37°C for 18–20 hours before cell plating. Recombinant human laminin 5 (Ln5; Reprocell Inc., Kanagawa, Japan, https://www.reprocell.com/en/) was also used to coat the culture plates at a concentration of 0.1 μg/cm^2 and incubated at 37°C for 18–20 hours before cell plating.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature (22°C–24°C). Immunostaining was carried out with the standard protocol. The following primary antibodies were used: goat anti-Sox17 (1:200; R&D Systems), mouse anti-GFP (1:1,000; Medical and Biological Laboratories Co., Ltd., Nagoya, Japan, http://www.mbl.co.jp/e/index.html), goat anti-Foxa2 (1:300; Santa Cruz Biotechnology), mouse anti-insulin (1:1,000; Sigma-Aldrich), and mouse anti-glucagon (1:1,000; Sigma-Aldrich). Alexa 488- or Alexa 568-conjugated secondary antibodies were used at 1:500 dilution (Molecular Probes). Cells were counterstained with 4′,6-diamidino-2-phenylindole (Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com). Images were taken using fluorescence microscopy.

Quantification of Insulin-Positive Cells

Cells labeled with anti-insulin antibody in 96-well plates were counted manually using a fluorescence microscope, and the number was expressed as a percentage of the total cell number counted in each sister well. Similarly, the Ins1-GFP-positive cell number per well of 96-well plates was counted manually, and the percentage was calculated.

Quantitative Reverse Transcription PCR Analysis

Total RNA was extracted from cells using an RNeasy micro kit (Qiagen, Valencia, CA, http://www.qiagen.com) according to the manufacturer's instructions. CDNA was prepared by reverse transcription of 500 ng total RNA using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan, http://www.takara-bio.com). The resulting cDNAs were amplified using a SYBR Green PCR kit (Takara). The primer sequences for each primer set are shown in supplemental online Table 1. MRNA expression data were normalized against actin expression in a corresponding sample.

C-Peptide Secretion Assay

C-peptide release was measured by incubating the cells in Krebs-Ringer solution containing bicarbonate and Hepes (KRBH: 129 mM sodium chloride, 4.8 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM monopotassium phosphate, 1.2 mM magnesium sulfate, 5 mM sodium bicarbonate, 10 mM Hepes, 0.1% bovine serum albumin). The cells were incubated in KRBH buffer for 1 hour to wash them. They were then incubated in KRBH buffer with 2.5 mM D-glucose for 1 hour and KRBH buffer with 20 mM D-glucose for 1 hour. The C-peptide levels in culture supernatants were measured using the mouse C-peptide enzyme-linked immunosorbent assay kit (Shibayagi, Gunma, Japan, http://www.shibayagi.co.jp/index-E.htm). Pancreatic islets were isolated from ICR mice using collagenase digestion as described by Noguchi et al. [22]. Twenty-five islets were hand picked and seeded on 24-well plates. The C-peptide secretion assay was performed as described previously.

Treatment With Streptozotocin and Transplantation in Mice

C.B-17/Icr-SCID/SCID Jcl mice (CLEA Japan, Tokyo, Japan, http://www.clea-japan.com), aged 8–10 weeks, were used as recipients of transplantation. All recipient mice were made diabetic by an intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich), 150 mg/kg body weight per day, freshly dissolved in citrate buffer (pH 4.5), on two consecutive days for a total dose of 300 mg/kg. Once nonfasting blood glucose levels exceeded 250 mg/dl for at least two consecutive days, the mice were considered to be diabetic and were used as transplant recipients. On day 17, the end of differentiation into insulin-producing cells, entire cells in culture were dissociated with 0.25% trypsin; then, 1×10^7 cells, suspended in 50 μl culture medium, were injected under the kidney capsules of recipient mice using a syringe with a 23-gauge needle (Nipro, Tokyo, Japan, http://www.nipro.co.jp/en/). Nonfasting blood glucose levels were measured at 7, 21, 35, 49, 77, and 105 days after transplantation. At 35 days after injection, grafted mice were sacrificed. The grafts were recovered from the kidney and subjected to immunofluorescence or reverse transcription PCR (RT-PCR) analysis.

Adult mouse islets were used as a positive control. Islets were isolated from ICR mice aged 8–10 weeks as follows: under general anesthesia induced by pentobarbital sodium (50 mg/kg), mice were injected with 2 ml Hanks' balanced salt solution (Invitrogen) containing 2 mg/ml collagenase, type V (Sigma-Aldrich), into the common bile duct. The distended pancreas was removed and incubated at 37°C for 16 minutes. The islets were purified by centrifugation (2,000 rotations per minute for 10 minutes) with Histopaque 1077 (Sigma-Aldrich) and RPMI medium gradient. Individual islets, free of attached acinar, vascular, and ductal tissues, were selected and removed with a Pasteur pipette under a dissecting microscope, yielding highly purified islets for transplantation. The number of islets was determined by counting manually. One hundred islets, suspended in 50 μl culture medium, were injected under the kidney capsules of recipient mice using a syringe with a 23-gauge needle in the same way as differentiated cells. Similarly, nonfasting blood glucose levels were measured at 7, 21, 35, 49, 77, and 105 days after transplantation.

Generation of Human iPS Cells and Differentiation Into NGN3-Expressing Cells

The human iPS cells were produced as reported previously [32, 33]. Human fibroblast BJ cells were plated at 5×10^5 cells per well in six-well plates and infected with SeV vectors (OCT3/4, SOX2, KLF4, and c-MYC; DNAVEC Corporation, Tsukuba, Japan, http://www.dnavec.co.jp/en/) at a multiplicity of infection (MOI) of 3. Infected cells were transferred onto mitomycin C-treated MEF feeder cells on day 6 after induction. When ES-like colonies appeared, the medium was changed to DMEM/F12 HAM (Sigma-Aldrich) supplemented with 100 μM NEAA, 2 mM L-Gln, 20% KnockOut serum replacement, 50 U/ml penicillin and 50 μg/ml streptomycin, 100 μM β-ME, and 5 ng/ml basic FGF. To establish the iPS cell lines, colonies were manually transferred onto MEF feeder cells.

For pancreatic differentiation, human iPS cells were dissociated with 2 U/ml dispase (Invitrogen) and then replated onto MEF feeder cells. At 70%–80% confluence, differentiation was initiated according to a protocol described by D'Amour et al. [34], modified by omitting stage 5.

Animal Care

All procedures involving mice were performed in compliance with the National Institutes of Health guidelines and were approved by the animal ethics committee of Kumamoto University (approval ID: C23-310).

Statistics

Data are shown as the mean plus or minus standard error of the mean. Student's *t* test was used to identify significant differences between two conditions, and one-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey-Kramer's post hoc analysis was used to compare multiple conditions. A *p* value of $<.05$ was considered significant.

