Genes to Cells

Neural cells play an inhibitory role in pancreatic differentiation of pluripotent stem cells

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Pancreatic endocrine β-cells derived from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have received attention as screening systems for therapeutic drugs and as the basis for cell-based therapies. Here, we used a 12-day β-cell differentiation protocol for mouse ES cells and obtained several hit compounds that promoted β-cell differentiation. One of these compounds, mycophenolic acid (MPA), effectively promoted ES cell differentiation with a concomitant reduction of neuronal cells. The existence of neural cell-derived inhibitory humoral factors for β-cell differentiation was suggested using a co-culture system. Based on gene array analysis, we focused on the Wnt/β-catenin pathway and showed that the Wnt pathway inhibitor reversed MPA-induced β-cell differentiation. Wnt pathway activation promoted β-cell differentiation also in human iPS cells. Our results showed that Wnt signaling activation positively regulates β-cell differentiation, and represent a downstream target of the neural inhibitory factor.

Introduction

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, which exhibit an unlimited self-renewal capacity and can differentiate into virtually any adult cell type, have received attention as a source for cell-based therapies. Numerous studies have been conducted on the efficient generation of neural cells (Kondo *et al.* 2014), cardiomyocytes (Mummery *et al.* 2012) and pancreatic β-cells (D'Amour *et al.* 2006; Nostro *et al.* 2011; Schulz *et al.* 2012; Pagliuca *et al.* 2014; Rezania *et al.* 2014) from ES and iPS cells. Stem cell researchers are recently getting closer to the successful generation of functional cells. Clinical trials using human ES- or iPS-derived cells for treating age-related macular degeneration are in progress (Schwartz *et al.* 2012, 2014; Cyranoski 2013). The biotechnology company Viacyte developed a large-scale culture system to produce pancreatic progenitor cells from human ES cells for the treatment of diabetes (Schulz *et al.* 2012) and launched a clinical trial to deliver encapsulated ES cell-derived pancreatic progenitor cells for patients with diabetes. Studies on pancreatic development have showed genes and signals that participate in the fate decision of the pancreatic endocrine lineages (Kim *et al.* 1997; Hebrok *et al.* 1998; Scharfmann 2000; Lammert *et al.* 2001; Gittes 2009). These discoveries have been applied to *in vitro* differentiation of human ES or iPS cells into pancreatic β-cells (D'Amour *et al.* 2006; Nostro *et al.* 2011). The human ES- or iPS-derived insulin-positive cells have been characterized as poly-hormonal cells with gene

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expression profiles resembling those of the embryonic pancreas, exhibiting immature β-cell features and lacking glucose-stimulated insulin secretion (GSIS) ability *in vitro* (D'Amour *et al.* 2006; Nostro *et al.* 2011; Basford *et al.* 2012; Bruin *et al.* 2014; Hrvatin *et al.* 2014). Nevertheless, the human ES cell-derived pancreatic progenitor cells matured into functional β-cells *in vivo* after a period of 3–4 months after implantation into mice (Kroon *et al.* 2008; Schulz *et al.* 2012). The generation of functional β-cells *in vitro* from human iPS and ES cells was recently reported, and these cells successfully reversed hyperglycemia on implantation into mice (Pagliuca *et al.* 2014; Rezania *et al.* 2014).

In addition to cell replacement therapy, ES and iPS cell-derived differentiated cells are receiving attention as tools for modeling human diseases and establishing novel therapeutic applications. ES and iPS cell-derived β-cells are useful screening systems for therapeutic drugs, but their complex culture protocols featuring long duration and multiple steps present an obstacle for large-scale chemical screenings. The establishment of a shorter differentiation protocol would therefore prove beneficial.

We have previously described the generation of functional pancreatic β-cells from mouse and human ES and iPS cells using 17-day or 28-day multistage protocols, respectively (Sakano *et al.* 2014; Shahjalal *et al.* 2014). To allow for rapid chemical screening of small molecular compounds, we optimized the culture condition and established a 12-day β-cell differentiation protocol. We obtained several hit compounds that promoted β-cell differentiation, and one of these compounds, mycophenolic acid (MPA), effectively promoted β-cell differentiation with a concomitant reduction of neuronal cells. We studied the underlying molecular mechanisms and concluded that neural cells secrete an inhibitory factor for β-cell differentiation. We also showed that the Wnt pathway plays an important role in potentiating β-cell differentiation.

Results

Establishment of a rapid β-cell differentiation system for chemical screening

The mouse ES cell line SK7, which bears green fluorescent protein (GFP) expression driven by the *pancreatic and duodenal homeobox gene 1* (*Pdx1*) promoter (Shiraki *et al.* 2008; Sakano *et al.* 2014), was used to establish a 3-step 12-day differentiation protocol that allows rapid screening of pro-differentiation factors (Fig. 1a). In our novel 12-day differentiation protocol, in addition to a shortened culture period, secreted growth factors such as bFGF, FGF10 and GLP-1 are removed, and the use of synthetic matrixes is replaced by gelatin, thereby enabled us to perform screening in a rapid and cost-effective manner. In this culture system, ES cells were first exposed to an activin-containing Medium 1 for 4 days to trigger definitive endoderm differentiation. The expression of *octamer-binding transcription factor 3/4* (*Oct3/4,* also known as *POU domain, class 5, transcription factor 1; Pou5f*) in the undifferentiated ES cells was rapidly down-regulated on day 5 of differentiation (Fig. 1b), whereas the expression of *sex determining region Y-box 17* (*Sox17*) was up-regulated (Fig. 1c). The medium was switched to retinoic acid (10 μM)-containing Medium 2 on day 5 and to Medium 3 on day 6. On day 7, a marked increase in *Pdx1* transcript was observed, which rapidly decreased again but was maintained at a substantial level thereafter (Fig. 1d). In contrast, the increase in *neurogenin 3* (*Neurog3*) transcripts was transient on day 7 (Fig. 1e). *NK6 homeobox1* (*Nkx6.1*) transcript gradually increased from day 8 (Fig. 1f). *Insulin1* transcript was detectable from day 7 and gradually increased to a substantial level on day 12 (Fig. 1g), when insulin-positive cells became detectable by immunochemical analysis (Fig. 1h). The sequential expression pattern of *Sox17*, *Pdx1*, *Neurog3* and *insulin1* resembled the results in our previous 17-day differentiation protocol (Sakano *et al.* 2014). Of the insulin-positive cells yielded, 61% turned out to be insulin single-positive cells.

