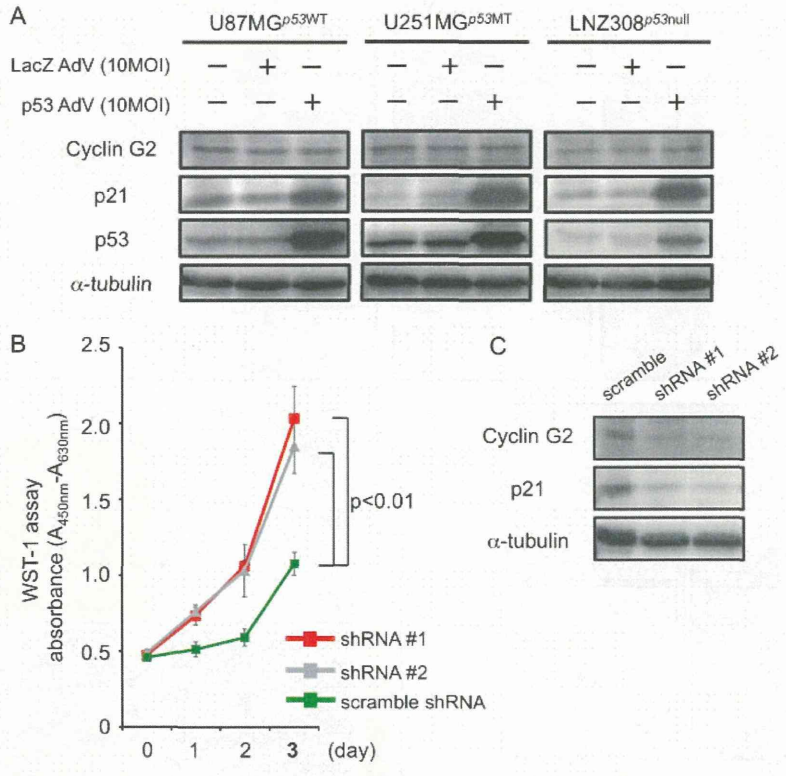

Figure W3. (A) Western blot analyses of U87MG (p53^{WT}), U251MG (p53^{Mut}), and LNZ308 (p53^{Null}) cells infected with *lacZ* or p53 adenovirus. These results show that the level of p53 does not affect that of cyclin G2 and forced expression of p53 does not induce cyclin G2 expression in GBM. (B) WST-1 assay showing the effect of cyclin G2's reduction on cell-cycle regulation in U87MG cells. (C) Cyclin G2 reduction resulted in p21 suppression.

