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Neural differentiation of mouse induced pluripotent stem cells using cadherin gene-engineered PA6 feeder cells

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Investigating neural differentiation of pluripotent stem cells, including induced pluripotent stem (iPS) cells, is of importance for studying early neural development and providing a potential source of cells for nerve regeneration. Stromal cell-derived inducing activity (SDIA) using PA6 stromal cells promotes neural differentiation of iPS cells. Thus, we hypothesized that cadherin gene-engineered PA6 feeder cells will enhance the performance of SDIA by facilitating cell–cell interactions. Consequently, we created cadherin gene-engineered PA6 cells. Efficiency of neural differentiation from mouse iPS cells on PA6 feeder cells overexpressing E-cadherin gene (46%) or N-cadherin gene (27%) was significantly higher compared with parental PA6 feeder cells (19%). In addition, efficiency of motor neuron differentiation from mouse iPS cells on cadherin-gene engineered feeder cells (E-cadherin, 7.4%; N-cadherin, 11%) was significantly higher compared with parental PA6 feeder cells (4.1%). Altogether, these results indicate that cadherin gene-engineered feeder cells are a potent tool for promoting neural differentiation of pluripotent stem cells.

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[**Key words:** Cadherin; iPS cell; Motor neuron; Neural differentiation; PA6 feeder]

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are an important tool for regenerative medicine and biological research (1). To study early neural development and also offer a potential source of cells for nerve regeneration, investigating neural differentiation of ES/iPS cells is of important significance (2). Embryoid bodies (EBs) are commonly used for neural differentiation of ES/iPS cells (3,4). However, EBs derived from ES/iPS cells contain various types of cells including non-neuronal subtypes and undifferentiated cells (5). To improve neural differentiation efficiency of ES/iPS cells, several protocols have been developed including inhibition of the BMP/SMAD signaling pathway (6,7), overexpression of Nurr1 as a critical transcription factor (8), and feeder cell-mediated induction (9). Among them, one of the most efficient *in vitro* neural differentiation methods is based on stromal cell-derived inducing activity (SDIA) by co-culture of ES/iPS cells with mouse stromal PA6 cells as a feeder layer (9). The SDIA method offers advantages over other methods, especially in terms of its technical simplicity and high efficiency. Furthermore, by timely exposure to patterning factors, Mizuseki et al. (10) have established SDIA-based methods for systematic induction of the neural crest, and dorsal-most central nervous system (CNS) and ventral-most CNS differentiation. Using the SDIA method, addition of sonic hedgehog (Shh) protein promotes differentiation of ventral CNS tissue, while additional retinoic acid (RA) treatment induces motor neurons (10).

Regulation of stem cell development depends on cell–cell and cell–matrix interactions that are involved in guiding the extracellular matrix (ECM) and adhesion molecule signaling (11). Among cell adhesion molecules, cadherins are a class of type-1 transmembrane homophilic adhesion proteins. E-cadherin and N-cadherin are involved in ES/iPS cell pluripotency and neural cell development, respectively (12). During neural differentiation, ES/iPS cells show an E- to N-cadherin switch (13). Haque et al. (14) established a cadherin-based artificial ECM substrate by immobilizing both E-cadherin and N-cadherin. Consequently, they demonstrated specific differentiation of mouse ES/iPS cells into neural cells. Alternatively, cell–cell interactions between ES/iPS cells and co-cultured cells are thought to have major implications for tissue differentiation (15,16). We hypothesized that cadherin gene-engineered feeder cells will enhance the performance of co-culture by facilitating cell–cell interactions. Previously, we established E-cadherin-expressing STO cells as a feeder layer to support undifferentiated culture of mouse ES/iPS cells. Accordingly, we demonstrated improved performance that was comparable to a conventional mouse embryonic fibroblast (MEF) feeder layer (17,18). In the present study, we investigated whether E- or N-cadherin gene transfer to PA6 cells can improve SDIA and induce accelerated neural differentiation of mouse iPS (miPS) cells.

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MATERIALS AND METHODS

Cell culture Mouse embryonic fibroblast (MEF) cells were isolated from fetuses of 14-d pregnant BALB/c mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G potassium and 50 μg/mL streptomycin sulfate). Animal experiments were approved by the Ethics Committee for Animal Experiments of Faculty of Engineering, Kyushu University (A28-173-1). Undifferentiated miPS cells (Ips-MEF-Ng-20S-17 iPS cell line; Riken BioResource Center, Ibaraki, Japan) (19) were maintained on mitotically inactivated MEF feeder cells, which were treated with 1 μg/mL mitomycin C for 2 h. Cells were cultured in iPS medium composed of Knockout-DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 4 mM L-glutamine, non-essential amino acids (Invitrogen), 100 μM 2-mercaptoethanol, antibiotics, 15% Knockout Serum Replacement (Invitrogen), and 1000 U/mL leukemia inhibitory factor (LIF) (ESGRO; Millipore, Billerica, MA, USA). Mouse stromal PA6 cells (Riken BioResource Center) were grown in alpha-MEM (Invitrogen) supplemented with 10% FBS and antibiotics. Mouse myoblast C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS and antibiotics (i.e., myoblast growth medium). To induce myogenic differentiation, the medium was changed to DMEM supplemented with 2% calf serum and antibiotics (i.e., myogenic differentiation medium). Cells were cultured at 37 °C in a 5% (v/v) CO_2 incubator.