Results

Protein Transduction Into Mouse ES Cells

To transduce proteins into mouse ES cells, we designed recombinant forms of rat Pdx1, rat NeuroD, and mouse MafA, as shown in Figure 1A. Pdx1 and NeuroD were not fused with any CPPs because they have a PTD [22, 23]. MafA was fused with 11R as a CPP [24, 25, 35]. These recombinant proteins were expressed and purified from *Escherichia coli,* as shown in Figure 1A. The predicted molecular mass of Pdx1, NeuroD, and MafA was 31 kDa, 40 kDa, and 38 kDa, respectively. The recombinant Pdx1 protein migrated at approximately 45 kDa, apparently different from the predicted size (Fig. 1A), whereas both the recombinant and endogenous Pdx1 migrated at 45 kDa independent of post-translational modification [36]. We examined the efficiency of protein transduction into mouse ES cells using 11R fused with enhanced GFP (11R-EGFP), and 11R-EGFP was detected in almost all of the cells 6 hours after transduction, whereas EGFP without 11R was not detected (supplemental online Fig. 1). We next examined the time-dependent transduction of Pdx1, NeuroD, and MafA-11R in mouse ES cells by Western blotting (Fig. 1B). Pdx1 and NeuroD were detected 1 hour after the application of each protein, whereas MafA-11R was detected 3 hours after transduction. Pdx1 and MafA-11R reached a peak at 6–12 hours and were retained for up to 48 hours, whereas NeuroD reached a peak at 48 hours (Fig. 1B). Protein transduction efficiency was also analyzed using Alexa 568-labeled proteins. Each protein was observed in almost all of the mouse ES cells 6 hours after treatment (Fig. 1C). To verify whether the proteins had transcriptional activity, Hela cells were transfected with rat insulin promoter-driven reporters, and luciferase activity was measured 6 hours after the transduction of Pdx1, NeuroD, or MafA-11R. Each protein significantly induced luciferase activity, and the combined treatment of these three proteins increased luciferase activity more than each protein alone. It is suggested that the transduced proteins had transcriptional activity (Fig. 1D).

Protein Transduction Facilitates the Differentiation of Mouse ES Cells Into Insulin-Producing Cells

We developed a stepwise differentiation protocol under feeder-free conditions, as shown in Figure 2A. On day 0, mouse ES cells were cultured on a three-dimensional synthetic matrix and converted into insulin-producing cells using three types of growth medium with supplements, as indicated in Figure 2A. It was confirmed that the expression of Sox17, a definitive endoderm marker, was not detected in undifferentiated cells but was markedly induced at day 7 during differentiation. That signal weakened after day 7 (Fig. 2B). Foxa2, a marker of pancreatic progenitors, began to be expressed on day 7, and that signal also appeared in later periods (Fig. 2C). Pdx1 expression, also a marker of pancreatic progenitors, was detected on days 11 and 17 (Fig. 2D). On day 17, the end of this differentiation protocol, several insulin-positive cells were detected (Fig. 2E). RT-PCR analysis revealed that the expression dynamics of Sox17, Pdx1, and Ins1 during differentiation (Fig. 2F) was similar to that of pancreatic β-cell development [37, 38].

Using this differentiation protocol, we next examined whether the transduction of Pdx1, NeuroD, and MafA-11R facilitated the differentiation of mouse ES cells into insulin-producing cells. To determine the most effective timing of protein transduction and combination of delivered proteins, we divided the transduction period into three periods as first (days 5–9), second (days 9–11), and third (days 13–17) under our stepwise differentiation protocol (Fig. 3A) and examined which timing or combination most effectively induced differentiation into insulin-producing cells. Protein transduction with Pdx1 during the first period, NeuroD during the second period, and MafA-11R during the third period most significantly increased the percentage of insulin-positive cells in the SK7 mouse ES cell line compared with phosphate-buffered saline-treated cells as a control (Fig. 3B, 3C). The transduction of one or two proteins had little effect on the number of insulin-positive cells (Fig. 3C). These results suggest that the three proteins act sequentially on pancreatic β-cell differentiation, whereas the treatment did not induce differentiation into definitive endoderm (Sox17-positive cells) on day 7 or pancreatic progenitors (Pdx1-GFP- and Foxa2-positive cells) on day 11 (supplemental online Fig. 2A–2C). These results suggest that this treatment facilitated differentiation into endocrine progenitors and/or insulin-producing cells. To confirm that protein transduction increases insulin production, we used an Ins1-GFP mouse ES cell line, ING112, established from a transgenic mouse line bearing the insulin promoter driving GFP expression [28, 29]. Transduction of the three proteins induced Ins1-GFP expression (Fig. 3D) and was most effective for Ins1 gene expression (Fig. 3E). We also examined whether protein transduction could induce the differentiation of a mouse iPS cell line, 20D17. On day 17, this treatment also significantly increased the percentage of insulin-positive cells and Ins1 gene expression in mouse iPS cells (supplemental online Fig. 3A, 3B). Next, to examine whether these proteins function via each PTD, we created PTD-deleted proteins of Pdx1 and NeuroD and intact MafA protein not fused with 11R (supplemental online Fig. 3C). Transduction of these proteins without each PTD did not induce Ins1 gene expression (supplemental online Fig. 3D).

ECM Derived From 804G Cells Facilitates Differentiation of Mouse ES Cells Into Insulin-Producing Cells

To increase the efficiency of differentiation into insulin-positive cells, we focused on ECM derived from 804G cells. The ECM contains Ln5 and fibronectin and has a beneficial effect on rat pancreatic β-cell survival, proliferation, and insulin secretion in response to glucose [31, 39–41]. 804G ECM treatment increased the number of Ins1-GFP-positive cells, and the transduction of Pdx1, NeuroD, and MafA increased it even further (Fig. 4A, 4B). The effects of 804G ECM and protein transduction were supported