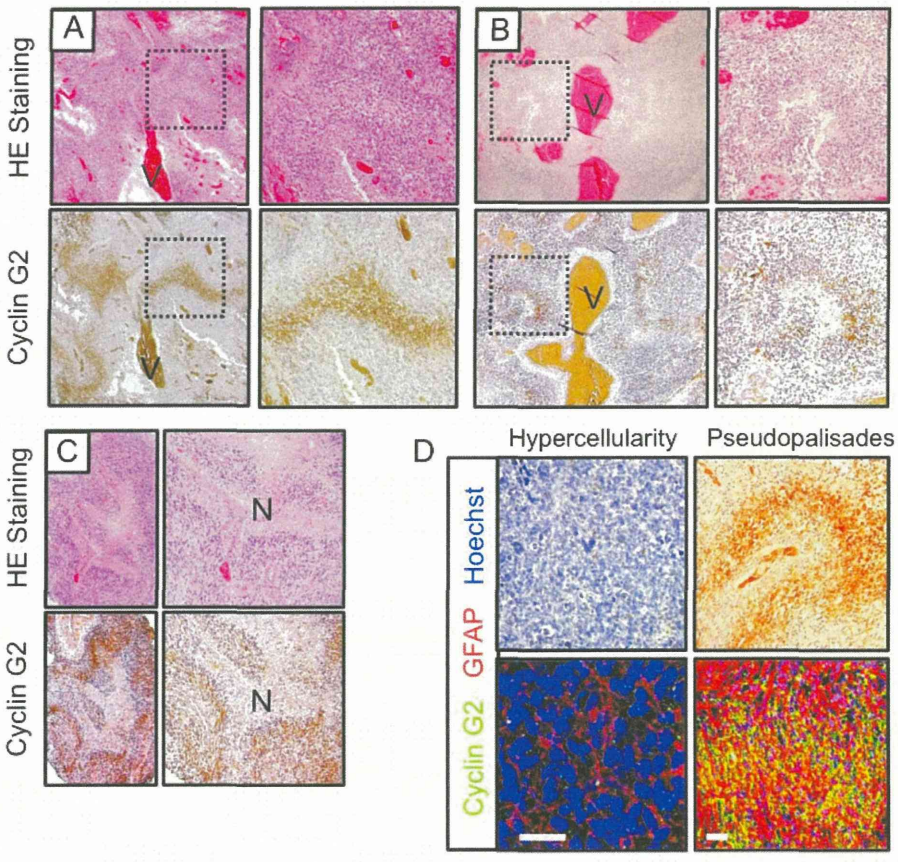

Figure W4. Cyclin G2 is abundant in pseudopalisade-forming glioma cells. (A–C) Cyclin G2 expression in pseudopalisades is observed in various types of GBM specimens. (D) Cyclin G2 is absent in high cellularity/high mitotic regions in GBM. The scale bars represent 10 μm in D. "V" and "N" indicate vessel and necrosis, respectively.

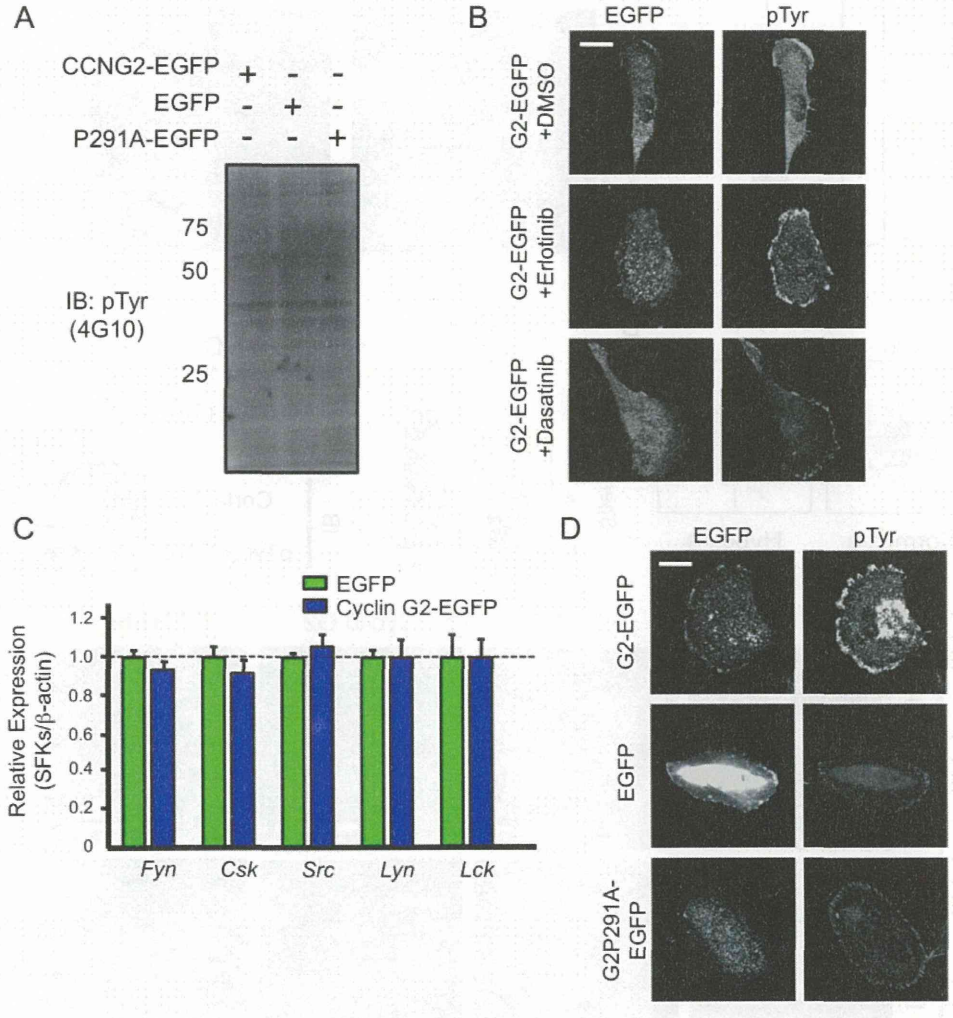

Figure W5. Cyclin G2 induces the restricted tyrosine phosphorylation of cortactin in an SFK-dependent manner. (A) Ectopic expression of cyclin G2 does not alter the total amount of tyrosine phosphorylation. (B) Dasatinib inhibits the phosphorylation induced by cyclin G2. Note that dasatinib, but not erlotinib, decreases the peripheral signals of phosphotyrosine induced by cyclin G2. The scale bar represents 10 μm in B. (C) Cyclin G2 does not enhance transcription of SFK mRNA. (D) Exogenous cyclin G2 increases, whereas the P291A mutant impairs, tyrosine phosphorylation signals at the juxtamembrane. The scale bar represents 10 μm in D.