Construction of E-cadherin or N-cadherin gene-engineered PA6 cells The Tet-On system (Clontech, Mountain View, CA, USA) was incorporated into retroviral vectors for inducible expression of the E- or N-cadherin gene. Fragments of full-length murine E-cadherin (20) or N-cadherin (21) cDNA were obtained from the RIKEN Bioresource Center, and ligated into pQMSCV/EGFP-TREtight-LETF-WPRE vector (22) to generate pQMSCV/EGFP-TRE-Ecadherin-WPRE or pQMSCV/EGFP-TRE-Ncadherin-WPRE, respectively. For retroviral vector production, two retroviral vector plasmids were used: pQMSCV/EGFP-CMV-rtTA-WPRE (22) and either pQMSCV/EGFP-TRE-Ecadherin-WPRE or pQMSCV/EGFP-TRE-Ncadherin-WPRE. pQMSCV/EGFP-CMV-rtTA-WPRE encodes a constitutive expression cassette for a transactivator (rtTA) that is activated by addition of doxycycline (Dox), while pQMSCV/EGFP-TRE-Ecadherin-WPRE and pQMSCV/EGFP-TRE-Ncadherin-WPRE include a tet-responsive element and encode expression cassettes for the E- and N-cadherin gene, respectively. Consequently, cadherin gene expression is induced via rtTA activation by Dox addition. Furthermore, these plasmids include an enhanced green fluorescent protein (EGFP) gene under control of the viral long terminal repeat (LTR) promoter. Retroviral vectors pseudo-typed using vesicular stomatitis G protein (VSV-G) were produced by transient transfection of 293FT cells with Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) and the retroviral vector plasmids, pcDNA4-gag/pol and pLP/VSV-G. For retroviral infection, PA6 cells were cultured for 24 h, and then the medium was replaced with retroviral solution containing retroviral vectors encoding the rtTA gene and inducible cadherin gene expression cassettes. Cells were then cultured in the presence of polybrene (Sigma–Aldrich, St. Louis, MO, USA) for 7 h, resulting in generation of PA6 cells capable of Dox-inducible E- or N-cadherin gene expression: PA6/E or PA6/N cells, respectively. Viral titers against PA6 cells were determined by flow cytometry using a cell sorter (SH800; Sony, Tokyo, Japan). Viral titers were approximately 1.2×10^6 IU/mL (multiplicity of infection, 40).

Immunocytochemical analysis Cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, permeabilized with 0.2% Triton-X-100, washed three-times with phosphate buffered saline (PBS), and blocked in 1% bovine serum albumin solution in PBS for 30 min. Primary antibodies were added and samples were incubated for 3 h. After washing three-times with PBS, samples were incubated with Alexa Fluor 488- and/or 546-conjugated secondary antibody (Life Technologies) for 45 min followed by washing three-times with PBS. The following primary antibodies were used: anti-E-cadherin monoclonal antibody (36/E-Cadherin; BD Biosciences, San Jose, CA, USA), anti-N-cadherin monoclonal antibody (H-63; Santa Cruz Biotechnology, Dallas, TX, USA), anti-neuron specific β-III tubulin (TuJ) monoclonal antibody (TuJ-1; R&D Systems, Minneapolis, MN, USA), anti-HB9 polyclonal antibody (bs-11320R; Bioss, Boston, MA, USA), and anti-sarcomeric α-actinin antibody (EA-53; Sigma–Aldrich). For visualization of acetylcholine receptors (AChRs), cells were incubated with Alexa Fluor 643-conjugated α-bungarotoxin (Life Technologies) for 1 h. In some experiments, samples were also stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were observed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) or Fluoview FV10i confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Neural differentiation Neural differentiation of miPS cells based on the SDIA method was performed according to a published method (9). PA6/E(+) and PA6/N(+) cells were prepared by adding Dox (1 μg/mL) at two days before the beginning of neural differentiation culture (day −2). On day −1, parental PA6 cells, PA6/E cells without Dox addition (PA6/E(−)), PA6/N cells without Dox addition (PA6/N(−)), PA6/E(+) cells, or PA6/N(+) cells were seeded (2.0×10^5 cells/well) as feeder cells onto wells of collagen-coated 6 well-plates (AGC Techno Glass, Shizuoka, Japan). On day 0, iPS cells were seeded (1.1×10^3 cells/well) onto confluent feeder cells and cultured in iPS medium without LIF addition (i.e., neural differentiation medium). The medium was replaced every other day until day 6. Cell clusters with diameters >100 μm were defined as a colony. On day 6, TuJ-positive (TuJ^+) colonies were counted in five fields of view from three individual wells per sample. Single colony areas were measured using BZ-II measurement module software (Keyence). Neural differentiation efficiency was determined by the following equation:

$$\text{Differentiation efficiency (\%)} = (\text{number of TuJ}^+ \text{ colonies})/(\text{number of initially seeded cells}) \times 100) \quad (1)$$

For long-term culture, a papain dissociation system (Worthington Biochemical, Lakewood, NJ, USA) was used for detachment of iPS cell-derived colonies. On day 6 of neural differentiation culture, cells were detached from the culture surface and pre-plated twice to remove feeder cells. Next, iPS-derived cells were passaged to freshly prepared feeders (parental PA6 cells, PA6/E(+) cells, or PA6/N(+) cells) and co-cultured in neural differentiation medium with N2 supplement (Thermo Fisher Scientific, Waltham, MA, USA) until day 10. TuJ^+ area in each well was measured using BZ-II measurement module software (Keyence).