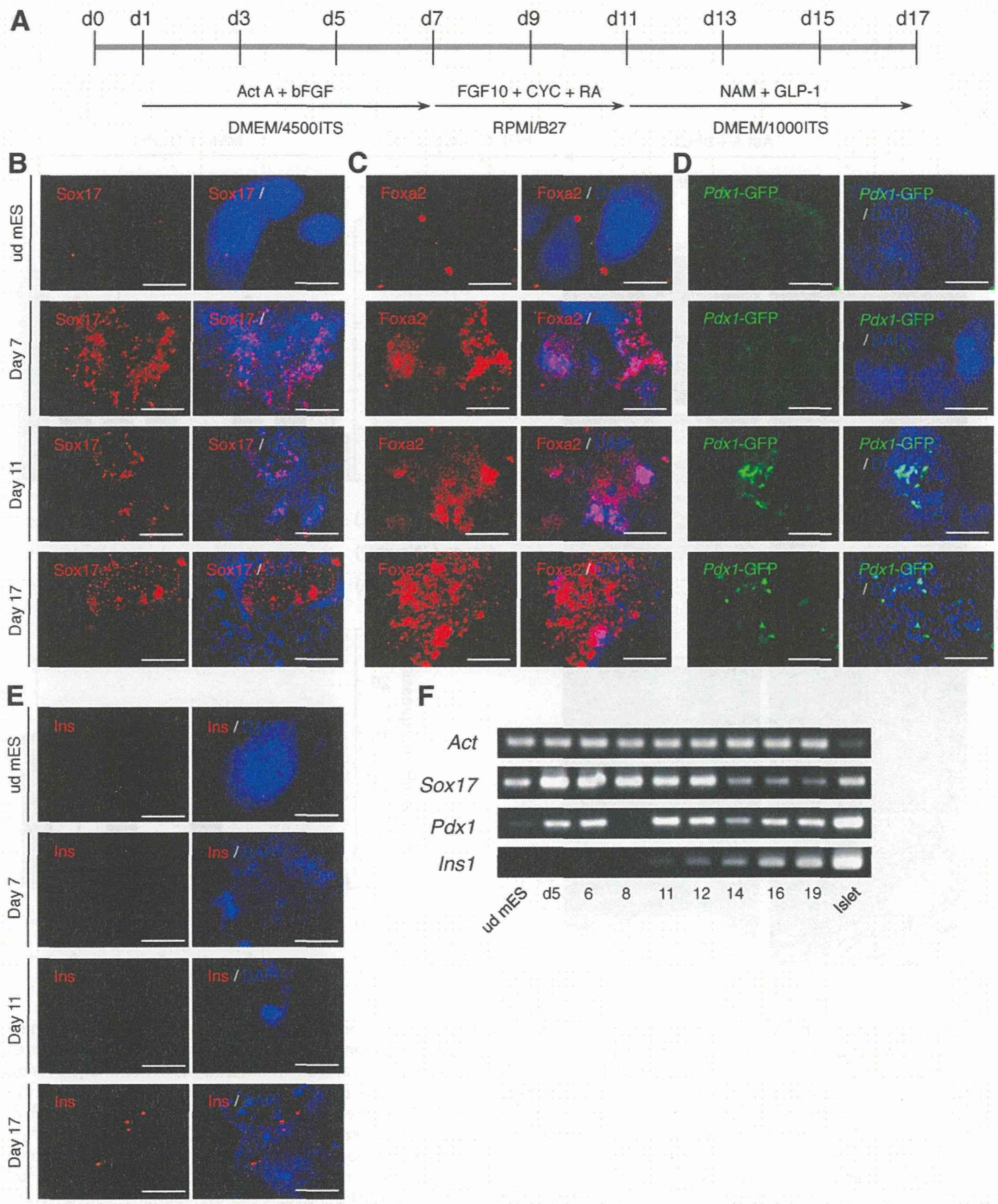

Figure 2. Differentiation of mouse embryonic stem cells into insulin-producing cells by a stepwise protocol. **(A):** Scheme of the timeline of the differentiation protocol. Three types of growth medium with supplements were used, as indicated. **(B–E):** Immunofluorescence analysis of the Pdx1-GFP mouse embryonic stem cell line SK7 for Sox17 **(B)**, Foxa2 **(C)**, Pdx1-GFP **(D)**, and insulin **(E)** was performed on days 0 (undifferentiated), 7, 11, and 17 of differentiation, respectively. Scale bars = 200 μm. **(F):** RNA was extracted from differentiated cells in the period indicated. Reverse transcription polymerase chain reaction analysis was performed on Act, Sox17, Pdx1, and Ins1. Abbreviations: Act, actin; Act A, activin A; bFGF, basic fibroblast growth factor; CYC, KAAD-cyclopamine; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GLP-1, glucagon-like peptide; Ins, insulin; Ins1, insulin 1; Islet, islets isolated from ICR mice; ITS, insulin-transferrin-selenium; NAM, nicotinamide; RA, retinoic acid; ud mES, undifferentiated mouse ES cells.

by an increase in Ins1 expression (Fig. 4C). We further examined whether 804G ECM and the transduction of the three proteins increased the number of Pdx1-positive pancreatic progenitor cells using a mouse ES cell line, SK7, containing a Pdx1-promoter-driven GFP reporter transgene. Protein transduction had no effect on the number of Pdx1-GFP-positive cells, whereas 804G ECM raised the number of positive cells (Fig. 4D). In differentiated cells treated with 804G ECM and protein transduction, a few cells expressed glucagon; however, coexpression with Ins1-GFP was not observed (Fig. 4E). We next examined the expression of marker genes of pancreatic β cells in differentiated cells treated with the protein transduction and 804G ECM. Combination