MPA promotes β-cell differentiation of mouse ES cells

A commercially available library of 303 bioactive pharmacologically defined small molecules arrayed as single compounds was tested using the new differentiation system. The compounds were added to the ES cell culture during differentiation days 6–12 at 0.625 or 2.5 μM. Twelve hit compounds yielding >1.5-fold increase in the number of β-cells, defined as insulin- and GFP (Pdx1) double-positive, were selected (Table S1 in Supporting Information). Of these compounds, MPA increased the number of β-cells in a concentration-dependent manner (Fig. 2a,b), as well as the expression of β-cell-related genes such as *Pdx1*, *Nkx6.1* and *insulin1*, on day 12 (Fig. 2c–e).

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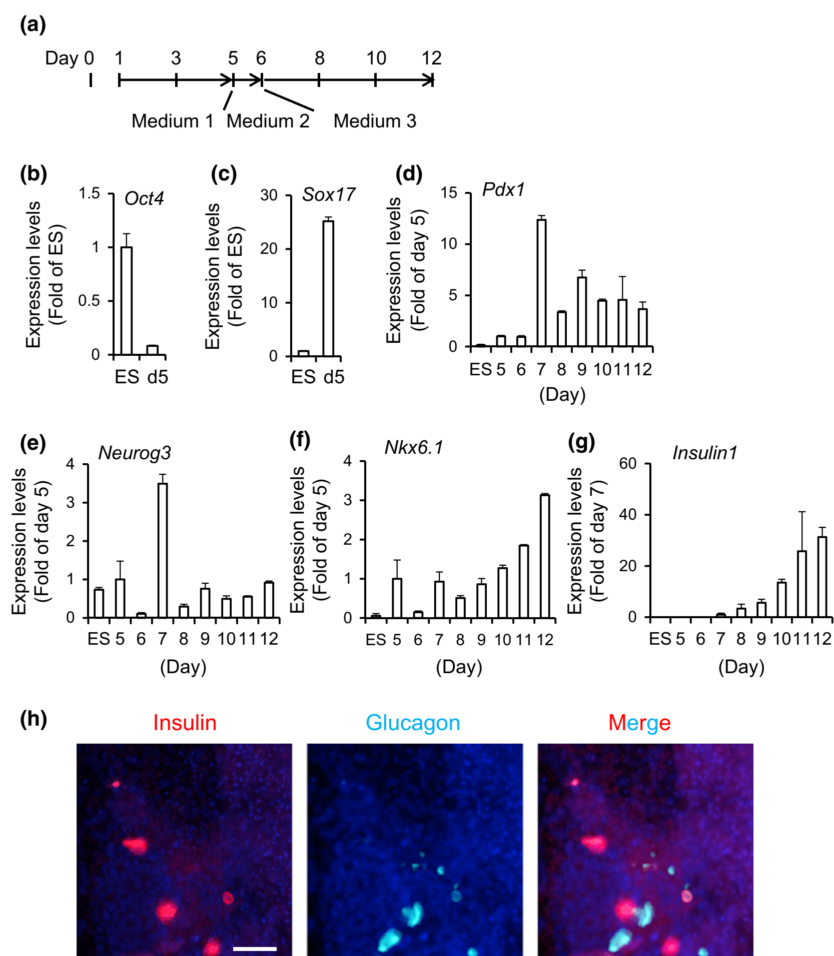

Figure 1 Rapid system for the differentiation of mouse embryonic stem (ES) cells into pancreatic β-cells. A 12-day rapid screening system for chemicals that potentiate mouse ES cell differentiation into pancreatic β-cells was established. (a) Schematic diagram of the 3-step differentiation system. (b–f) Real-time polymerase chain reaction analysis of time-dependent expression of *Oct4* (b), *Sox17* (c), *Pdx1* (d), *Neurog3* (e), *Nkx6.1* (f) and *insulin1* (g). (h) Immunostaining of insulin (red) and glucagon (cyan) on day 12. Data represent mean ± SEM (n = 3). Scale bar indicates 50 μm.

To determine the effective time window for the treatment, we compared the MPA treatment of cells over different time periods: days 1–3, days 3–5, days 5–6, days 6–8, days 8–10 and days 10–12 (Fig. 3a). When ES cells were treated with MPA at 0.4 or 2 μM on days 5–6 or days 6–8, β-cell numbers significantly increased (Fig. 3b). Addition of MPA during days 1–3 or 3–5 was also tested but turned out to be toxic (RN, unpublished). We then focused on days 5–6 (Fig. 3c), because it appeared as the most effective time window (Fig. 3b). MPA triggered a marked increase in *Pdx1, Neurog3* and *insulin1* transcripts relative to the controls (Fig. 3d–f), indicating the potentiating effect of MPA on β-cell differentiation. Treatment with 2 μM MPA on days 5–6 increased insulin-positive cells that were also positive for GFP (Pdx1) and Nkx6.1 (Fig. 3g). Insulin- and GFP (Pdx1) double-positive β-cells that co-expressed Nkx6.1 were 43% and 67%, in control and MPA-treated cells, respectively.

We also tested the effect of MPA on ING112 ES cells, another mouse ES cell line bearing *insulin1*-promoter driving GFP expression (Higuchi *et al.* 2010), and found that the differentiation into GFP (insulin)-positive cells was also promoted by MPA in a dose-dependent manner (Fig. S1 in Supporting Information).