For differentiation of miPS cells to motor neurons, 0.2 μM RA (Wako Pure Chemical Industries, Osaka, Japan) and 1 μM purmorphamine (Sigma–Aldrich) (Shh signaling pathway agonist (23)) were added to neural differentiation medium on day 4 (10), and cells cultured until day 9. The number of both TuJ- and HB9-double positive (TuJ^+HB9^+) colonies was counted in five fields of view from three individual wells per sample. Single colony areas were measured using BZ-II measurement module software (Keyence). Differentiation efficiency of iPS cells to motor neurons was determined by the following equation:

$$\text{Differentiation efficiency (\%)} = (\text{number of TuJ}^+\text{HB9}^+ \text{ colonies})/(\text{number of initially seeded cells}) \times 100 \quad (2)$$

Co-culture assay with myotubes On day 8 of motor neuron differentiation, iPS cell colonies were detached from feeder cells by papain treatment and pre-plated onto 100-mm collagen coated dishes (AGC Techno Glass) for 1 h. Next, 3.2×10^5 of these cells along with 3.2×10^5 cells C2C12 cells were plated into 35-mm tissue culture dishes, and cultured in myogenic differentiation medium. In some experiments, agrin (100 μg/mL; R&D System), a proteoglycan involved in neuromuscular junction (NMJ) development during embryogenesis (24), was added to myogenic differentiation medium. After incubating for 4 days in myogenic differentiation medium, immunocytochemical staining of cells and measurement of contractile activity of myotubes were performed. Width of α-actinin-positive myotubes was measured from images using BZ-II measurement module software (Keyence). To estimate mean myotube width, the five largest myotubes from five fields in each of three separate wells per sample were chosen and measured. To assay contractile activity of myotubes, electrical pulse stimulation was applied to cells. Carbon electrodes were placed 18 mm apart at opposite sides of a tissue culture dish. Cells were stimulated with electric pulses for 30 min with the following properties: voltage, 0.3 V/mm; width, 4 ms; and frequency, 1 Hz (25). After a 30-min electrical pulse stimulation period, electric pulses were applied again, and myotube movement recorded at a speed of 15 frames/s for 25 s using a BZ-9000 fluorescence microscope (Keyence). For synapse blocking experiments, a nicotinic cholinergic antagonist, (+)-tubocurarine chloride pentahydrate (also known as curare) (50 μM; Sigma–Aldrich) (26), was added to the medium to block

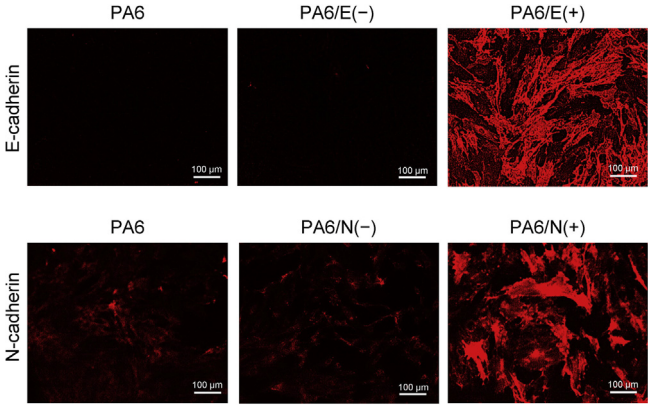

FIG. 1. Generation of cadherin gene-engineered PA6 cells. Immunocytochemical staining of E-cadherin and N-cadherin expression in PA6 cells with anti-cadherin antibodies. PA6, parental PA6 cells; PA6/E(−), E-cadherin gene-transduced PA6 cells without doxycycline addition; PA6/N(−), N-cadherin gene-transduced PA6 cells without doxycycline addition; PA6/E(+), E-cadherin gene-transduced PA6 cells with doxycycline addition; and PA6/N(+), N-cadherin gene-transduced PA6 cells with doxycycline addition.

AChRs present in NMJs. A single myotube was set in the captured image, and tracked during electrical pulse stimulation. Displacement was analyzed using motion analyzer software (Keyence). For estimating displacement range, the three myotubes displaying highest contractile activity in each of three fields in three separate dishes were chosen and measured using motion analyzer software (Keyence).

Statistical analysis Statistical comparisons were performed using the Mann−Whitney *U* rank sum test. Values of $P < 0.05$ were considered to indicate significant differences.

RESULTS

Cadherin expression in genetically engineered PA6 feeder cells The E-cadherin or N-cadherin gene with a Dox-inducible expression cassette was introduced into stromal PA6 cells (designated PA6/E or PA6/N cells). Consequently, transgene expression was induced by Dox addition to the medium (PA6/E(+) or PA6/N(+)). Genetically engineered PA6 cells were evaluated for E-cadherin and N-cadherin expression (Fig. 1). Immunocytochemical staining revealed that parental PA6 cells exhibited extremely low levels of E-cadherin expression with slight N-cadherin expression. In contrast, PA6/E(+) cells and PA6/N(+) cells exhibited greater expression levels of E-cadherin and N-cadherin, respectively.