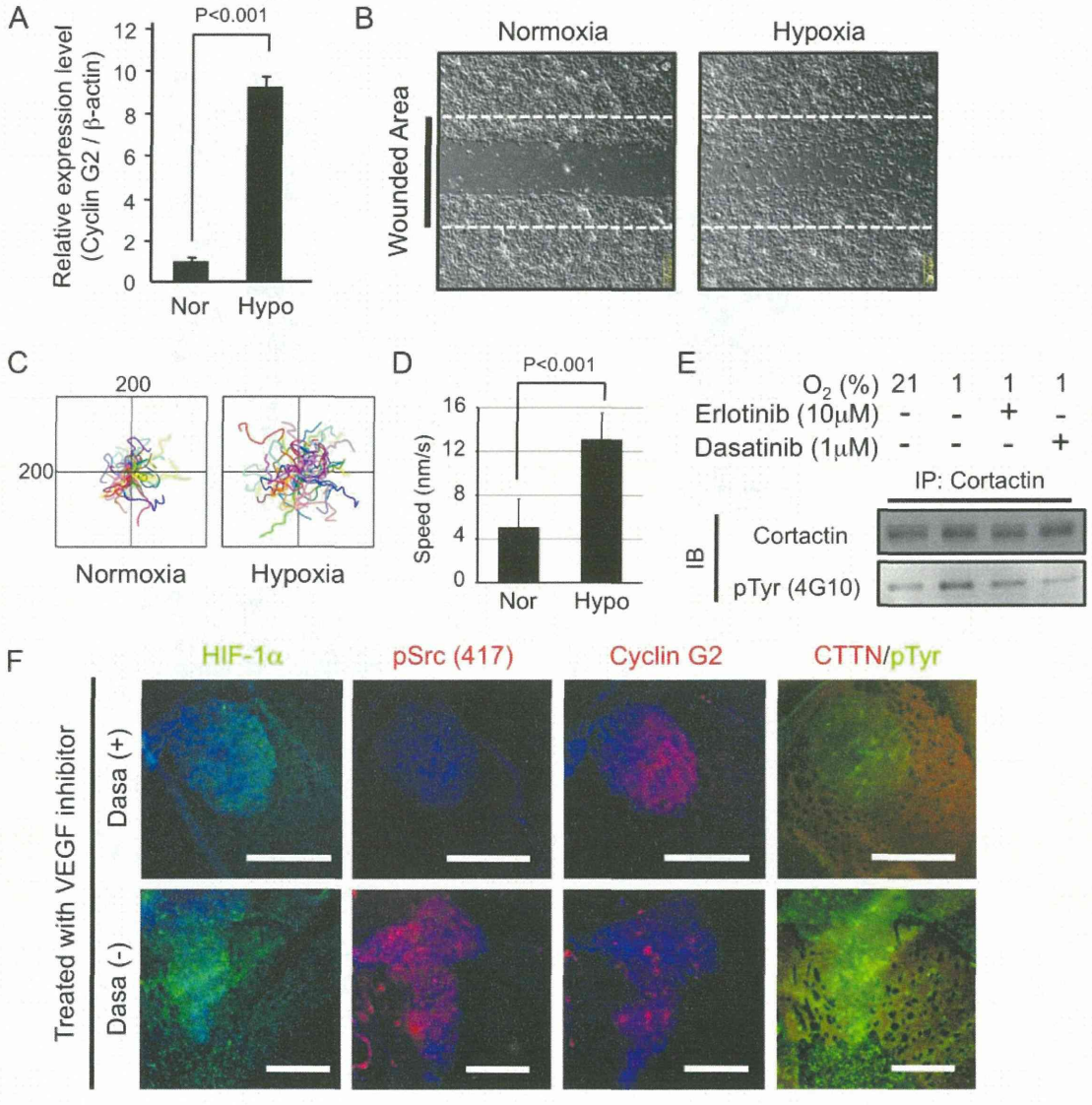

Figure W6. The effectiveness of dasatinib on the expansion of glioma-initiating cells. (A) Cyclin G2 expression is enhanced in response to hypoxia in murine glioma-initiating 005 cells. (B–D) Hypoxia stimulates the motility of 005 cells. (E) Tyrosine phosphorylation of cortactin is enhanced in response to hypoxic stimulation in 005 cells and dasatinib attenuates it. Note that 005 cells show the phosphorylation in a normoxic and steady state. (F) Dasatinib attenuates the hypoxia-driven local invasion of 005 cells. Note that dasatinib treatment inhibits the phosphorylation of src and cortactin. The scale bars represent 200 μm (F).

