta2/neuroD* gene, we incubated β-TC3 cells with 2 μmol/l TAT/ngn3 and obtained RNA samples for quantitative RT-PCR analysis at different time points. Figure 2*D* shows that there is a sharp increase in *beta2/neuroD* expression 5 h after addition of the protein. The signal decreases to noninduced levels after 16 h. These results are consistent with the observed half-life of the protein in vitro (Fig. 2*E*). Collectively, these data demonstrate that physiologically active TAT/ngn3 does migrate to the nucleus after uptake and is able to activate a downstream target gene in vitro in a manner similar to that expected of native ngn3.

Treatment of early pancreatic explants with TAT-ngn3 results in preferential differentiation into glucagon-producing cells. During murine development, the first endocrine cell type (glucagon positive) is observed as early as e9. Premature differentiation of pancreatic progenitor cells caused by forced expression of *ngn3* under the control of the *Ipf1/Pdx1* promoter results primarily in the generation of glucagon-expressing cells (6). We predicted that TAT/ngn3 would have a comparable effect on pancreatic progenitors in cultured e9.5 whole-gut explants.

In *Ipf1/ngn3* transgenic animals, the premature differentiation of pancreatic progenitors occurs at the expense of pancreatic progenitor cell expansion and later differentiation of other pancreatic cell types (6), such as insulin-expressing cells and exocrine cell types, that effectively appear first around e13. To see whether TAT/ngn3 would promote the generation of glucagon-positive cells at the expense of insulin-positive cells when applied to early pancreatic anlagen, e9.5 whole-gut explants were cultured for 2 or 6 days in the presence or absence of TAT/ngn3 (2 μmol/l). After 2 days, all four explants in the control group, but only one of five in the TAT/ngn3 group, had insulin-positive cells (Fig. 3). After 6 days, four of seven (57%) explants in the control group, but none (of seven) in the TAT/ngn3 group showed insulin expression (Fig. 3).

Although the above experiment suggests that TAT/ngn3 promotes the differentiation of glucagon-expressing cells at the expense of insulin-expressing cells, the scarcity of insulin-positive cells in the control explants leaves open the possibility that their reduced appearance rate is not a direct consequence of enhanced glucagon cell differentiation. To further explore this issue, we next determined the amount of glucagon-producing cells in TAT/ngn3-exposed explants compared with that found in controls. Then, e9.5 entire guts were dissected and cultured for 48 h in the presence of TAT/ngn3 or TAT peptide alone. Explants were subsequently fixated and immunostained for glucagon. Confocal planes of each embryonic pancreas were ob-