Neural differentiation efficiency of miPS cells on cadherin gene-engineered PA6 feeder cells miPS cells were cultured on a PA6 feeder monolayer and allowed to differentiate for 6 days (Fig. 2). Control miPS cells in feeder-free cultures were negative for the neuron specific marker, TuJ. Meanwhile, TuJ^+ colonies formed on PA6/E(+) feeders, with PA6/N(+) feeder cells being larger than

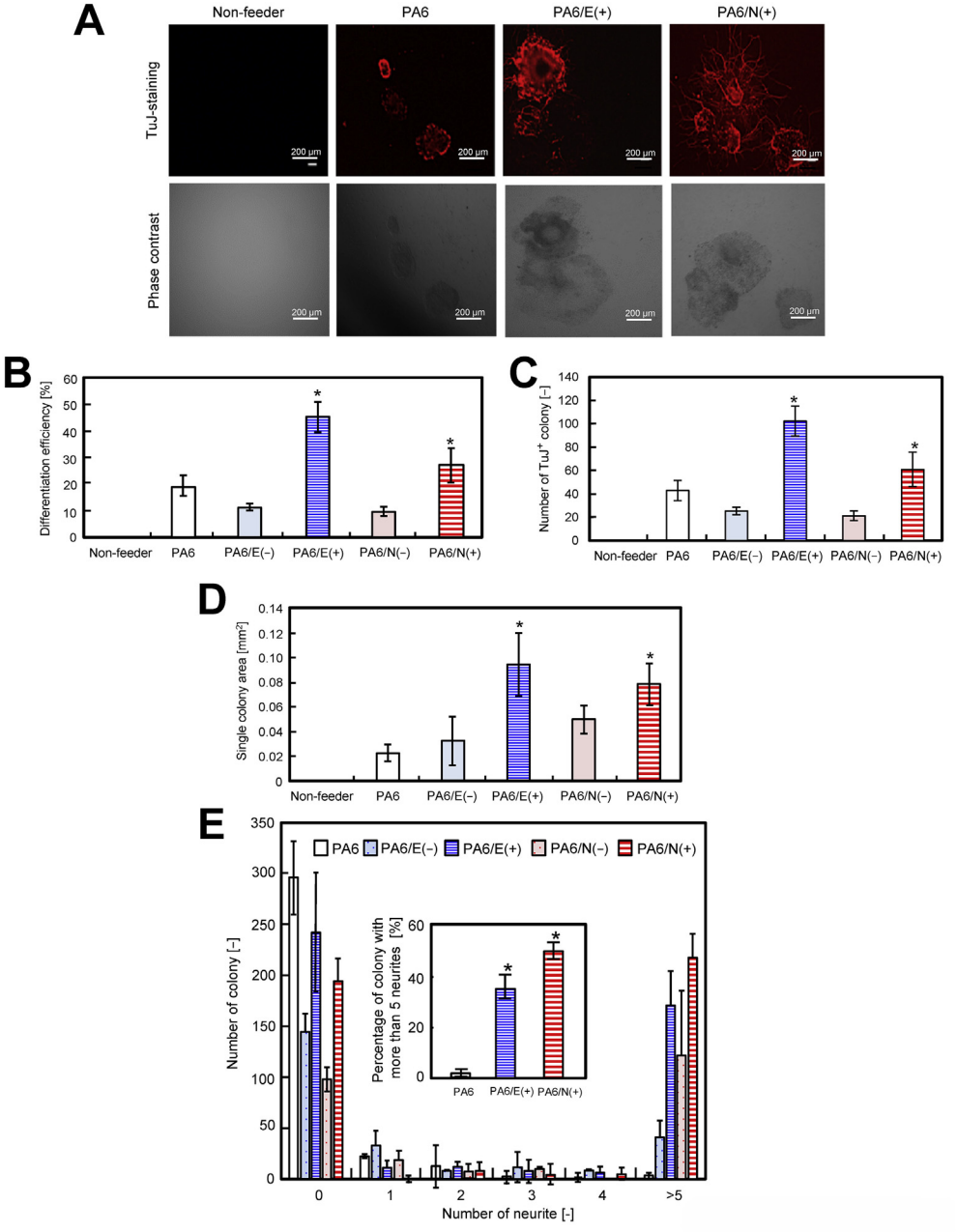

FIG. 2. Neural differentiation of induced pluripotent stem (iPS) cells on PA6 feeder cells for 6 days. (A) Anti-neuron specific β-III tubulin (TuJ) staining of iPS cells cultured on PA6 feeders. (B) Quantitative analysis of differentiation efficiency, (C) number of TuJ-positive colonies, (D) single colony area, and (E) number of colonies with neurite outgrowth. Inset graph of E shows the percentage of colonies with outgrowth of more than 5 neurites. Data are expressed as mean ± SD of triplicate experiments. $^*P < 0.05$ versus parental PA6 feeder.

parental PA6 cells and exhibiting distinctive neurite outgrowth on day 6 (Fig. 2A). Quantitative analysis revealed that differentiation efficiency (Fig. 2B), number of TuJ$^+$ colonies (Fig. 2C) and TuJ$^+$ colony area (Fig. 2D) were significantly higher on PA6/E(+) and PA6/N(+) feeders compared with parental PA6 feeder, suggesting that cadherin gene-engineered feeder cells enhance SDIA.

Further, PA6/E(+) feeder cells showed the greatest effects on both differentiation efficiency and TuJ$^+$ colony growth. The effect of cadherin expression on neural differentiation was further evaluated by quantifying the number of neurites formed by iPS-derived cells on PA6 feeders (Fig. 2E). miPS cells cultured on the parental PA6 cell layer formed an extremely low number of TuJ$^+$ colonies with neurite outgrowth, whereas both PA6/E(+) and PA6/N(+) feeders increased the number of TuJ$^+$ colonies with more than five neurites. Specifically, the percentage of TuJ$^+$ colonies with more than 5 neurites was 35.5 ± 4.4% and 49.9 ± 3.3% for PA6/E(+) and PA6/N(+) feeders, respectively, suggesting that PA6/N(+) feeder cells are effective for inducing neurite outgrowth. For long-term neural differentiation culture, the co-culture period was extended to day 10. iPS-derived cells cultured on a PA6 feeder layer were reseeded onto a freshly prepared PA6 feeder layer on day 6, and co-cultured until day 10 (Fig. 3). Elongated neurites were observed on day 10 (Fig. 3A). As shown in Fig. 3B, TuJ$^+$ area was significantly larger in co-cultures with cadherin gene-engineered PA6 feeders compared with the parental PA6 feeder. The most effective protocols involved iPS-derived cells cultured on PA6/E(+) feeder and reseeded onto either PA6/E(+) feeder (Protocol E→E) or PA6/N(+) feeder (Protocol E→N) on day 6 and co-cultured until day 10.