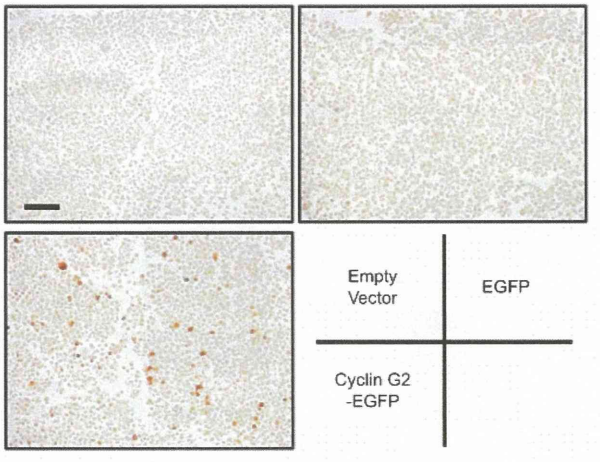

Figure W7. Goat anti-human cyclin G2 antibody (Santa Cruz Biotechnology, Inc) successfully recognized ectopic cyclin G2 in paraffin-embedded HEK293 cells that were transfected with cyclin G2–EGFP. For other applications including immunoblot analysis, see references. The scale bar represents 100 μm.

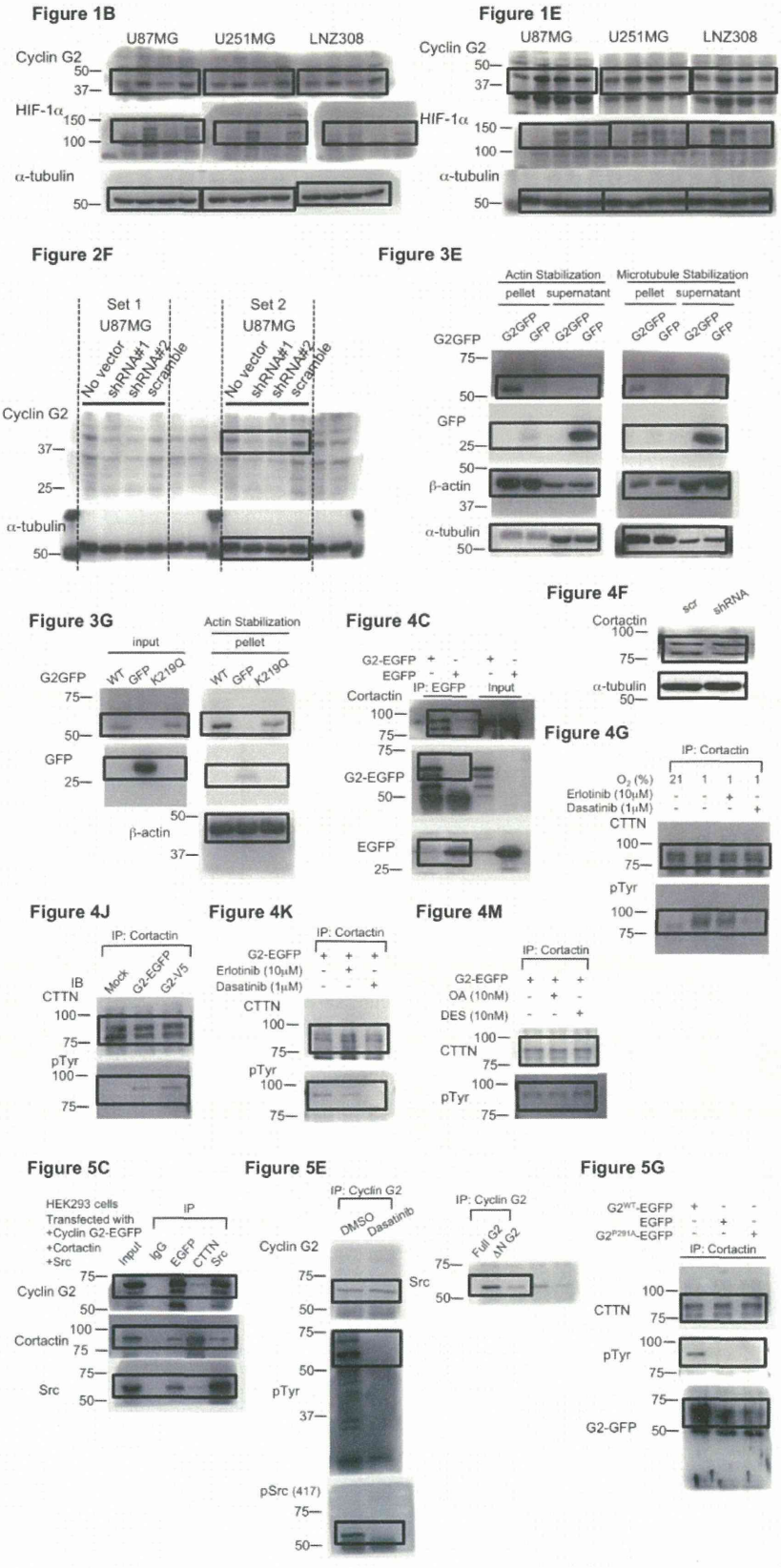

Figure W8. Full scans of the immunoblots shown in the figures. Boxes indicate the parts used in the figures, and numbers indicate the molecular weights. All data were obtained with the VersaDoc Imaging System (Bio-Rad).

Generation of Functional Insulin-Producing Cells From Mouse Embryonic Stem Cells Through 804G Cell-Derived Extracellular Matrix and Protein Transduction of Transcription Factors

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Generation of Functional Insulin-Producing Cells From Mouse Embryonic Stem Cells Through 804G Cell-Derived Extracellular Matrix and Protein Transduction of Transcription Factors

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Key Words. Diabetes • Embryonic stem cells • Pancreatic differentiation • Transcription factors • Induced pluripotent stem cells

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ABSTRACT

Embryonic stem (ES) and induced pluripotent stem (iPS) cells have potential applications to regenerative medicine for diabetes; however, a useful and safe way to generate pancreatic β cells has not been developed. In this study, we tried to establish an effective method of differentiation through the protein transduction of three transcription factors (Pdx1, NeuroD, and MafA) important to pancreatic β cell development. The method poses no risk of unexpected genetic modifications in target cells. Transduction of the three proteins induced the differentiation of mouse ES and mouse iPS cells into insulin-producing cells. Furthermore, a laminin-5-rich extracellular matrix efficiently induced differentiation under feeder-free conditions. Cell differentiation was confirmed with the expression of the insulin 1 gene in addition to marker genes in pancreatic β cells, the differentiated cells secreted glucose-responsive C-peptide, and their transplantation restored normoglycemia in diabetic mice. Moreover, Pdx1 protein transduction had facilitative effects on differentiation into pancreatic endocrine progenitors from human iPS cells. These results suggest the direct delivery of recombinant proteins and treatment with laminin-5-rich extracellular matrix to be useful for the generation of insulin-producing cells. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:114–127*

INTRODUCTION