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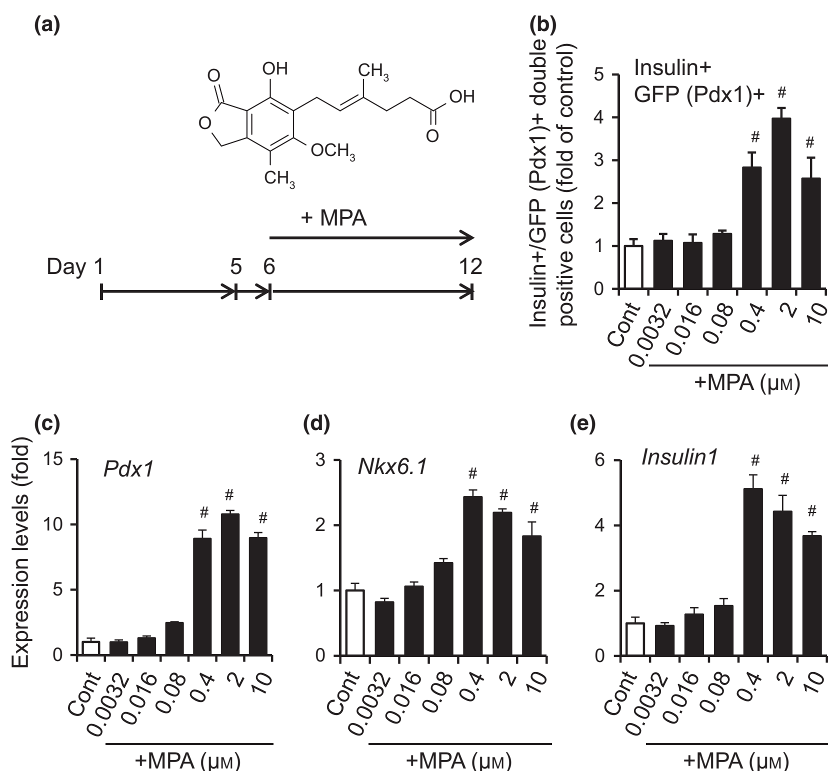

Figure 2 Mycophenolic acid (MPA) potentiates β-cell differentiation from mouse embryonic stem (ES) cells in a concentration-dependent manner. MPA was identified as the top hit chemical to potentiate ES cell differentiation into pancreatic β-cells, and its dose dependency was confirmed. (a) Schematic diagram for the screening system that identified MPA, which was used to study its dose-responsive action on β-cell differentiation. The chemical structure of MPA is also shown. (b) The proportion of insulin- and GFP (Pdx1) double-positive β-cells on day 12 (n = 6). (c–e) Real-time polymerase chain reaction analyses of *Pdx1* (c), *Nkx6.1* (d) and *insulin1* (e) on day 12 (n = 3). Data represent mean ± SEM. $^{\#}P < 0.05$, MPA treatments versus control by Dunnett's test.

MPA treatment promotes mouse ES cell differentiation into β-cells with glucose-stimulated c-peptide secretion activity

We next examined the GSIS activity of the ES cell-derived β-cells. We previously reported dibutyryl adenosine 3′,5′-cyclic AMP (dBu-cAMP) as a factor that stimulate differentiation of ES cells into β-cells with GSIS activity (Sakano *et al.* 2014). We supplemented Medium 3 with dBu-cAMP and GLP-1 (Fig. 4a) and confirmed that MPA supplementation markedly increased C-peptide contents (Fig. 4b) as well as GSIS activity (Fig. 4c) in the ES cell-derived β-cells.

The resultant MPA-treated β-cells showed a c-peptide content of 1.86 ng/mg protein and secreted 7.83 ng c-peptide/mg protein at high glucose. The adult mouse islets secrete c-peptide at 400 ng/mg protein at high glucose (Sakano *et al.* 2014). The proportions of β-cells in ES cell-derived cells treated with DMSO control or 2 μM MPA were 0.3% or 1.3%, respectively. Considering that β-cells are 80% of the islets, insulin secretion ability of the ES cell-derived β-cells was roughly equivalent to that of the adult mouse islets, although the c-peptide content still requires improvement.

MPA mediates IMPDH inhibition and neuronal cell apoptosis potentiates β-cell differentiation

The targets of MPA were investigated by microarray analysis of cells treated on day 5 with MPA and harvested on day 6. We carried out Ingenuity Pathway analysis (IPA) to identify the down-regulated or up-regulated genes (Tables S2 and S3 in Supporting Information). The analysis showed that genes categorized for their involvement in the development and function of the nervous system were down-regulated (Table S4 in Supporting Information). We next

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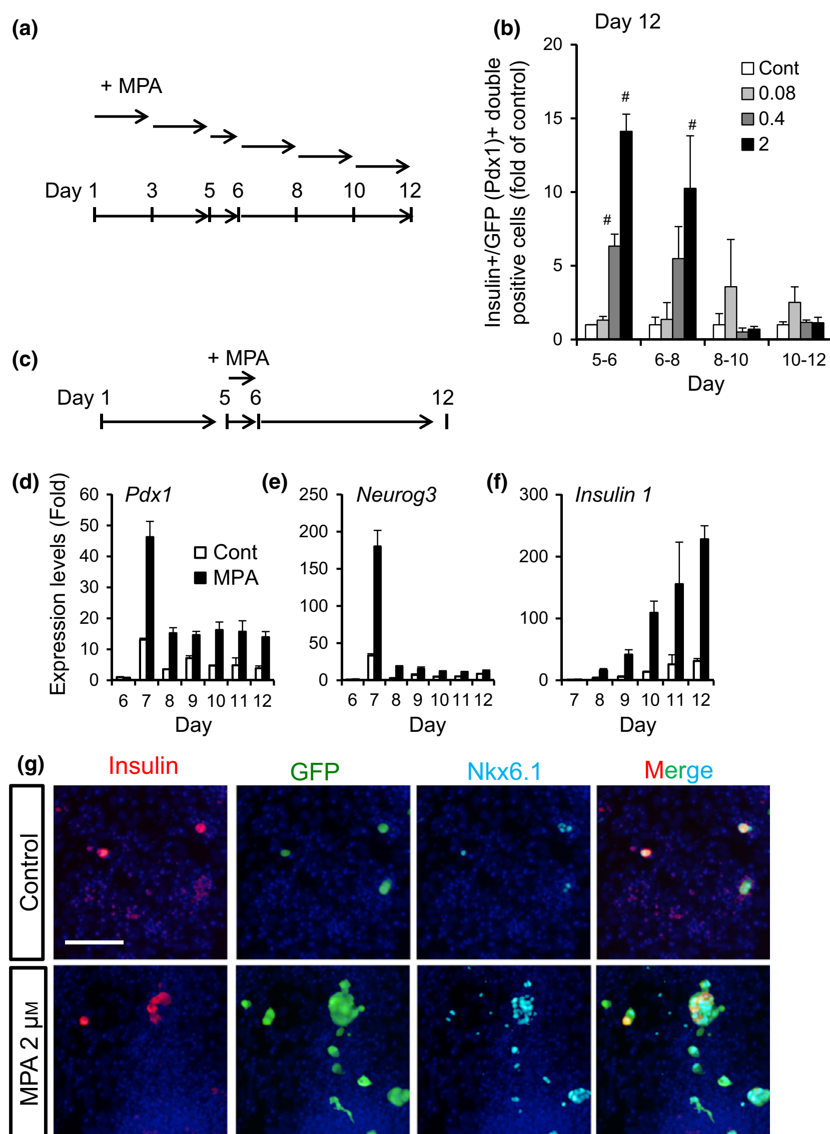