Effect of cadherin gene-engineered PA6 feeder cells on miPS cell differentiation into motor neurons To investigate the effect of PA6/E(+) and PA6/N(+) feeder cells on differentiation into motor neurons, SDIA treatment of miPS cells was combined with addition of purmorphamine and RA (SDIA/purmorphamine/RA treatment). Cells were co-cultured until day 9 (Fig. 4). A larger number of TuJ$^+$HB9$^+$ colonies with neurite outgrowth formed on PA6/E(+) and PA6/N(+) feeders compared with parental PA6 feeder (Fig. 4A). Quantitative analysis revealed that PA6/N(+) feeder had the greatest effect on differentiation of miPS cells into motor neurons (Fig. 4B) and TuJ$^+$HB9$^+$ colony growth (Fig. 4C). For functional analysis, motor neurons generated with SDIA/purmorphamine/RA treatment on PA6/N(+) feeder cells were co-cultured with C2C12-derived myotubes (Fig. 5). As shown in Fig. 5A and B, myotube width was increased by co-culture with SDIA/purmorphamine/RA-induced cells. In co-cultures, AChR clustering alongside TuJ$^+$ neurites was observed (Fig. 5C). For assay of myotube contractile activity, electrical pulse stimulation was applied, and myotube movement measured (Fig. 5D). In co-cultures, SDIA/purmorphamine/RA-induced cells enhanced contractile activity of myotubes. Further, agrin, an AChR clustering agent, slightly enhanced displacement of myotubes under electrical pulse stimulation. To assess the role of the neurotransmitter acetylcholine in these observed contractions, an

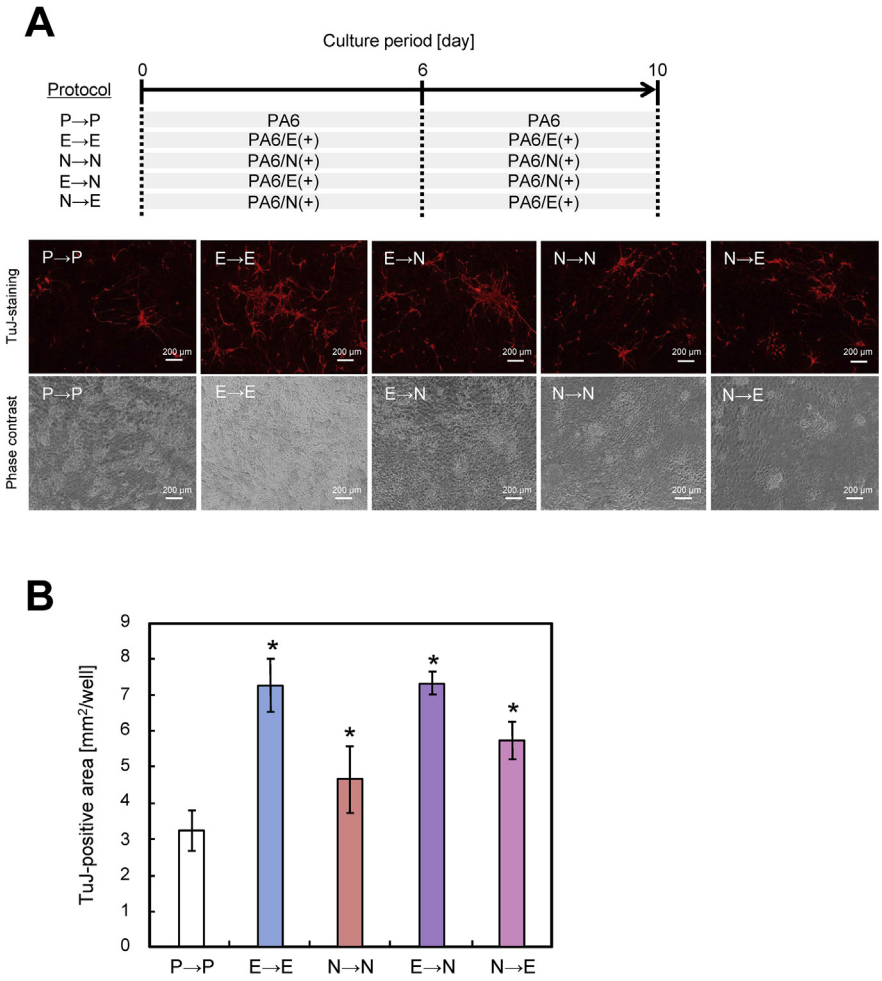

FIG. 3. Neural differentiation of induced pluripotent stem (iPS) cells on PA6 feeder cells for 10 days. (A) Protocols and anti-neuron specific β-III tubulin (TuJ) staining of iPS cells cultured on PA6 feeders. (B) Quantitative analysis for TuJ-positive area. Data are expressed as mean ± SD of triplicate experiments. *$P < 0.05$ versus Protocol P→P.