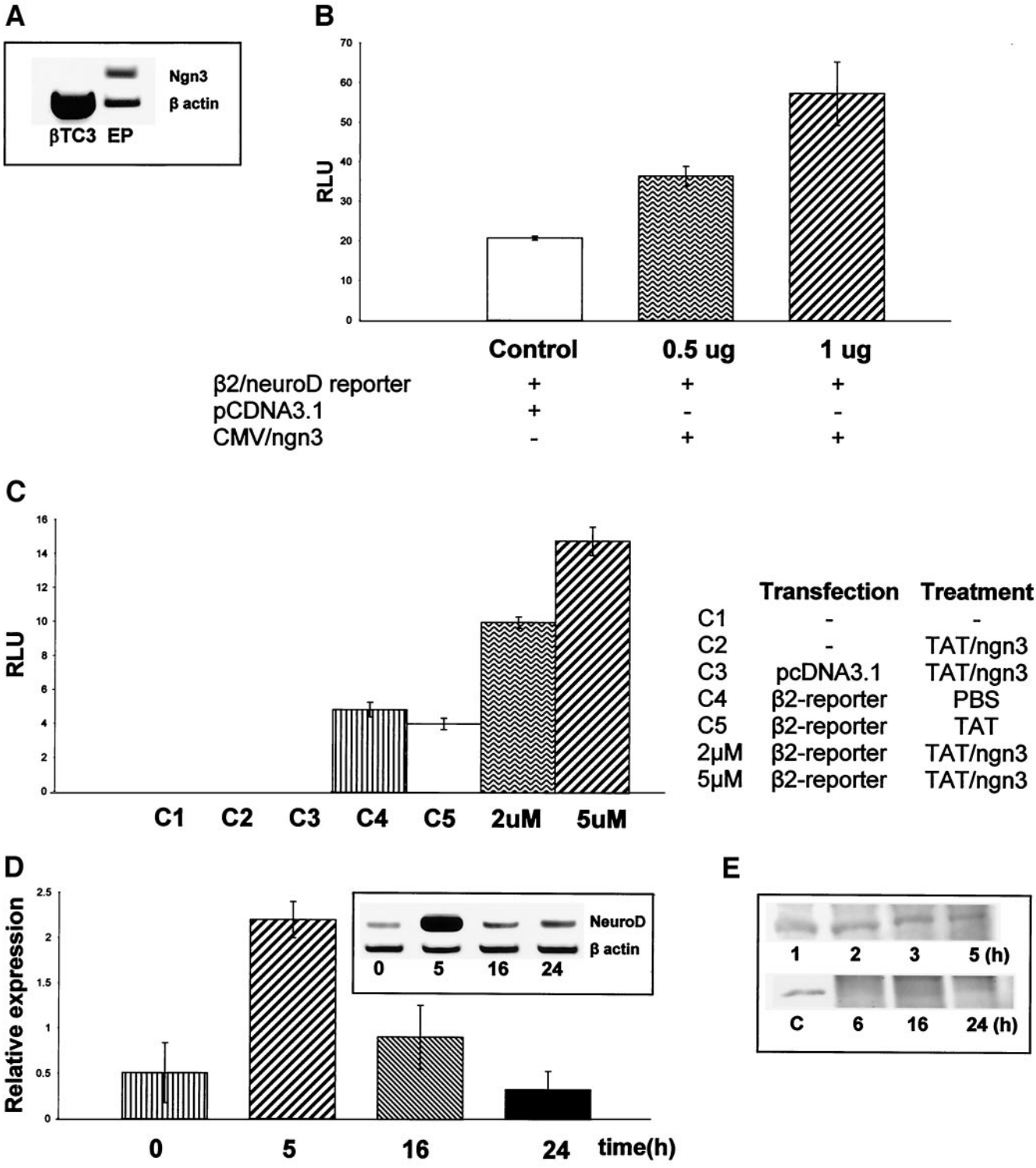


FIG. 2. TAT/ngn3 activates a nuclear reporter gene in vitro. *A*: RT-PCR shows that *ngn3* is not actively expressed in β-TC3 cells. Positive control, e13.5 embryonic pancreas (EP). Loading control, β-actin. *B*: Cytomegalovirus (CMV)-*ngn3* activates a *beta2/neuroD2* reporter gene. Transfection of β-TC3 cells with a *beta2/neuroD2*-luciferase reporter gene (day 1) and a ngn3 expression vector (CMV-*ngn3*; day 2) induces luciferase expression. Y bars: SE for each group. Control, cells transfected with a mock plasmid (pcDNA 3.1; day 2); RLU, random luminescence units. *C*: TAT/ngn3 activates a *beta2/neuroD* reporter gene. Incubation of β-TC3 cells with TAT/ngn3 stimulates luciferase expression under the control of the *beta2/neuroD2* promoter. Cells were transiently transfected with a *beta2/neuroD2*-luciferase reporter vector and then incubated for 24 h with 2 or 5 μmol/l of TAT/ngn3. C1, control 1, cells untransfected and untreated; C2, control 2, cells untransfected and treated with TAT/ngn3; C3, control 3, cells transfected with a mock DNA molecule and then treated with TAT/ngn3 (2 μmol/l); C4, control 4, cells transfected with the reporter vector (*beta2/neuroD2*-luciferase) and then treated with PBS; C5, control 5, cells transfected with the reporter vector and then with TAT peptide. Y bars: SE for each group. *D*: TAT/ngn3 induces expression of endogenous *beta2/neuroD*. TAT/ngn3 (2 μmol/l) induces expression of the endogenous *beta2/neuroD* gene, as evidenced by real-time and conventional (*inset*) RT-PCR of samples taken at time points 0 (before addition), 5, 16, and 24 h. Y bars: SE for each group. All values were normalized to β-actin expression. *E*: GelCode-stained polyacrylamide gel showing TAT/ngn3 band at different time points of incubation in serum-containing medium at 37°C. The protein is stable for up to 5 h and then is progressively degraded. *C*: Fresh protein control.