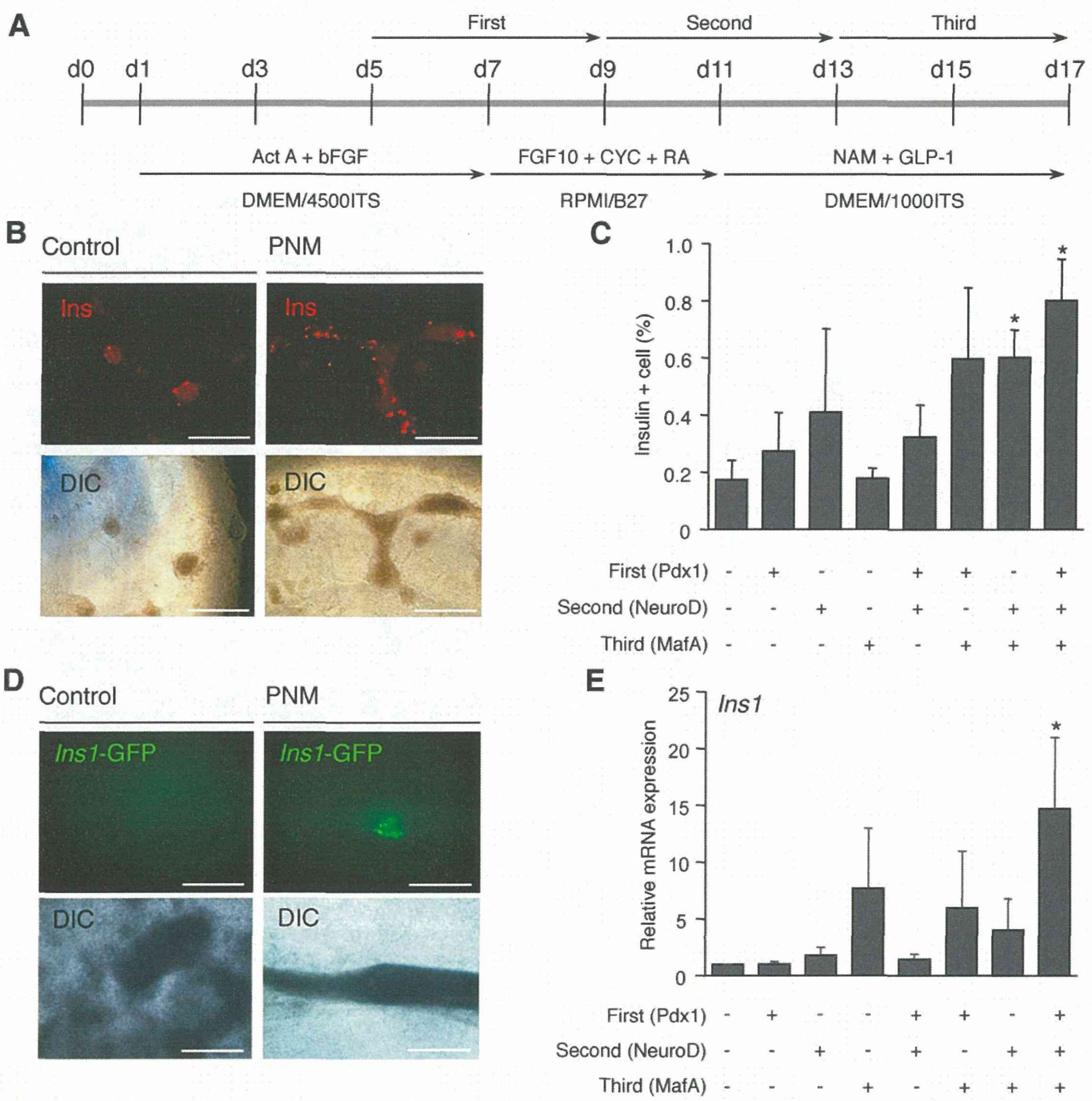

Figure 3. Protein transduction-induced generation of insulin-producing cells from mouse embryonic stem (ES) cells. **(A):** The timeline of protein transduction. The timing of protein transduction was divided into three periods (first, second, and third). Recombinant proteins were added to cells at a final concentration of 1 μM. **(B):** SK7 mouse ES cells were induced to differentiate, and an immunofluorescence analysis for insulin was performed on day 17. Scale bars = 200 μm. **(C):** Values are the percentage of insulin-positive cells per well of a 96-well plate. Data are the mean $\pm$ SEM. $*$, $p < .05$ versus corresponding control (one-way analysis of variance [ANOVA] followed by Tukey-Kramer's post hoc analysis), $n = 4$ each. **(D):** The Ins1-GFP mouse ES cell line ING112 was induced to differentiate, and GFP fluorescence was measured on day 17. Scale bars = 200 μm. **(E):** Quantitative reverse transcription polymerase chain reaction analysis of Ins1 expression in mouse ES cells treated with indicated proteins. Data are shown as the mean plus or minus standard error of the mean. $*$, $p < .05$ versus corresponding control (one-way ANOVA followed by Tukey-Kramer's post hoc analysis), $n = 8$ each. Abbreviations: Act A, activin A; bFGF, basic FGF; CYC, KAAD-cyclopamine; DIC, differential interference contrast; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GLP-1, glucagon-like peptide; Ins1, insulin 1; ITS, insulin-transferrin-selenium; NAM, nicotinamide; PNM, Pdx1, NeuroD, and MafA-11R; RA, retinoic acid.

treatment increased the expression of *Pdx1*, *NeuroD*, *MafA*, *Glut2*, and *Kir6.2* (Fig. 5).

Because Ln5 is a major component of 804F ECM, we examined whether the effect of 804G ECM could be achieved with recombinant Ln5. Mouse ES cells were cultured on dishes coated with Ln5 in differentiation medium for 17 days. The percentage of Ins1-GFP-expressing cells and levels of Ins1 were significantly increased by treatment with recombinant Ln5 (Fig. 6A, 6B), suggesting that the effect of 804G ECM is related to Ln5. We next examined the potential for glucose-stimulated secretion of C-peptide in cells treated with Ln5 and transduced with Pdx1, NeuroD, and MafA-11R. The amount of C-peptide released by the cells stimulated with high glucose (20 mM) was approximately 3.7-fold greater than after basal (2.5 mM) stimulation, in a manner similar to that in adult mouse islets (4.9-fold; Fig. 6C).

The physiological competence of differentiated cells to maintain glucose homeostasis in vivo was evaluated by transplantation into mice with STZ-induced diabetes. ING112 mouse ES cells were transduced with Pdx1, NeuroD, and MafA and treated with 804G ECM. After 17 days, the cells were collected by trypsin treatment, and 1×10^7 cells were transplanted into kidney capsules of severe combined immunodeficient (SCID) mice in which diabetes was