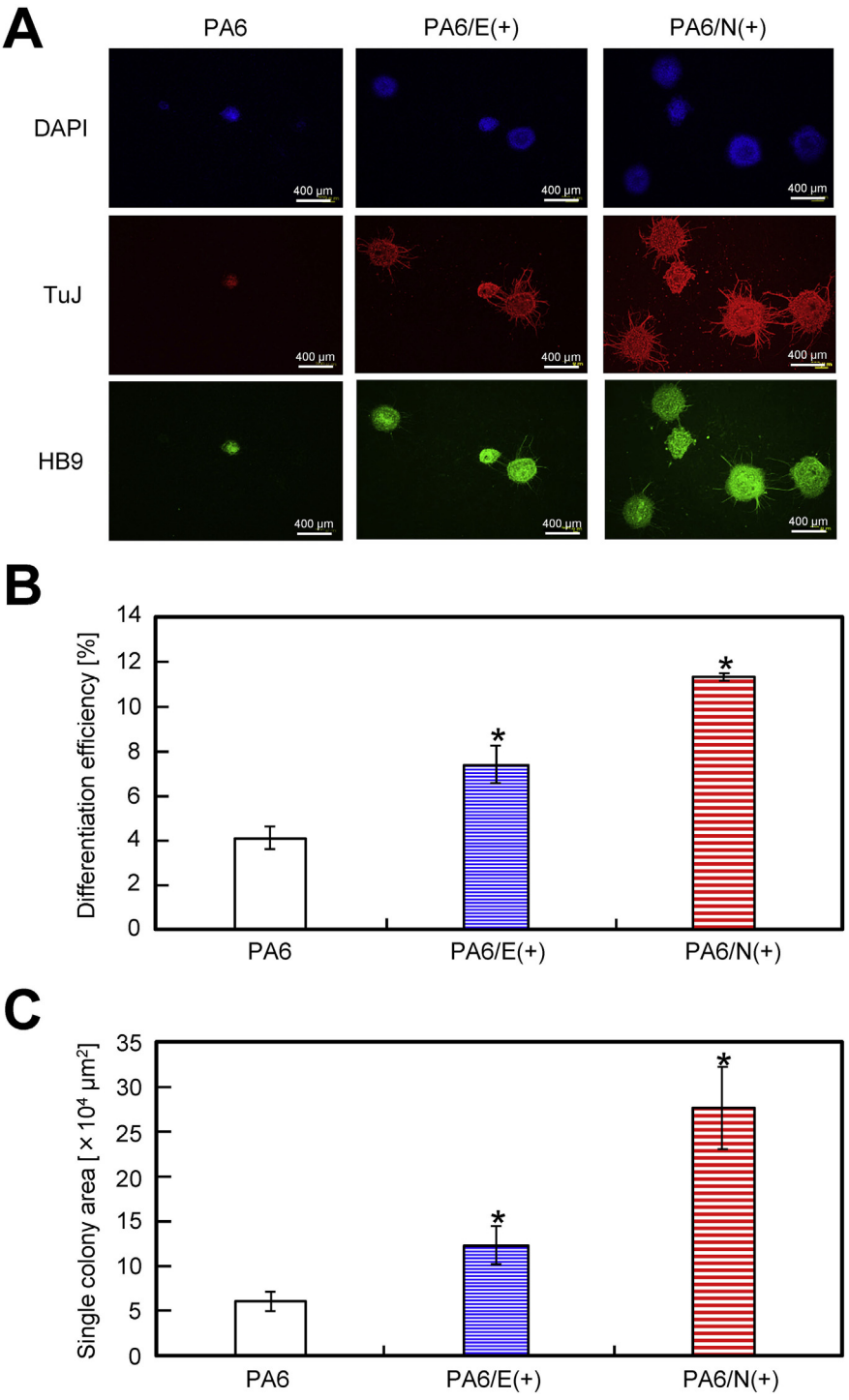

FIG. 4. Motor neuron differentiation of induced pluripotent stem (iPS) cells on PA6 feeder cells. (A) Immunocytochemical staining of iPS cells cultured on PA6 feeders. (B) Quantitative analysis of motor neuron differentiation efficiency and (C) single colony area. Data are expressed as mean ± SD of triplicate experiments. *$P < 0.05$ versus parental PA6 feeder.

AChR antagonist, curare, was used. Displacement of myotubes co-cultured with SDIA/purmorphamine/RA-induced motor neurons under electrical pulse stimulation was significantly decreased by curare treatment. These results suggest that motor neurons generated with SDIA/purmorphamine/RA treatment are functional, innervating C2C12-derived myotubes and causing AChR-dependent muscular contraction.

DISCUSSION

In this study, we have demonstrated that cadherin gene transfer into stromal PA6 cells significantly improves the performance of SDIA for neural differentiation of iPS cells. As feeder cells for neural differentiation of ES cells, Kawasaki et al. (9) screened various cells (such as MDCK, MEF, COS, OP9, and NIH3T3 cells), and