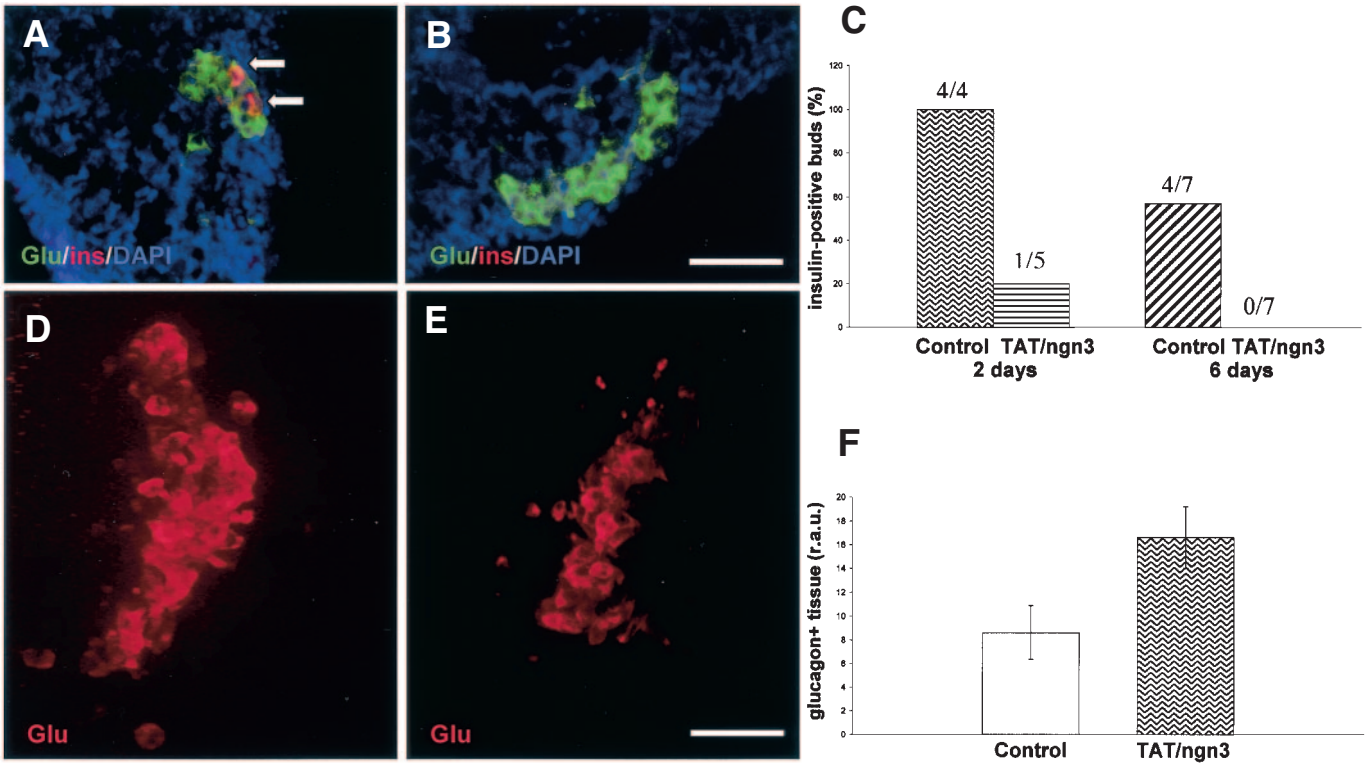


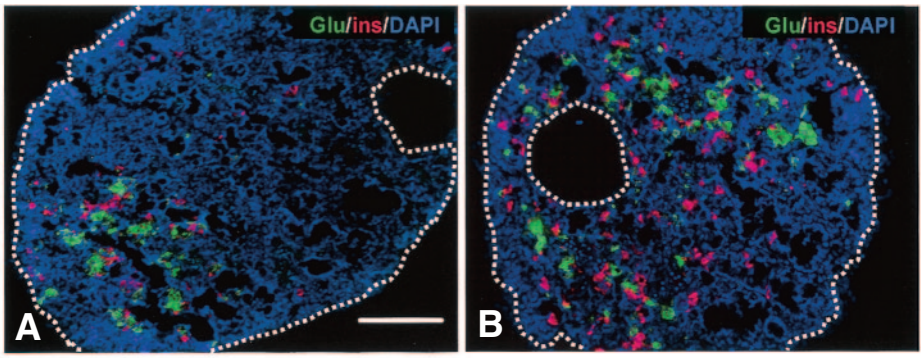
FIG. 3. Effect of TAT/ngn3 on e9.5 pancreatic buds in vitro. *A*: Glucagon (green) and insulin (red, arrows) in e9.5 embryonic gut cultured with native ngn3 (6 days). DAPI (4,6-diamidino-2-phenylindole)-stained nuclei (blue) are shown. *B*: Insulin$^+$ cells are normally absent from e9.5 embryos cultured for 6 days with TAT/ngn3. *C*: TAT/ngn3 on e9.5 embryos: incubation experiments (2/6 days). *y*-axis: percent of explants with insulin-positive cells. Insulin-positive embryos/embryos per group are indicated above each column. *D* and *E*: Three-dimensional reconstruction of glucagon-positive clusters (red) in e9.5 guts cultured with (*D*) or without (*E*) TAT/ngn3. *F*: TAT/ngn3 on e9.5 embryos. TAT/ngn3-treated embryos had a twofold average increase in glucagon-positive cells compared with their TAT-treated counterparts. Original magnification: 320×. Size bars: 50 μm. r.a.u., random area units.