Diabetes mellitus is a devastating disease, and the World Health Organization expects the number of diabetic patients to increase to 300 million by the year 2025. Although insulin therapy has proved useful for the treatment of diabetes, it is hoped that β-cell-replacement therapy will be even more effective; however, there is a limited supply of donor β cells. The generation of new β cells from expandable stem cell sources is necessary for regenerative medicine. Human embryonic stem (ES) cells hold promise as a source of new β cells. Furthermore, the discovery that adult stem cells can be reprogrammed to revert back to induced pluripotent stem (iPS) cells has raised the possibility of generating patient-specific cell types [1].

Several groups have reported ways of generating pancreatic cell lineages from ES and iPS cells [2–4]. These methods induce definitive endoderm differentiation in the first stage and then pancreatic specialization and maturation in subsequent stages, using combinations of growth factors and small molecules; however, the differentiated cells are immature or not fully functional in culture. Although delivering a specific combination of genes for transcription factors using adenoviral vectors can reprogram differentiated pancreatic exocrine cells in adult mice to become cells that closely resemble β cells [5], this method may be of limited use for patient treatment because of the risk of unexpected genetic modifications by exogenous DNA.

The cellular delivery of various biological compounds such as bioactive proteins has been improved recently by conjugating the compounds to short peptides known as cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) [6–8]. The initial discovery of CPPs or PTDs originated from the unexpected observation that certain full-length proteins or protein domains can translocate across the plasma membrane. This was first shown for the HIV Tat transactivator [9, 10] and for the homeodomain of the *Drosophila melanogaster* transcription factor Antennapedia [11] and has since expanded to include "nonnatural" peptides that share this property. CPPs and PTDs are widely used in research, and, impressively, multiple clinical trials are testing the PTD-mediated delivery of macromolecular drug conjugates in patients with a variety of

diseases [12]. In the research field of regenerative medicine, it was shown that protein transduction with CPPs is useful for the generation of iPS cells from human and mouse fibroblasts [13, 14]. Moreover, protein transduction has been shown to be useful for pancreatic differentiation. Pancreatic transcription factors containing PTD drive mouse ES cells toward endocrine pancreas [15]. In addition, Vargas et al. showed that Tat-mediated transduction of MafA protein in utero enhanced pancreatic insulin production [16].