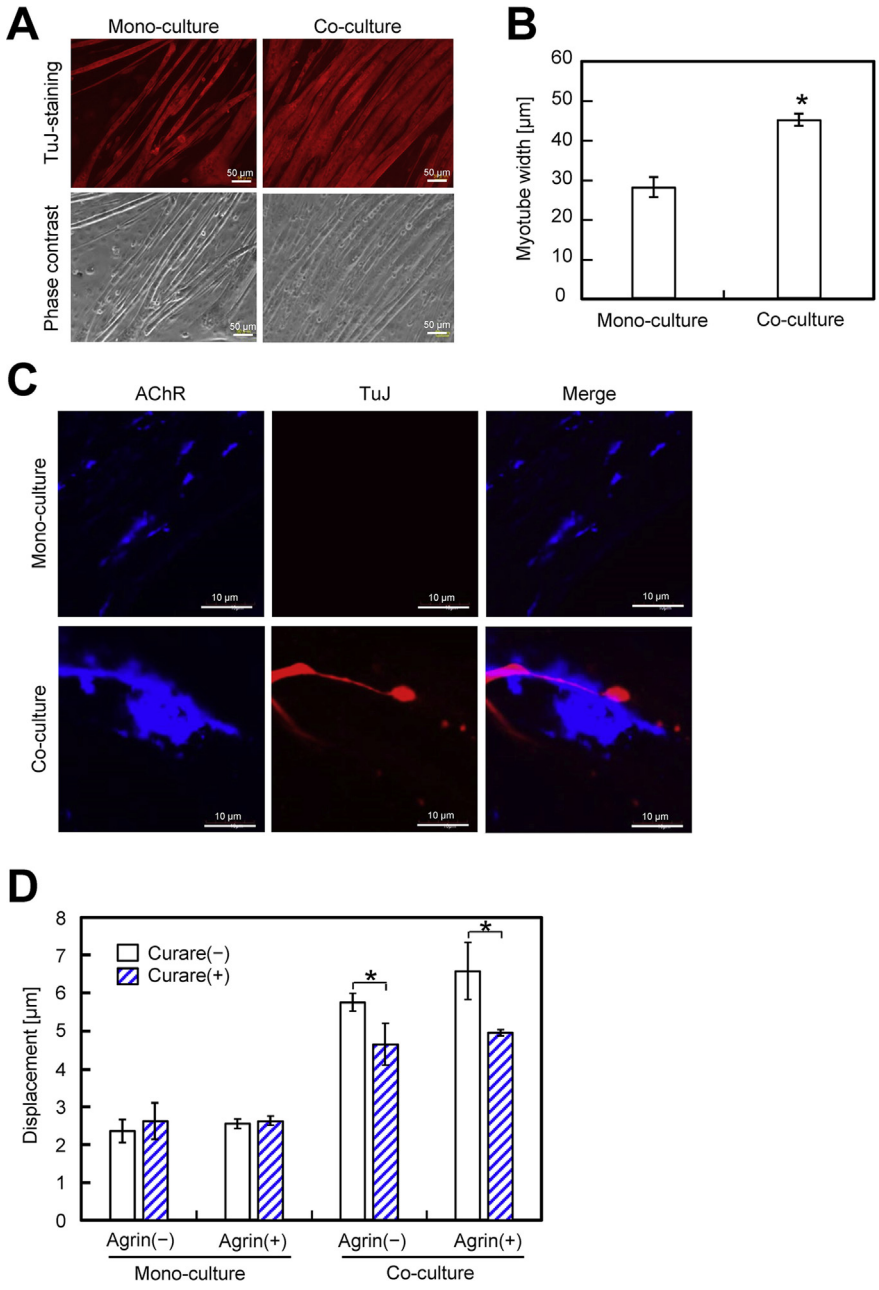

FIG. 5. Co-culture assay with myotubes. (A) Immunocytochemical staining of C2C12 myotubes. (B) Quantitative analysis of myotube width. Data are expressed as mean ± SD of triplicate experiments. *$P < 0.05$ versus monoculture. (C) Co-immunocytochemical staining of α-bungarotoxin that is specific for acetylcholine receptors (AChRs), and anti-neuron specific β-III tubulin (TuJ) for neurite outgrowth. (D) Quantitative analysis of displacement range for myotube contractile activity with (+) or without (−) agrin/curare treatment. Data are expressed as mean ± SD of triplicate experiments. *$P < 0.05$.

demonstrated that PA6 cells induced remarkably efficient neural differentiation. Feeder cells support target cells via paracrine interactions (e.g., cytokine release) and/or juxtacrine interactions (e.g., integrin/cadherin binding) (16). Interestingly, PA6 cells are reported to retain SDIA even after fixation with PFA (9), suggesting that SDIA of PA6, is partially mediated through the ECM of the cell surface and/or membrane-tethered soluble growth factors. In contrast, Akaike's group developed cadherin-coated plates (14,27,28), and demonstrated that recombinant E- and N-cadherin proteins improve neural differentiation of mouse ES/iPS cells (14). Cadherin binding is known to cause intracellular signaling through the β-catenin pathway (11) and inhibits catenin-based regulation of proliferation genes such as cyclin D1 (29), suggesting that cadherins are a molecular switch from growth to differentiation. Taken together, these observations suggest two possibilities regarding the mechanism by which cadherin gene-engineered PA6 cells improve SDIA performance for neural differentiation. First, SDIA may be mediated by secreted growth factors that are tethered to the ECM on the cell surface. Kawasaki et al. (9) reported that when PA6 cells are physically separated from co-cultured ES cells using the filter membrane of cell culture inserts, PA6 cells are still able to induce TuJ^+ colonies from ES cells, indicating that PA6 cells produce soluble growth factors for neural differentiation. However, efficiency was reduced to around one-third the levels with co-culture of ES cells on PA6 cells. Consistently, in the present study, co-culture with PA6/E(+) using a filter membrane of cell culture inserts induced a small number of TuJ^+ colonies from mouse iPS cells (data not shown). Moreover, Kawasaki et al. (9) also reported that PA6-

conditioned medium was unable to induce Tuj^+ colonies from ES cells. In the present study, conditioned medium derived from PA6/E(+) or PA6/N(+) cells as well as parental PA6 cells was unable to induce Tuj^+ colonies from mouse iPS cells (data not shown).