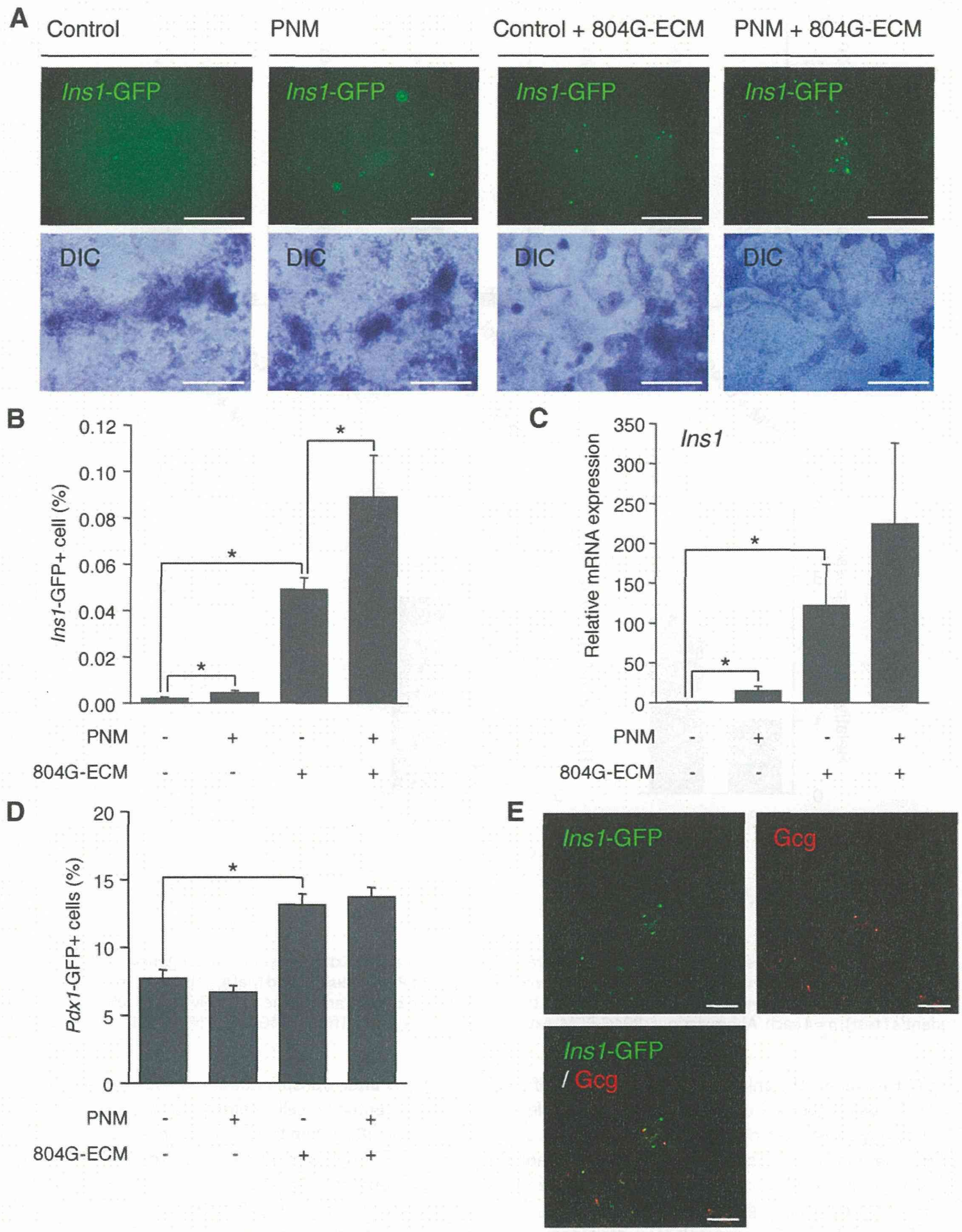

Figure 4. The 804G-ECM treatment facilitates the differentiation of mouse embryonic stem (ES) cells into insulin-producing cells. **(A):** Ins1-GFP fluorescence on day 17. On day 0, ING112 mouse ES cells were plated on untreated or 804G ECM-treated wells and induced to differentiate. Recombinant proteins were added to cells at a final concentration of 1 μM. Scale bars = 200 μm. **(B)** Values are the percentage of Ins1-GFP-positive cells per well of a 96-well plate. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (one-way analysis of variance [ANOVA] followed by Tukey-Kramer's post hoc analysis), $n = 5$ each. **(C):** Quantitative reverse transcription polymerase chain reaction analysis of Ins1 expression in ING112 mouse ES cells at day 17. Data are shown as the mean plus or minus standard error of the mean. *, $p < .05$ versus corresponding control (one-way ANOVA followed by Tukey-Kramer's post hoc analysis), $n = 9$ each. **(D):** Pdx1-GFP-positive cells differentiated from SK7 mouse ES cells were quantified by flow cytometry at day 11. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (one-way ANOVA followed by Tukey-Kramer's post hoc analysis), $n = 4$ each. **(E):** Immunofluorescence analysis of ING112 mouse ES cells for Ins1-GFP and glucagon was performed on day 17 of differentiation. Scale bars = 200 μm. Abbreviations: 804G-ECM, extracellular matrix derived from 804G cells; DIC, differential interference contrast; GFP, green fluorescent protein; Ins1, insulin 1; PNM, Pdx1, NeuroD and MafA-11R.

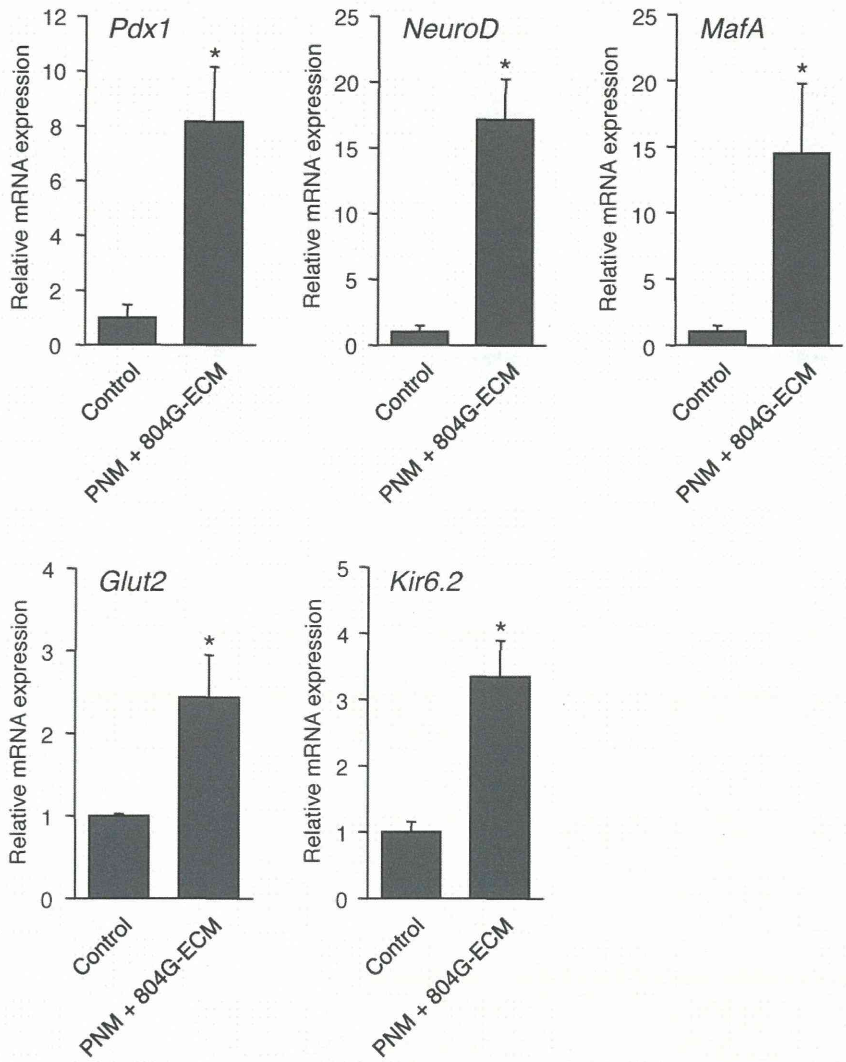

Figure 5. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of pancreatic marker genes in differentiated ING112 mouse embryonic stem cells treated with 804G and transduced with recombinant Pdx1, NeuroD, and MafA. RNA was extracted from the cells on day 17 of differentiation. Quantitative RT-PCR was performed on the indicated genes. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (Student's *t* test), $n = 4$ each. Abbreviations: 804G-ECM, extracellular matrix derived from 804G cells; PNM, Pdx1, NeuroD, and MafA-11R.

induced by STZ treatment. The animals transplanted with differentiated cells showed reversed diabetes (blood glucose level <200 mg/dl) by day 21 in three of the nine recipient mice, and normoglycemia was maintained up to 105 days after transplantation, whereas no recipient showed decreased blood glucose levels in the sham-operated group (Fig. 6D). As a positive control, 100 islets isolated from adult mice were transplanted into kidney capsules of STZ-treated SCID mice. With our protocol, transplantation with mouse islets decreased blood glucose levels in three of the nine recipient mice (Fig. 6D). Next, to examine insulin expression in the grafts, SCID mice were treated in the same way as described. At 35 days after transplantation, the grafts were extracted and examined for Ins1-GFP fluorescence and Ins1 expression. Several Ins1-GFP-positive cells were found in the grafts (supplemental online Fig. 4A), and the grafts kept expressing Ins1 after transplantation (supplemental online Fig. 4B). In contrast, no Ins1 expression was detected in the right kidney, the nontransplanted side. Quantitative RT-PCR showed that Ins1 expression in the grafts after transplantation was 11-fold higher than that in the differentiated cells before transplantation (supplemental online Fig. 4B). When the grafts were removed by unilateral nephrectomy from mice that showed restored normoglycemia in the differentiated cell group, these mice became hyperglycemic (supplemental online Fig. 4C). This result confirms that the decreased blood glucose level of STZ-treated mice was related to the transplanted cells.