tained every 25 μm, from the first (top) to the last (bottom) sections positive for glucagon. We observed that in TAT/ngn3-treated embryos ($n = 6$), clusters of glucagon-producing cells were generally thicker and denser than in control guts (Fig. 3). Metamorph image analysis was used to quantify the overall amount of glucagon-positive cells in each embryo. As shown in Fig. 3, TAT/ngn3-treated guts contained approximately twice as much glucagon-producing tissue as controls ($n = 5$). An ANOVA test indicated that this increase was statistically significant ($F = 5.16$; $P = 4.95 \times 10^{-2} < 0.05$). These results are consistent with our hypothesis that TAT/ngn3 stimulates α-cell differentiation at the expense of other pancreatic cell types in e9 explants.

Treatment of e13.5 pancreatic explants with TAT-ngn3 enhances endocrine differentiation. Although glucagon-producing cells appear throughout development, it is thought that the inductive microenvironment found in the pancreatic bud at e12–e13 favors the differentiation of insulin-producing cells (6). Therefore, ectopic expression (or administration) of ngn3 at this time is likely to result in enhanced differentiation of cycling progenitors, preferentially into insulin-expressing cells. To test this hypothesis, e13.5 dorsal pancreatic buds were cultured for 48 h in the presence of TAT/ngn3 (2 μmol/l, $n = 23$). A control group ($n = 14$) was treated either with TAT peptide (2 μmol/l, $n = 8$) or native ngn3 protein (2 μmol/l, $n = 6$). At termination, each bud was individually fixed, frozen, sectioned (5 μm), and immunostained for insulin and glucagon. Metamorph image analysis software was used to quantitate relative amounts of insulin and glucagon cells in each section, and values were averaged for each single pancreatic bud. Because the mean values obtained in each of the control subgroups (TAT peptide and native ngn3 protein) were statistically similar, we combined them into one single group for the sake of simplicity. As shown in Fig. 4, there is a 1.93-fold increase in the overall number of endocrine cells (insulin + glucagon) in the study group compared with the control group (ANOVA $F = 7.42$, $P = 1.9 \times 10^{-2} < 0.05$). Although the number of glucagon-positive cells is higher in the study group than in the controls, the increase in insulin-expressing cells is markedly superior (2.07-fold, ANOVA $F = 6.45$, $P = 2.27 \times 10^{-2} < 0.05$). The ratio of insulin- to glucagon-expressing cells is also enhanced in the TAT/ngn3 group, but such an increase is not statistically significant. The use of TAT/ngn3 in buds explanted at a slightly earlier developmental stage (e12.5) also resulted in a similar enhancement in endocrine differentiation (data not shown). Together, these results indicate that TAT/ngn3 stimulates overall endocrine differentiation, especially that of insulin-producing cells, in explanted e12–e13 pancreatic buds.

DISCUSSION

Although TAT-mediated transportation of proteins is a well-established technology (15,36), its application to deliver transcription factors is less well documented (28). The observation that TAT-fused proteins are internalized