Similarly, although MEF feeder cells produce many growth factors that are important for self-renewal of ES/iPS cells, conditioned medium derived from MEF culture cannot maintain pluripotency of ES/iPS cells (17), indicating that the amount of growth factors secreted from MEF is not enough for high performance. We previously reported that magnetic accumulation of magnetically labeled STO feeder cells onto mouse ES/iPS cells improves undifferentiated growth of pluripotent stem cells (30). These observations suggest that physical contact (specifically, tight and close) between target cells and feeder cells is essential for efficient provision of growth factor signals. Another possible mechanism is the effect of the β-catenin pathway through cadherin binding. In the present study, we successfully constructed gene-engineered PA6 cells expressing E-cadherin and N-cadherin (Fig. 1). However, the amount of cadherin on the cell surface may be much lower than cadherin-coated plates using E-cadherin- or N-cadherin-Fc fusion protein, as reported by Haque et al. (14). We cannot exclude either possibility, but the former appears to be supportive at present. Further detailed mechanisms will be analyzed using newly established PA6/E(+) and PA6/N(+) clones with different levels of cadherin gene expression.

Interestingly, PA6/E(+) feeder cells showed higher efficiency of both Tuj^+ neural differentiation (Fig. 2B) and colony growth (Fig. 2C) than PA6/N(+) feeder cells. In contrast, PA6/N(+) feeder cells showed higher efficiency of inducing neurite outgrowth than PA6/E(+) feeder cells (Fig. 2D). Karpowicz et al. (31) demonstrated the importance of E-cadherin adhesion for neural stem cell differentiation, in which neuronal differentiation was reduced by blocking cell–cell interactions through E-cadherin. In contrast, Haque et al. (32) reported that N-cadherin reduced Rho/ROCK activation and β-catenin expression, which led to stimulation of neurite outgrowth. These results suggest that PA6/E(+) feeder cells induce relatively high growth of neural differentiating cells, whereas PA6/N(+) feeder cells tend to guide neurite elongation. Neural differentiation of miPS cells is reportedly associated with an E- to N-cadherin switch (13,14), and consistent with these previous reports, western blot analysis revealed that undifferentiated iPS cells show E-cadherin expression on day 0, while SDIA-induced iPS cells were positive for N-cadherin on day 6 (data not shown). Based on these results, we hypothesized that replacing PA6/E(+) feeder cells with PA6/N(+) feeder cells during the culture time course will be effective for SDIA using cadherin gene-engineered PA6 feeders. However, unexpectedly, no significant difference was observed in Tuj^+ area between Protocols E→N and E→E (Fig. 3). These results suggest that close contact with PA6 feeder cells via E-cadherin at an early developmental state is effective for neural differentiation of miPS cells.

In contrast, PA6/N(+) feeder cells showed higher efficiency of Tuj^+HB9^+ motor neuron differentiation (Fig. 4B) and colony growth (Fig. 4C) than PA6/E(+) feeder cells. Although the precise mechanism of how PA6/N(+) feeder cells effectively enhance motor neuron differentiation under SDIA/purmorphamine/RA treatment remains to be elucidated, physical contact (tight and close) of iPS-derived cells with PA6 feeder cells may be effective, because both PA6/N(+) and PA6/E(+) feeders showed higher efficiency of Tuj^+HB9^+ motor neuron differentiation compared with parental PA6 feeder (Fig. 4B). When Tuj^+HB9^+ neural cells generated with SDIA/purmorphamine/RA treatment on PA6/N(+) feeder were co-cultured with C2C12-derived myotubes, myotube width was significantly increased (Fig. 5A and B), suggesting induction of muscle hypertrophy. Stimulation from motor neurons via NMJs is the most important cue for skeletal muscle development (33), including muscle hypertrophy. As visualized by co-immunostaining (Fig. 5C), Tuj^+ neurites co-localized with α-bungarotoxin$^+$ AChR clusters, which indicates NMJ formation. Ko et al. (34) reported that myotubes treated with agrin, an AChR clustering agent, formed more synapses with neurites. We added agrin into co-cultures, but only slight enhancement of myotube contractile activity upon electrical pulse stimulation was observed (Fig. 5D). This is consistent with a recent study showing that agrin treatment alone slightly increases the number of AChR clusters (35). When the AChR antagonist curare was added to a monoculture of C2C12 cells, owing to the absence of motor neurons, myotube contractile activity was not decreased (Fig. 5D), even although AChRs were present on C2C12 cells (Fig. 5C). In contrast, curare reduced contractile activity of myotubes co-cultured with SDIA/purmorphamine/RA-induced motor neurons (Fig. 5D), indicating that functional NMJs had formed. We preliminarily varied the concentration of curare for blocking NMJs, but displacement of myotubes in co-culture was not reduced to the same level of monocultured myotubes by curare treatment (at least <100 μM) (data not shown). These results suggest that enhancement of myotube contractile activity in co-cultures after curare treatment is independent of acetylcholine-mediated neurotransmission through NMJs.

In conclusion, we have shown that cadherin gene-engineered PA6 feeder cells can enhance SDIA for neural differentiation of miPS cells. Further, motor neurons generated with SDIA/purmorphamine/RA treatment are also functional. Recently, *in vitro* construction of NMJs has attracted attention for drug screening of neuromuscular diseases (36). miPS-derived motor neurons induced by SDIA/purmorphamine/RA treatment using cadherin gene-engineered PA6 feeders may be useful for studying NMJ formation. Investigating neural differentiation of miPS cells is of important significance to offer a potential source of cells for nerve regeneration. Cadherin gene-engineered PA6 feeders may be a powerful tool for increasing the yield of neural cells from miPS cells for preclinical investigations.

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