Differentiation of Human iPS Cells With Pdx1 Protein Transduction

Finally, we examined whether protein transduction could facilitate the differentiation of human iPS cells. The human iPS cell line iPSC23 was derived from human fibroblasts. The iPSC23 cells showed human ES cell-like morphology and expressed a typical pluripotency marker (supplemental online Fig. 5A–5C). Histological examination of teratomas derived from iPSC23-transplanted

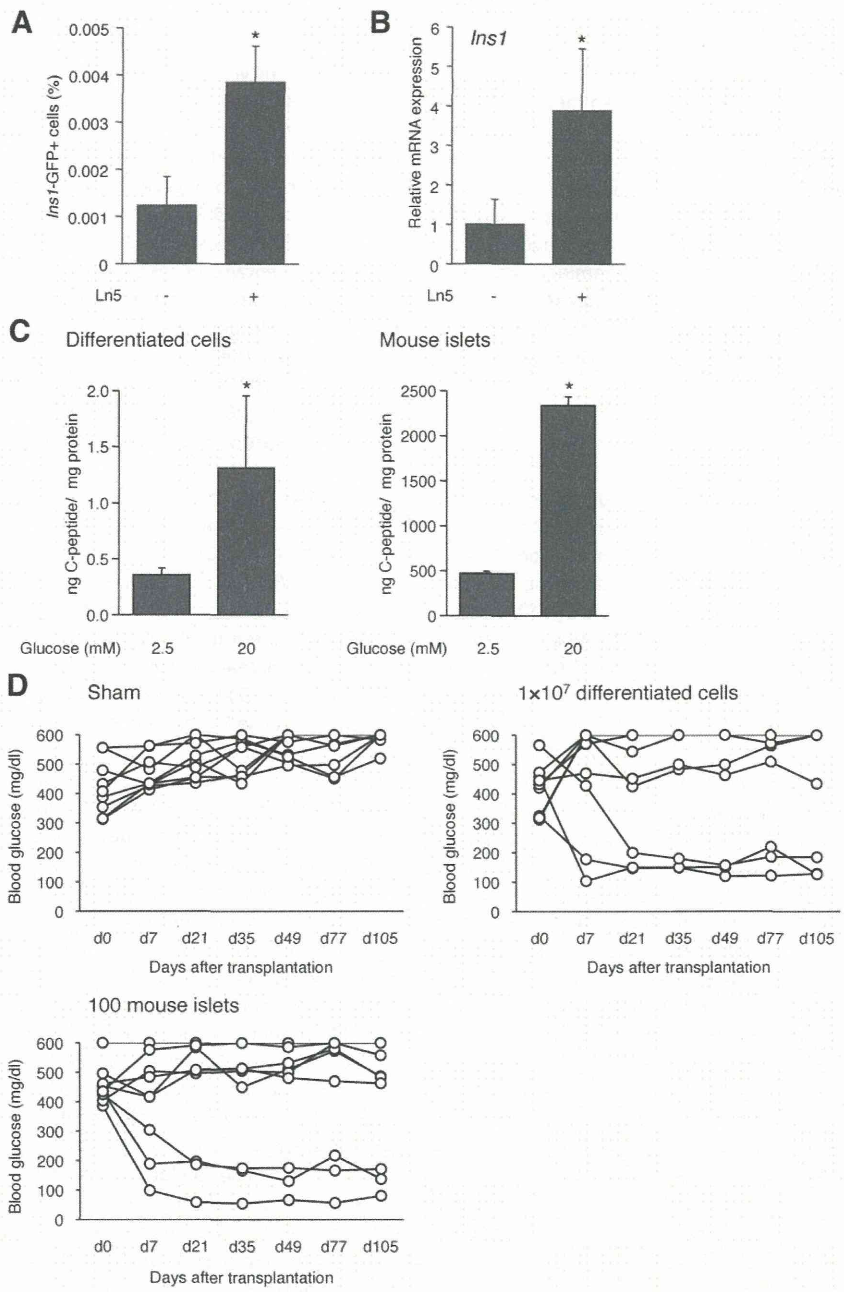

Figure 6. Recombinant Ln5 facilitates the differentiation of mouse embryonic stem cells into insulin-producing cells. **(A):** Values are the percentage of Ins1-GFP-positive cells per well of a 96-well plate on day 17 of differentiation. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (Student's *t* test), $n = 3$ each. **(B):** Quantitative reverse transcription polymerase chain reaction analysis of Ins1 expression in the differentiated cells treated with Ln5. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (Student's *t* test), $n = 3$ each. **(C):** Secretion of glucose-responsive C-peptide. On day 17, differentiated cells treated with Ln5 and protein transduction were subjected to a C-peptide secretion assay. The medium was replaced with Krebs-Ringer solution, and the cells were stimulated with 2.5 mM and 20 mM D-glucose. The amount of mouse C-peptide released in the medium was analyzed by enzyme-linked immunosorbent assay and normalized to the total amount of protein. Adult mouse islets were also subjected to the C-peptide secretion assay (right graph). Data are the mean ± SEM. *, $p < .05$ versus corresponding control (Student's *t* test), $n = 4$ each. **(D):** Diabetic mice were generated by streptozotocin treatment (150 mg/g, twice). Differentiated cells (1×10^7) derived from ING112 mouse embryonic stem cells treated with extracellular matrix derived from 804G cells and protein transduction were transplanted under the kidney capsule of severe combined immunodeficient (SCID) mice. As a positive control, islets were isolated from ICR mice, and 100 islets were picked and transplanted into streptozotocin-treated SCID mice. Sham refers to sham-operated mice given culture medium only. Blood glucose levels were measured on day 0–105 after transplantation. Each line represents one recipient mouse. Abbreviations: Ins1, insulin 1; Ln5, laminin 5.