Figure 3 Mycophenolic acid (MPA) treatment during an early time window effectively potentiates β-cell differentiation from mouse embryonic stem (ES) cells. Dose-dependent potentiating effects of MPA on β-cell differentiation from ES cells were tested during different time windows. MPA treatment on days 5–6 was most effectively potentiated β-cell differentiation. (a,c) Schematic diagrams of the time windows for treatments with MPA in a β-cell differentiation system. (b) The proportions of insulin- and GFP (Pdx1) double-positive β-cells on day 12. (n = 6) (d–f) Real-time polymerase chain reaction analysis of *Pdx1* (d), *Neurog3* (e) and *insulin1* (f) on day 12 (n = 3). (g) Immunostaining of insulin (red), GFP (green) and Nkx6.1 (cyan) on day 12. Data represent mean ± SEM. $^{\#}P < 0.05$, MPA treatments versus control by Dunnett's test. Scale bar indicates 100 μm.

examined time-dependent expression of several neuronal cell-related gene transcripts, namely *tubulin β 3 class III* (*Tubb3*), *hairy and enhancer of split-5* (*Hes5*), *microtubule-associated protein 2* (*Map2*), *paired box 3* (*Pax3*) and *Zic family member 1* (*Zic1*), by real-time polymerase chain reaction (PCR) analysis. As expected, these gene transcripts were down-regulated (Fig. 5b–f). Our results therefore suggest a down-regulation of neuronal cells by MPA treatment. MPA treatment seemed to be specific to the neuronal cells. Immunochemical analysis unexpectedly showed a large population of Tuj1-positive neuronal cells existed in culture. In contrast, very few Flk1-, Pdgfra- or CD31-positive mesodermal cells, or

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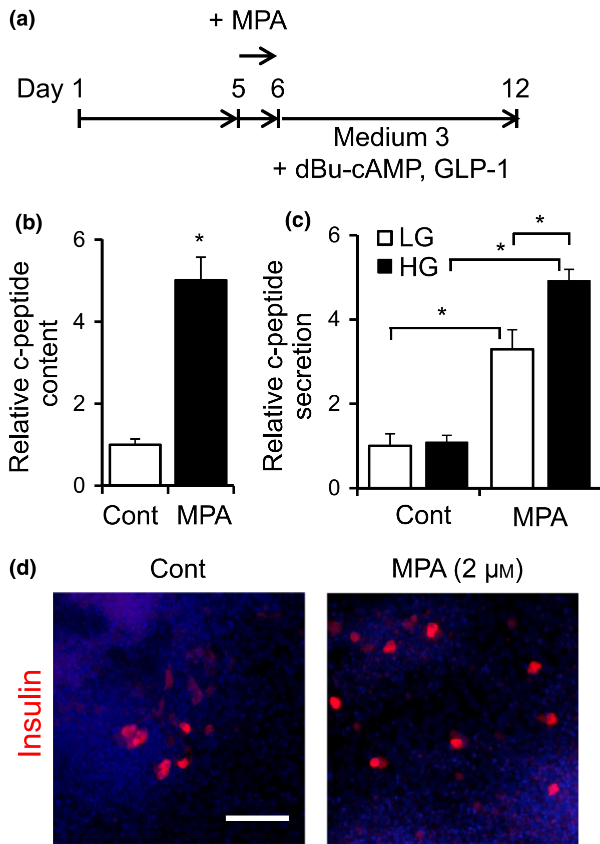

Figure 4 Mycophenolic acid (MPA) treatment potentiates differentiation of mouse embryonic stem (ES) cells into functional β-cells. ES cell-derived cells treated with MPA differentiated into functional pancreatic β-cells with greater C-peptide contents and GSIS activity. (a) Schematic diagram of ES cell differentiation with MPA treatment (2 μM) on differentiation day 5 and dBu-cAMP and GLP-1-containing Medium 3 treatment from differentiation day 6. (b) C-peptide contents on day 12 ($n = 8$). (c) Glucose-stimulated c-peptide secretion on day 12. LG, 5.5 mM glucose ($n = 4$); HG, 25 mM glucose ($n = 4$). (d) Representative immunostaining images of insulin on day 12. Data represent mean ± SEM. $*P < 0.05$, by Student's *t*-test. Scale bar indicates 100 μm.

p63-, CK14- or CK18-positive surface ectodermal cells (Tadeu & Horsley 2013) were observed in culture on day 5 (Fig. S2 in Supporting Information).

We then examined the time-dependent effects on neuronal and endodermal cells. We found that a large proportion of Tuj1-positive neuronal cells existed in culture, which decreased rapidly after 5 h MPA treatment. However, Sox17-positive endodermal cells slowly increased after 24 h MPA treatment (Fig. 5g–i). To confirm that this reduction of neuronal cells is a direct effect of MPA, we next derived neural cells from ES cells and treated these cells with MPA (Fig. 5j). MPA treatment (0.4 or 2 μM) reduced Tuj1-positive neuronal cells to 40% or 20% of the control levels, respectively (Fig. 5k,m). Concomitantly, TUNEL-positive cells were increased by MPA treatment (Fig. 5l,m). Therefore, our results suggest that MPA treatment indeed reduced the neuronal cell population via induction of apoptosis.