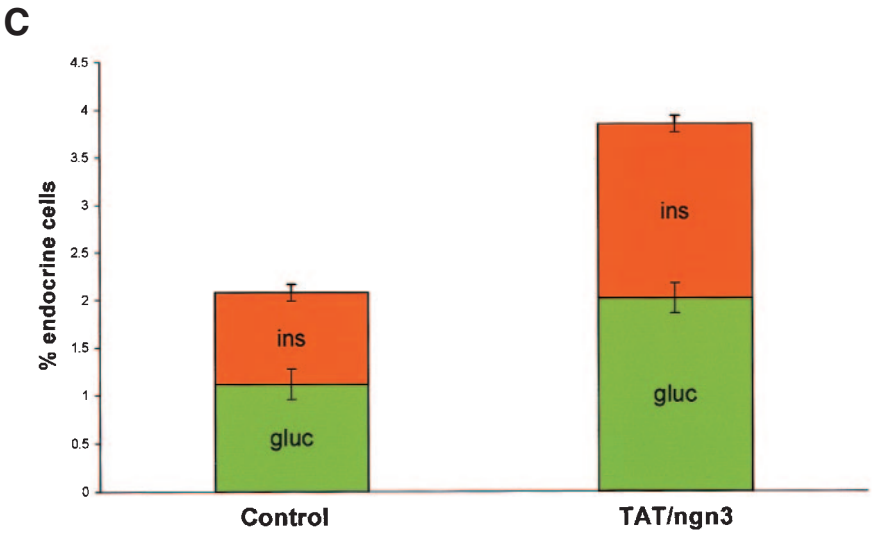


FIG. 4. Effect of TAT/ngn3 on e13.5 explants in vitro. Dorsal buds were explanted and cultured for 48 h with TAT-ngn3 (2 μmol/l) and either TAT peptide or native ngn3 protein (both at 2 μmol/l). *A*: Section of an e13.5 bud, representative of the average percentages of glucagon (green) and insulin (red)-expressing cells found in control (TAT-treated) cultures (~2% of endocrine cells). *B*: Section of an e13.5 bud with the average percentage of endocrine cells (~4%) after incubation with TAT/ngn3. *C*: Effect of TAT/ngn3 on e13.5 pancreatic buds (insulin and glucagon). Y bars: SE for each group. Original magnification: 100×. Size bars: 100 μm.

by endocytosis, a mechanism commonly associated with cytoplasmic degradation (36), as well as the need for the protein to translocate across several cellular membranes (outer, vesicular, and nuclear), have been cited among the theoretical concerns for the use of TAT to transport nuclear factors. Indeed, there is evidence suggesting that TAT-mediated membrane translocation might require unfolding and subsequent renaturation of the protein (36,37), which might decrease the overall efficiency of the process and therefore the amount of protein in the nuclear compartment available for immunodetection. However, nuclear translocation itself is probably not a rate-limiting step here because native nuclear factors are naturally transported to the nucleus after they are synthesized in the cytoplasm. TAT/ngn3 seems to accumulate preferentially in the cytoplasm, but some diffuse staining can also be detected in the nucleus. Our observation that TAT/ngn3 enhances expression of both a reporter gene placed under the control of the *beta2/neuroD* promoter (a natural downstream target of the native protein) and the endogenous *beta2/neuroD* gene confirms that the recombinant protein reaches the nucleus in a biologically active conformation. Our experiments in embryonic explants further support this conclusion. It is known that early expression of *ngn3* under the *Ipf1/Pdx1* promoter in transgenic mice results in a premature differentiation of progenitor cells into glucagon-expressing cells (6). Such an increase in the number of glucagon-producing cells occurs at the expense of other terminally differentiated cell types, including insulin-expressing cells. In our experiments, insulin-producing cells were rarely spotted in TAT/ngn3-treated whole-gut explants compared with controls. Although there is some variability in the appearance of β-cells in vitro, the increased amount of glucagon-producing cells observed in the explants exposed to TAT/ngn3 is consistent with an effect of TAT/ngn3 at promoting endocrine differentiation. Treatment of e12 and e13 pancreatic explants with TAT/ngn3 also results in a net increment of endocrine cells. Although glucagon-expressing cells still appear (and will keep differentiating throughout development), our data suggest that the progenitor cells activated by TAT/ngn3 are preferentially recruited toward the β-cell lineage.

The half-life of the recombinant protein is short, which explains why a TAT/ngn3-induced gene (*beta2/neuroD*) recuperates original levels of expression in β-TC3 cells 16 h after the protein was added to the medium. In contrast, exposure of embryonic explants to TAT/ngn3 has a permanent effect, consistent with the irreversible induction of endocrine differentiation in predisposed progenitor cells. Our approach, therefore, seems uniquely suited to mimic in vitro the natural pattern of expression of genes that are only transiently expressed.

In summary, our data demonstrate that TAT/ngn3 promotes endocrine differentiation in vitro, in a manner consistent with the predicted biological function of the native protein. The use of protein transduction domains to deliver transcription factors at specific time points potentially represents a powerful tool for gain-of-function developmental

studies, circumventing the need for time-consuming and often unpredictable methods such as transgenesis or conditional gene targeting. This work is also the first study, to our knowledge, in which protein transduction domain–fused transcription factors are used to aid in the directed differentiation of progenitor cells. The results presented here suggest a novel way to design islet differentiation protocols, which would involve the precise in vitro recapitulation of islet development by means of the sequential administration of key transcriptional factors to stem cell cultures. Such an approach would be more advantageous and flexible than those based on gene transfer because it would allow for the precise timing of protein administration and removal when its function is no longer required.

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