Transcription factors involved in pancreatic development have been identified by gene knockout and cell-type-specific gene expression studies [17–19]. A specific combination of Pdx1, Ngn3, and MafA reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β cells [5]. Moreover, the combined expression of these transcription factors by adenoviral vectors in mouse ES cells improves the differentiation efficiency into insulin-producing cells [20]. Ngn3 functions as a transcriptional activator of NeuroD through multiple E boxes present within the minimal NeuroD promoter [21], suggesting that NeuroD can be substituted for Ngn3 [5]. It is thought that the delivery of Pdx1, NeuroD, and MafA into ES and iPS cells by protein transduction has the potential to generate pancreatic β cells.

In this study, we tried to develop an effective method of pancreatic differentiation through protein transduction using three transcription factors, Pdx1, NeuroD, and MafA. We previously showed that purified Pdx1 could be transduced into cells and that the 16 amino acids of Pdx1 truly form a PTD [22]. NeuroD protein also has an arginine- and lysine-rich PTD sequence and can permeate several cells [23]. It is expected that these two proteins would be easily transduced into ES or iPS cells via their own PTDs. MafA was fused with 11 polyarginines (11R) as a CPP [24, 25]. Protein transduction of the three transcription factors significantly induced the differentiation of mouse ES and mouse iPS cells into insulin-producing cells. We also found that the extracellular matrix (ECM) derived from 804G cells, a rat bladder carcinoma cell line, significantly induced differentiation into pancreatic progenitors and insulin-producing cells. The differentiated cells also secreted glucose-responsive C-peptide, and their transplantation restored normoglycemia in some diabetic mice. Furthermore, protein transduction of Pdx1 significantly increased *NGN3* expression in human iPS cells during pancreatic differentiation. These results suggest that the direct delivery of recombinant proteins is useful for the differentiation of ES and iPS cells into insulin-producing cells that are functionally similar to β cells.

Materials and Methods

Construction of Vectors and Purification of Recombinant Proteins

Construction of the pET21a (+) expression plasmid containing rat Pdx1 and rat NeuroD cDNA was reported previously [22, 23]. For the recombinant form of MafA fused with 11R, mouse full-length MafA cDNA was amplified by polymerase chain reaction (PCR) using appropriate linker primers, and the 11R sequence was subcloned into the XhoI-NotI sites of pGEX-6p-1. BL21 (DE3) cells transformed with each plasmid were grown at 37°C to an optical density at 600 nm (OD 600) of 0.8. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.1 mM, and the cells were incubated for 12 hours at 24°C. Cells were lysed and sonicated in nondenaturing lysis buffer (20 mM Hepes, pH 8.0, 100 mM sodium chloride, 1% TritonX-100, 0.1 mg/ml lysozyme), and the supernatant was recovered. For the purification of Pdx1 and NeuroD, the supernatant was applied to a column of TALON resin (Clontech, Mountain View, CA, http://www.clontech.com). The MafA-11R protein was purified using glutathione sepharose (GE Healthcare, Piscataway, NJ, http://www.gehealthcare.com), and the glutathione-*S*-transferase tag was cleaved off with PreScission protease (GE Healthcare). We tried to purify MafA-11R by using the 6-histidine tag and TALON resin but failed because of the formation of inclusion bodies for almost all proteins. Consequently, we used the glutathione-*S*-transferase tag and glutathione sepharose for purification of MafA-11R. In Figure 1C, purified proteins were conjugated using an Alexa 568 protein labeling kit (Molecular Probes, Eugene, OR, http://probes.invitrogen.com).