Mycophenolic acid is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme in guanosine *de novo* synthesis (Allison & Eugui 2000; Hedstrom 2009). We therefore supplemented guanosine simultaneously with the addition of MPA on day 5 (Fig. 6a). Guanosine supplementation significantly reversed the MPA-mediated enhancing effect on β-cell differentiation in a concentration-dependent manner with a complete reversal at 100 μM (Fig. 6b). Therefore, MPA-mediated potentiation of β-cell differentiation was apparently achieved through guanosine reduction by inhibition of IMPDH. Tuj1- or Sox17-expressing cells were immunostained on days 6 and 8 after MPA treatment with or without guanosine supplementation, and Tuj1-positive neuronal cells were found to be significantly reduced to approximately 40% of the control by MPA (Fig. 6c,e). Guanosine supplementation significantly reversed MPA-mediated neuronal cell reduction (Fig. 6c,e). Conversely, MPA addition significantly increased the Sox17-positive endodermal cells to approximately twofold compared to the control (Fig. 6c,f). On day 8, neurite-like outgrowth of the Tuj1-positive neuronal cells was observed in the control culture, and this was inhibited by the 2 μM MPA treatment (Fig. 6d). Guanosine supplementation reversed the MPA-induced reduction in the neurite-like outgrowth (Fig. 6d). Therefore, MPA treatment strongly reduced the neuronal cell population through its guanosine-depleting effects.

We then tested whether the effect of MPA on β-cell differentiation through neuronal inhibition is conserved, using other differentiation protocol. Using an embryoid body (EB) formation protocol (Fig. S3 in Supporting Information), we found that MPA triggered a decrease in Tuj1-positive neuronal cells, an increase in Sox17-positive endodermal cells, which led to an increase in insulin- and GFP (Pdx1) double-positive β-cells. Taken together, these results suggest that MPA treatment might potentiate β-cell differentiation through reduction of neuronal cells. Our present results show that neuronal cells exist in culture exert negative effects on β-cell differentiation.

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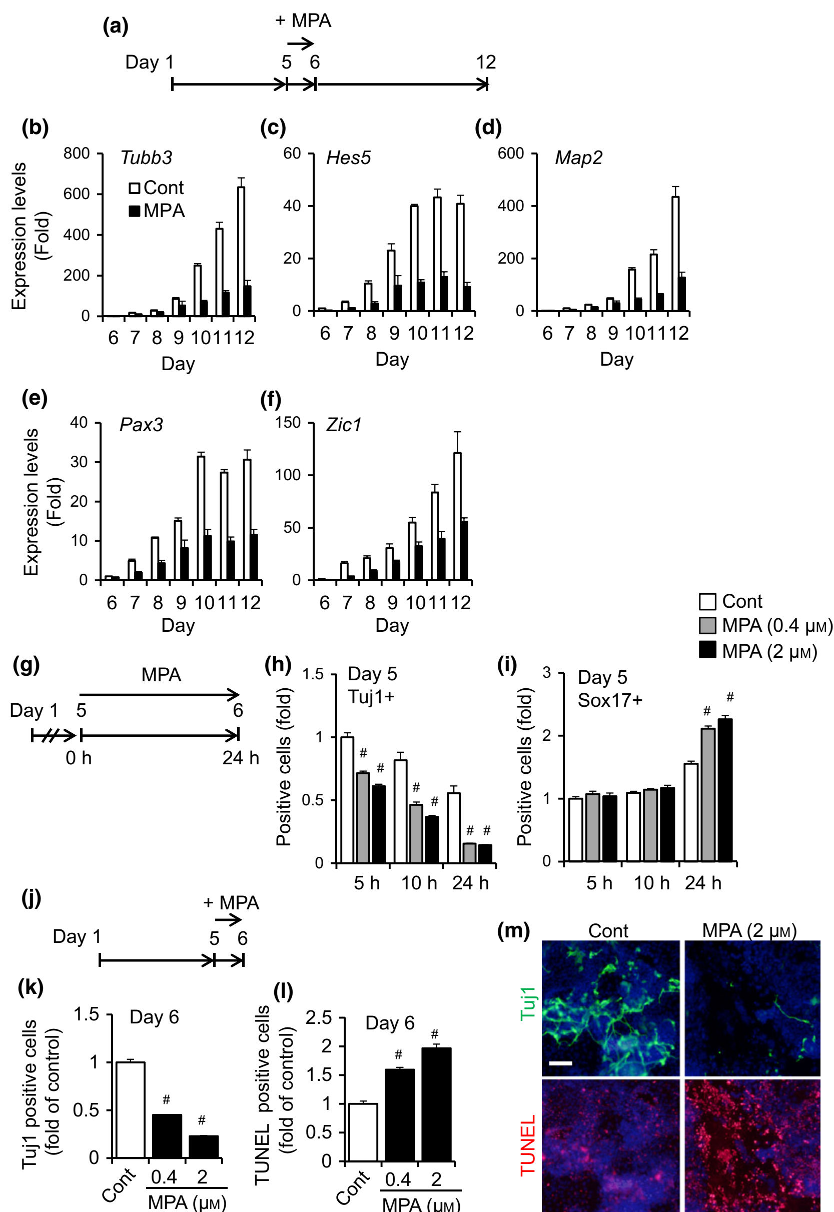

Figure 5 Mycophenolic acid (MPA) treatment induces neuronal cell reduction. MPA treatment reduced neuronal cell-related transcript and Tuj1-positive neuronal cells during β-cell differentiation and triggered neuronal cell apoptosis. (a) A schematic diagram of the time window for treatment with MPA (2 μM) in β-cell differentiation system. (b–f) Real-time polymerase chain reaction analysis of *Tubb3* (b), *Hes5* (c), *Map2* (d), *Pax3* (e) and *Zic1* (f) ($n = 3$). (g) A schematic diagram of the time window for treatment with MPA (2 μM). MPA was treated from day 5 (0 h). Cells were fixed and analyzed at 5, 10, or 24 h after treatment. (h,i) The proportions of Tuj1-positive neuronal cells (h) or Sox17-positive endodermal cells at each time point ($n = 4$). (j) Schematic diagram of the time window for treatment with MPA during neural cell differentiation. (k,l) The proportion of Tuj1-positive neuronal cell (k) and TUNEL-positive apoptotic cells (l) on day 6 ($n = 3$). (m) Tuj1 immunostaining for neuronal cells (upper panels) and TUNEL staining for apoptotic cells (lower panels) on day 6. Data represent mean ± SEM. $^{\#}P < 0.05$, by Dunnett's test. Scale bar indicates 100 μm.