SCID mice showed that the tumors contained various tissues (supplemental online Fig. 5D). To induce differentiation into pancreatic endocrine cells, we used the protocol reported by D'Amour et al. [34] (Fig. 7A). During differentiation, iPSC23 showed marked increases in *SOX17* and *FOXA2* expression on day 4. After stage 1, *PDX1* and *NGN3* expression began to peak on day 13 and 10, respectively (Fig. 7B). These gene expression patterns were consistent with previous reports [34]; however, *INS* gene expression was not observed, even after stage 5 (data not shown). Consequently, in this study, we examined whether transduction of Pdx1 protein affects *NGN3* expression levels, a marker of pancreatic progenitor. Treatment with Pdx1 during stages 2 and 3 caused a significant increase in NGN3 in both rat and human proteins (Fig. 7C).

Discussion

In the present study, we established a method of generating insulin-producing cells using 804G ECM treatment and the transduction of Pdx1, NeuroD, and MafA-11R. Transduction of the three proteins facilitated the differentiation of mouse ES and mouse iPS cells into insulin-producing cells. The 804G ECM treatment induced their differentiation into Pdx1-positive pancreatic progenitor cells. The differentiated cells secreted C-peptide in a glucose-dependent manner and, in some cases, restored normoglycemia when transplanted into diabetic mice.

Pdx1 and NeuroD can be transduced into cells via their own PTD [22, 23]. MafA was fused with 11R as a CPP, and MafA-11R was capable of penetrating cells. Pdx1 is a master regulator of both pancreatic development and the differentiation of progenitor cells into the β-cell phenotype [42, 43]. The expression patterns of Pdx1 in the developing pancreas are maintained throughout development and provide both spatial and temporal contributions to the commitment of endoderm to a pancreatic phenotype [44]. NeuroD is the basic helix-loop-helix transcription factor and is a key regulator of insulin gene transcription in pancreatic β cells [45]. Expression of the NeuroD gene is activated by Ngn3 [21]. Like Ngn3, ectopic expression of NeuroD induces premature endocrine differentiation in the pancreas [46]. MafA is part of a family of large Maf transcription factors and is crucial for glucose-responsive insulin gene transcription in β cells [47–50]. MafA is also important in the embryonic development of the pancreas and adult β-cell function [47, 51, 52]. Pdx1, Ngn3, and MafA are powerful inducers of the differentiation into β cells of non-β cells [5]. NeuroD is also an inducer of differentiation into β cells [5]. Transcription factors involved in regulation of the expression of key genes required in the developing endocrine pancreas have been identified [18]. The pancreas originates early in development on embryonic day 8.5 to embryonic day 9.5 in the mouse [53, 54]. Expression of Pdx1 begins in epithelial cells of the dorsal and ventral pancreatic anlage on embryonic day 8.5 [42, 55, 56]. NeuroD expression occurs in a subset of pancreatic epithelial cells as early as embryonic day 9.5 [57]. MafA is expressed in early β cells during the second developmental transition on embryonic day 14 [49, 58]. In the present study, we examined the combination of transduced proteins and timing of the transduction most able to induce differentiation. The use of Pdx1 on days 5 and 7, NeuroD on days 9 and 11, and MafA-11R on days 13 and 15 was found to be most effective. The order of transduction is similar to that for developmental expression in vivo. Thus the order in which Pdx1, NeuroD, and MafA-11R are transduced may be crucial to the differentiation of ES and iPS cells into insulin-producing cells. Treatment with these three proteins did not affect the yield of definitive endoderm and pancreatic progenitors, as shown in supplemental online Figure 2A–2C. It suggests that this treatment facilitated the latter period of differentiation, such as endocrine progenitors and insulin-producing cells. In Figure 3C, the percentage of insulin-positive cells is 0.799% after Pdx1, NeuroD, and MafA-11R treatment in the SK7 mouse ES cell line, whereas in the ING112 cell line, the percentage of Ins1-GFP-positive cells is 0.004% (Fig. 4B). There is some variation of differentiation efficiency between these two ES cell lines. One of the reasons for this variation might be differences in the genetic background. The SK7 line was established from B6CBAF1 mouse strains and the ING112 line from CD-1 mouse strains [26–29]. ES cell lines established from different mouse strains showed variability in endodermal differentiation capacity [59].

The 804G ECM derived from rat bladder carcinoma has been used for mouse, rat, and human pancreatic islet cell cultures [31, 39–41, 60–63]. The ECM is rich in Ln5 [64–66], a heterotrimer consisting of $\alpha 3$, $\beta 3$, and $\gamma 2$ subunits, and an essential component of several epithelial basement membranes [67, 68] and improves glucose-stimulated insulin secretion, survival, and proliferation of primary pancreatic β cells [31, 39–41]. In the present study, we showed that 804G ECM significantly increased the efficiency with which mouse ES cells and human iPS cells differentiate into insulin-producing cells. The finding that the ECM increased the number of Pdx1-GFP-positive cells on day 8 (Fig. 4D) may indicate that it predominantly facilitates the differentiation into pancreatic progenitors. In pancreatic development, precursor cells of endocrine pancreas coexpress insulin and glucagon [69]. In the case of in vitro differentiation from ES cells, coexpression of insulin and glucagon was observed in some reports and was often referred to as "immature β cells" [34, 70]. In this study, coexpression of these two hormones did not occur in differentiated cells (Fig. 4E). This suggests that insulin-producing cells generated with our protocol were differentiated similarly to mature β cells. Experiments performed with recombinant Ln5 suggested that the protein is the major component of 804G ECM responsible for facilitating differentiation (Fig. 6A, 6B). The differentiated cells obtained using protein transduction and Ln5 treatment had the ability to secrete C-peptide in response to glucose stimulation. Furthermore, when transplanted, the differentiated cells restored normoglycemia in some diabetic mice. In this study, we transplanted 1×10^7 cells of entire cultures as differentiated cells. Because the efficiency of differentiation to insulin-producing cells was approximately 0.1% in the case of ING112 cells, as shown in Figure 4B, transplanted cells corresponded to 1×10^4 insulin-producing cells (0.1% of 1×10^7). Moreover, we showed that Ins1 gene expression was increased by approximately 10-fold in the grafts after transplantation (supplemental online Fig. 4B). Consequently, we speculate that the transplanted cells corresponded to 1×10^5 β cells (approximately 100 islets). To compare the effect of differentiated cells with adult mouse islets, 100 islets were transplanted and showed restored normoglycemia in three of the nine recipient mice (Fig. 6D). This efficacy is equivalent to the efficacy of differentiated cells and corresponds to a previous report that only 33% of diabetic mice showed normoglycemia after transplantation with 100 islets [71]. These results suggest that the insulin-producing cells generated in this study are functionally similar to mature pancreatic β cells.