Mouse ES and iPS Cell Lines

The mouse ES cell line SK7, containing a Pdx1-promoter-driven green fluorescent protein (GFP) reporter transgene, was established by culturing blastocysts obtained from transgenic mice homozygous for the Pdx1-GFP gene [26, 27]. The mouse ES cell line, ING112, containing an insulin 1 (Ins1) promoter-driven GFP reporter transgene, was established by culturing the blastocysts obtained from transgenic mice homozygous for the Ins1-GFP gene [28, 29]. Both mouse ES cell lines were maintained on mouse embryonic fibroblast (MEF) feeders in Glasgow Minimum Essential Medium supplemented with 1,000 U/ml leukemia inhibitory factor (LIF), 15% KnockOut serum replacement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 1% fetal bovine serum (FBS), 100 μM nonessential amino acids (NEAA), 2 mM L-glutamine (L-Gln), 1 mM sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin (P/S), and 100 μM β-mercaptoethanol (β-ME). The mouse iPS cell line, 20D-17 [30], was maintained on MEF feeders in Dulbecco's modified Eagle's medium (DMEM) supplemented with LIF, 15% FBS, NEAA, L-Gln, P/S and β-ME.

Differentiation of Mouse ES Cells Into Insulin-Producing Cells

Before differentiation, mouse ES cells were passaged using 0.25% trypsin and seeded at 5,000 cells per well on 96-well plates with an Ultra-Web synthetic surface (Corning, Tewksbury, MA, http://www.corning.com/lifesciences). After overnight culture, the cells were cultured in high-glucose (4,500 mg/l) DMEM supplemented with 10 mg/l insulin, 5.5 mg/l transferrin, 6.7 μg/ml sodium selenite (insulin-transferrin-selenium G supplement; Invitrogen), 2.5 mg/ml ALBUMAX II (Invitrogen), NEAA, L-Gln, P/S, β-ME, 10 ng/ml activin A (R&D Systems, Minneapolis, MN, http://www.rndsystems.com), and 5 ng/ml basic fibroblast growth factor (FGF; Peprotech, Rocky Hill, NJ, http://www.peprotech.com) from day 1 to day 6. From day 7 to day 10, the culture medium was changed to RPMI supplemented with NEAA, L-Gln, P/S, β-ME, B27 supplement (Invitrogen), 50 ng/ml FGF10 (Peprotech), 250 nM KAAD-cyclopamine (Calbiochem, San Diego, CA, http://www.emdbiosciences.com), and 1 μM retinoic acid (Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com). At day 11, the medium was switched to low-glucose (1,000 mg/l) DMEM supplemented with insulin-transferrin-selenium G supplement, ALBUMAX II, NEAA, L-Gln, PS, β-ME, 10 mM nicotinamide (Sigma-Aldrich), and 10 nM glucagon-like peptide (Sigma-Aldrich), and the cells were cultured until day 17. Protein transduction was