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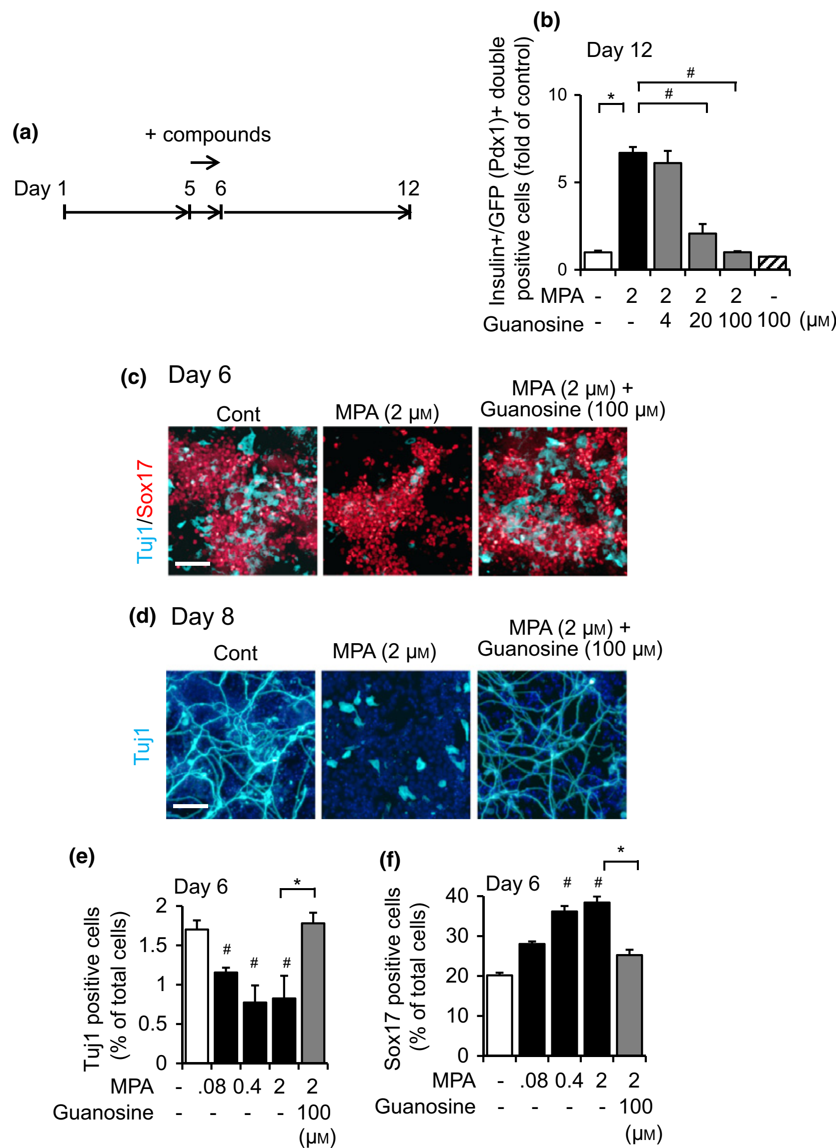

Figure 6 Mycophenolic acid (MPA)-mediated potentiation of β-cell differentiation and reduction of neural cells are caused by its guanosine-depleting effect. Adding guanosine to the MPA treatment abolished MPA-mediated β-cell differentiation and neural cell reduction. (a) Schematic diagram of embryonic stem (ES) cell differentiation with MPA and guanosine treatments on differentiation day 5. (b) The proportions of insulin- and GFP (Pdx1) double-positive β-cells on day 12 ($n = 3$). (c) Sox17 (red) and Tuj1 (cyan) immunostaining on day 6. (d) Tuj1 (cyan) immunostaining on day 8. (e,f) The proportion of Tuj1-positive neuronal cells (e) and Sox17-positive endodermal cells (f) on day 6 ($n = 3$). Data represent mean ± SEM. $*P < 0.05$, by Student's *t*-test or $^{\#}P < 0.05$, by Dunnett's test. Scale bars indicate 100 μm.

Small G protein activation increases neuronal cells and decreases β-cell differentiation

Intracellular depletion of guanosine inactivates small G protein. We therefore evaluated the impact of small G protein activity on neuronal cells and β-cell differentiation. ES cells were treated with Rho/Rac/Cdc42 activator I, a small G protein activator, during the first 5 h on differentiation day 5 (Fig. 7a). Rho/Rac/Cdc42 activator I constitutively activates Rho GTPase isoforms by blocking their intrinsic GTPase activity (Flatau *et al.* 1997; Schmidt *et al.* 1997; Lerm *et al.* 1999). The activator increased the Tuj1-positive neuronal cell population in a concentration-dependent manner by up to twofold compared to the control