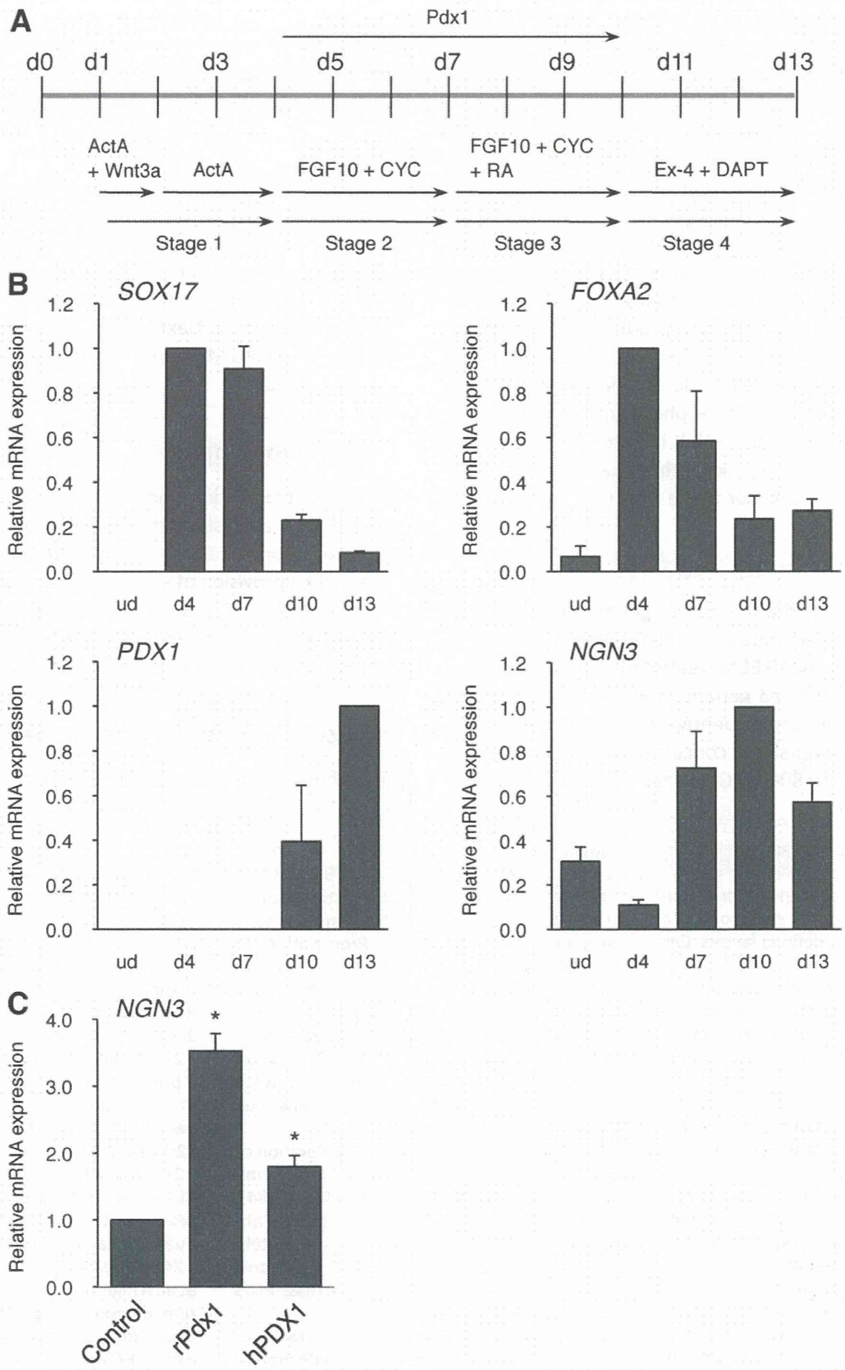

Figure 7. Protein transduction-induced generation of NGN3-expressing cells from human induced pluripotent stem (iPS) cells. **(A):** Scheme of pancreatic differentiation from human iPS cells and the timeline of protein transduction. Recombinant Pdx1 protein was added to cells at a final concentration of 1 μM from day 4 to day 10 of differentiation. **(B):** Dynamic gene expression patterns of *SOX17, FOXA2, PDX1,* and *NGN3* in human iPS cell line iPSC23 during differentiation were analyzed on days 0 (undifferentiated), 4, 7, 10, and 13 by quantitative reverse transcription polymerase chain reaction. Data are the mean ± SEM. $n = 3$ each. **(C):** Human iPSC23 cells were treated with rat Pdx1 or human PDX1 recombinant proteins at the time points shown in **(A)**. Total RNA was extracted on day 13 of differentiation, and *NGN3* expression was analyzed by quantitative reverse transcription polymerase chain reaction. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (one-way analysis of variance followed by Tukey-Kramer's post hoc analysis), $n = 3$ each. Abbreviations: Act A, activin A; CYC, KAAD-cyclopamine; DAPT, γ-secretase inhibitor; Ex-4, exendin-4; FGF, fibroblast growth factor; hPDX1, human PDX1; RA, retinoic acid; rPdx1, rat Pdx1; ud, undifferentiated.

Protein transduction is also useful for the pancreatic differentiation of human iPS cells. Treatment with Pdx1 protein significantly increased *NGN3* expression, a marker of pancreatic endocrine progenitors (Fig. 7C). Rat Pdx1 protein was more effective on *NGN3* expression than human PDX1 protein. It is considered that one of the reasons is the difference in purification efficiency between the two proteins. The rat recombinant was more efficiently purified than the human recombinant, and human PDX1 needed to be concentrated by centrifugal filters to adjust the concentration to the rat recombinant. These manipulations might lead to slight degradation of proteins and lower the transcriptional activity of proteins. Consistent with our result, rat Pdx1 is more efficiently translated than human PDX1 in in vitro transcription and translation [36].

In this study, we failed to detect insulin-positive cells using a previously reported differentiation protocol [34], even when treated with NeuroD and MafA-11R. Although several previous studies showed the generation of insulin-producing cells from human ES cells or human iPS cells in vitro [2–4, 34], various obstacles remain. The insulin-producing cells generated from these pluripotent cells tend to display immature phenotypes and in many instances are not fully functional [72]. It is necessary to develop a more efficient and nontumorigenic method that induces the differentiation of human iPS cells for tissue replacement therapy.

Conclusion

In this study, we developed a method of generating functional insulin-producing cells from mouse ES and mouse iPS cells using protein transduction and 804G ECM treatment. Protein transduction has no risk of unexpected genetic modifications by exogenous DNA in target cells. Consequently, the differentiated cells are expected to have fewer safety concerns such as tumorigenesis. We also found that 804G ECM treatment facilitated the expansion of Pdx1-positive pancreatic progenitors, leading to an increase in insulin-producing cells. This newly established method would provide an efficient and safe way to utilize patient-specific iPS cells for the treatment of diabetes.

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Author Contributions

T. Kaitsuka: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; H.N.: conception and design, provision of study material or patients; N.S. and S.K.: provision of study material or patients, data analysis and interpretation; T. Kubo, F.-Y.W., and F.H.: collection and/or assembly of data; K.T.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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