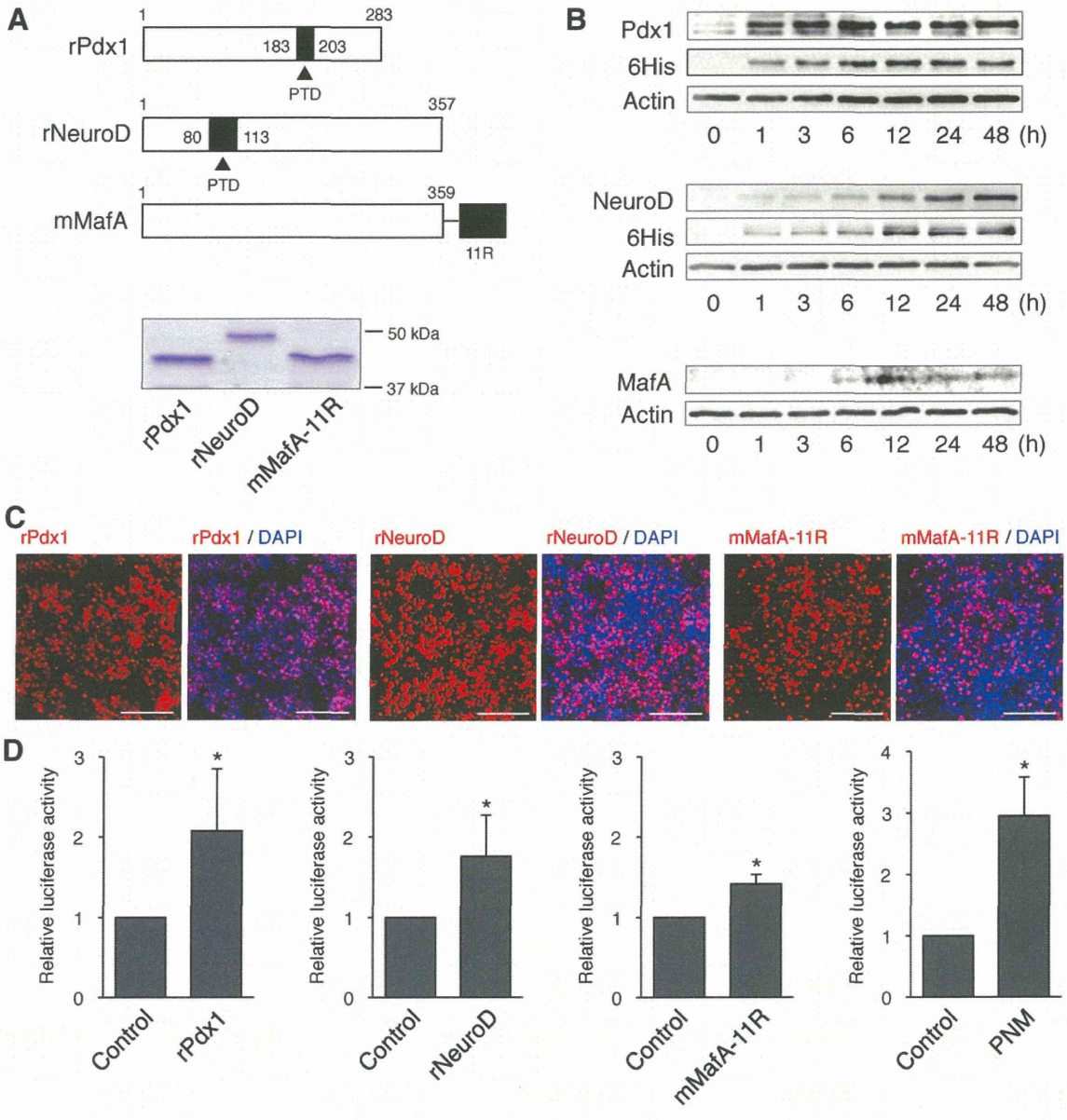

Figure 1. Protein transduction of Pdx1, NeuroD, and MafA-11R. **(A):** Upper panels show schematic representation of rat Pdx1, rat NeuroD, and mouse MafA-11R recombinant proteins. Pdx1 and NeuroD can permeate cells because of an arginine- and lysine-rich PTD. Because MafA does not have a PTD, a polyarginine sequence was fused at the C terminus. The lower panel shows purified proteins analyzed by Coomassie blue staining. **(B):** Time-dependent changes of rPdx1 (upper), rNeuroD (middle), and mMafA-11R (lower) protein transduction in mouse embryonic stem cells were analyzed by Western blotting using the indicated antibodies. Recombinant proteins were added to cells at a final concentration of 1 μM. Actin was used for a loading control. **(C):** Mouse embryonic stem cells were treated with each protein labeled with Alexa 568-fluorescence dye for 6 hours at a final concentration of 1 μM, and images were taken by confocal microscopy. Scale bars = 100 μm. **(D):** Hela cells were transfected with rat insulin promoter-driven reporters. After 24 hours, cells were treated with rPdx1, rNeuroD, mMafA-11R, or a combination of the three proteins at a final concentration of 1 μM for 6 hours, and luciferase activity was measured. Data are the mean ± SEM. *, $p < .05$ versus corresponding control (Student's *t* test), $n = 4$ each. Abbreviations: 6His, six histidines; 11R, 11 polyarginines; DAPI, 4′,6-diamidino-2-phenylindole; mMafA, mouse MafA; PNM, Pdx1, NeuroD, and MafA-11R; PTD, protein transduction domain; rNeuroD, rat NeuroD; rPdx1, rat Pdx1.

performed on days 5, 7, 9, 11, 13, and 15. Recombinant forms of Pdx1, NeuroD, and MafA-11R were added to cells at a final concentration of 1 μM. For the time-dependent analysis of transduced proteins, cells were washed with phosphate-buffered saline, and the whole cell lysates were extracted and subjected to Western blotting using anti-Pdx1 (Millipore, Billerica, MA, http://www.millipore.com), anti-NeuroD (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), anti-6His (Wako Chemical, Osaka, Japan, www.wako-chem.co.jp/english), and anti-MafA (Bethyl Inc., Montgomery, TX, http://www.bethyl.com).

Preparation of the 804G-ECM

ECM from medium conditioned by 804G cells was prepared and used as described previously [31]. The cells were grown in DMEM (Invitrogen), containing 10% FBS and 1,000 mg/l glucose. At confluence, they were rinsed and maintained for another 3 days in the same medium. Conditioned medium (referred to as "804G-ECM") was collected, centrifuged at 120*g* for 10 minutes to remove any detached cells and debris, filtered through a 0.22-μm Millipore filter, and frozen at −20°C for later use. Culture plates were coated with 804G-ECM. Plates were incubated at