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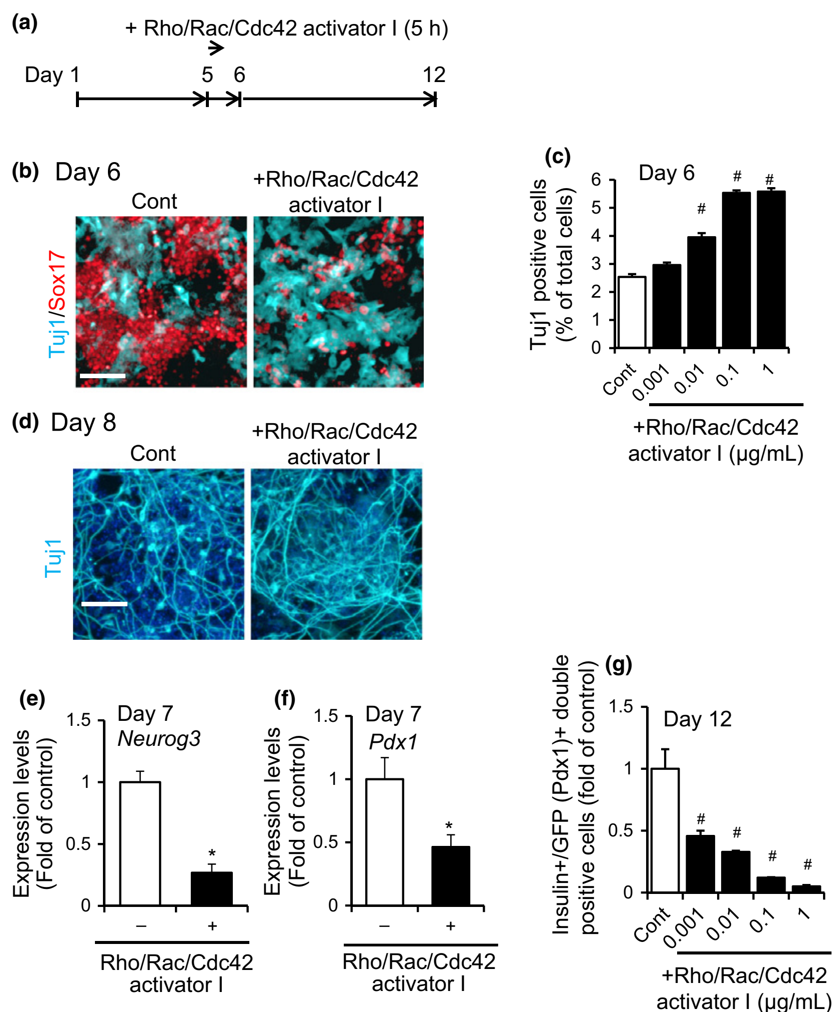

Figure 7 Small G protein activation increases neuronal cells and decreases β-cell differentiation. The small G protein activator Rho/Rac/Cdc42 activator I increased Tuj1-positive neuronal cell population in a concentration-dependent manner and concomitantly decreased β-cell differentiation. (a) Schematic diagram of the time window for Rho/Rac/Cdc42 activator I treatment during the first 5 h on differentiation day 5 in a β-cell differentiation system. (b) Sox17 (red) and Tuj1 (cyan) immunostaining on day 6. (c) The relative proportions of Tuj1-positive neuronal cells on day 6 ($n = 3$). (d) Tuj1 (cyan) immunostaining on day 8. (e,f) Real-time polymerase chain reaction analysis of *Neurog3* (d) and *Pdx1* (e) on day 7 ($n = 3$). (g) The proportions of insulin- and GFP (Pdx1) double-positive β-cells on day 12 ($n = 3$). Data represent mean ± SEM. $^{*}P < 0.05$, by Student's *t*-test. $^{\#}P < 0.05$, by Dunnett's test. Scale bars indicate 100 μm.

level at 0.1 and 1 μg/mL on day 6 (Fig. 7b,c). It further promoted neurite-like outgrowth observed on day 8 (Fig. 7d). Concomitantly, Rho/Rac/Cdc42 activator I treatment decreased *Neurog3* and *Pdx1* transcripts on day 7 (Fig. 7e,f), which seemed to contribute to the reduction of β-cells (Fig. 7g) in a concentration-dependent manner.

In summary, our results showed a negative correlation of β-cell differentiation with the neuronal cell population. These results strongly suggest that the promoting effects of MPA treatment on β-cell differentiation are caused by a reduced neuronal cell population via inhibition of an IMPDH/guanosine/small G protein activation pathway.

Co-culture with neural cells reverses MPA-mediated β-cell differentiation through inhibition of Wnt signaling

We next investigated the possibility of an involvement of soluble factors secreted from neural cells. We prepared ES cell-derived neural cells, consisting of

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approximately 80% Tuj1- or nestin-positive cells, with the absence of apparent FABP4-positive adipocytes (Fig. S4 in Supporting Information). During days 6–12, MPA-treated or control-cultured ES cell-derived definitive endodermal cells were co-cultured with the ES cell-derived neural cells or MEF through in a transwell system (Fig. 8a,b). β-cell differentiation was evaluated on day 12. Co-culture of ES cell-derived endodermal cells with neural cells markedly inhibited β-cell differentiation, both with and without MPA treatment. Nonetheless, no difference was observed when the cells were co-cultured with MEF (Fig. 8c). The results strongly suggested that neural cells secrete a humoral factor that inhibits β-cell differentiation.

We then attempted to characterize the inhibitory signal secreted from the neural cells. After the evaluation of the microarray analysis data on differentiation day 6, genes that increased by at least twofold were selected for the estimation of the upstream target by IPA. The Wnt/β-catenin pathway was identified as one of the top 10 up-regulated pathways (Table S2 in Supporting Information). We added the glycogen synthase kinase 3β (GSK-3β) inhibitor CHIR99021 to the neural cell co-culture during days 6–12 (Fig. 8a,c) and found that CHIR99021 (1 μM)

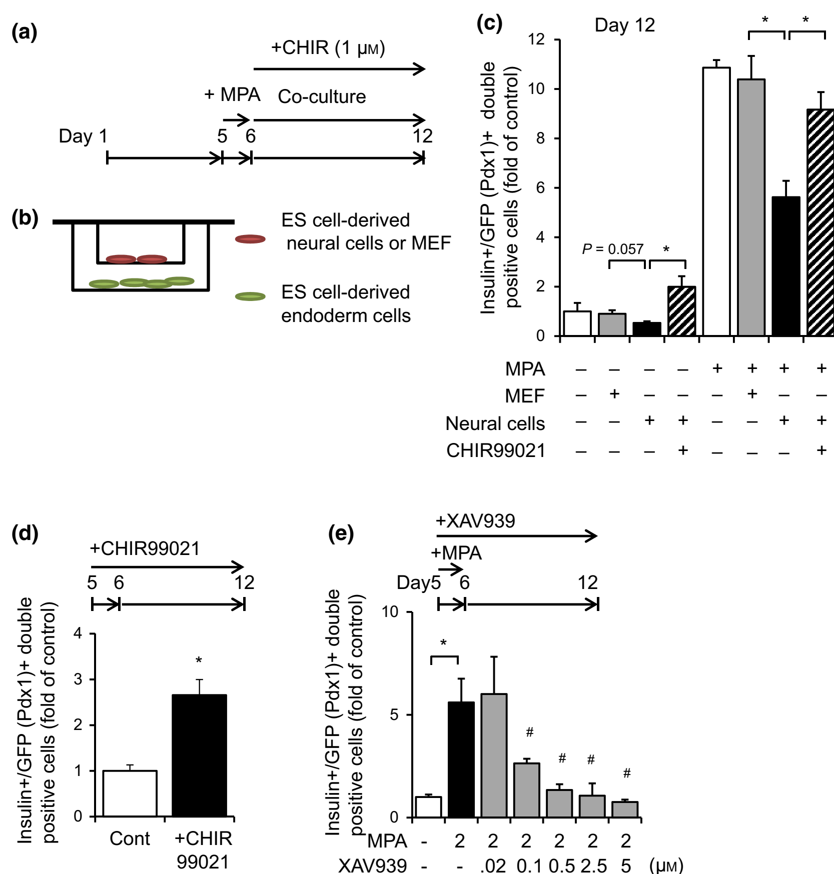

Figure 8 β-cell differentiation of mouse embryonic stem (ES) cells is inhibited by co-culture with neural cells and promoted by Wnt pathway activation. (a–c) β-cell differentiation from ES cells was tested by co-culture with neural cells derived from ES cells using a transwell culture. Schematic diagram for combining mycophenolic acid (MPA) (2 μM) with CHIR99021 (1 μM) treatment and with the neural cell co-culture (a), and a diagram showing the transwell culture (b). The proportion of insulin- and GFP (Pdx1) double-positive β-cells on day 12 ($n = 4$) (c). (d,e) The Wnt pathway activator CHIR99021 potentiated pancreatic differentiation, whereas MPA-mediated potentiation was inhibited by the Wnt pathway inhibitor XAV939. The proportion of insulin- and GFP (Pdx1) double-positive β-cells on day 12 ($n = 3$) after treatments with CHIR99021 (1 μM) (d) or MPA (2 μM) and XAV939 (0.02, 0.1, 0.5, 2.5 and 5 μM) (e). Data represent mean ± SEM. *$P < 0.05$, by Student's *t*-test. $^{\#}P < 0.05$, by Dunnett's test.

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reversed the inhibitory effects of neural cell co-culture and significantly promoted β-cell differentiation (Fig. 8c).

We then evaluated the effects of Wnt signaling activation on β-cell differentiation during days 5–12. As expected, CHIR99021 (1 μM) significantly promoted β-cell differentiation more than 2.5-fold compared to the control (Fig. 8d). We next asked whether MPA-induced β-cell differentiation is mediated through activation of a Wnt pathway by treatment of cells with the Wnt pathway inhibitor XAV939. MPA-induced β-cell differentiation was inhibited by XAV939 addition during days 5–12 in a concentration-dependent manner. XAV939 (5 μM) almost completely reversed MPA-induced β-cell differentiation (Fig. 8e). Therefore, MPA potentiated β-cell differentiation through reducing the neural cell-derived inhibitory signal, which then triggered activation of the Wnt/β-catenin pathway.

Wnt pathway activation potentiates β-cell differentiation in human iPS cells

We next evaluated the effects of neuronal cell co-culture on β-cell differentiation using the human iPS cell line Toe. We differentiated Toe iPS cells into β-cells based on the 5-stage differentiation protocol reported previously (Shahjalal *et al.* 2014) with minor modifications. We tested the effects of neural cell co-culture on β-cell differentiation. Co-culture of Toe iPS cell-derived endodermal cells with mouse ES cell-derived neural cells from differentiation days 3–19 significantly reduced the proportion of β-cells evaluated by insulin immunostaining (Fig. 9a–d). We next focused on later stage and added CHIR99021 (1 μM) during the final 6 days of β-cell differentiation. CHIR99021 treatment significantly increased the proportion of β-cells (Fig. 9e,g) without affecting somatostatin-positive δ-cells or glucagon-positive α-cells (Fig. 9g). By contrast, neural cell co-culture reduced the proportion of β-cells (Fig. 9f). Therefore, Wnt pathway activation was showed to promote β-cell differentiation in both mouse ES cells and human iPS cells.

Discussion

Embryonic stem cells and iPS cells are currently effectively differentiated into β-cells with protocol using a 5-stage differentiation system for duration of 15–25 days, although these cells do not display glucose-dependent insulin secretion (D'Amour *et al.* 2006; Nostro *et al.* 2011; Basford *et al.* 2012; Bruin *et al.* 2014; Hrvatin *et al.* 2014). These protocols require the addition of many recombinant growth factors, including Wnt3a, activin A, Fgf10, Noggin, Igf1 and Exendin4. Small compounds such as retinoic acid, gamma-secretase inhibitors, Shh inhibitors and ALK inhibitors are also used. Endocrine progenitor cells have been reported to turn into functional β-cells 3–4 months after transplantation in rodents (Kroon *et al.* 2008; Schulz *et al.* 2012). Recently, Pagliuca *et al.* and Rezania *et al.* achieved the generation of functional β-cells with GSIS activity after *in vitro* differentiation for a prolonged period of more than 30 days (Pagliuca *et al.* 2014; Rezania *et al.* 2014). We previously reported a 3-stage, 17-day differentiation system and screened for effective compounds to differentiate β-cells. The reduction of recombinant protein used and culture period presents an important factor in terms of cost and throughput for large-scale screening applications. We report here a 12-day rapid screening system with minimal recombinant factor use, limited to activin A and ITS supplement.

A chemical library with 303 bioactive compounds was used to search for candidates that could potentiate β-cell differentiation. We obtained 12 hit compounds, and Marimastat, an ADAM-17 inhibitor, was the second potent compound. Inhibition of ADAM-17 down-regulates the Notch pathway (Guo *et al.* 2013), resembling the effect of gamma-secretase inhibition, which has been reported to enhance β-cell differentiation (Nostro *et al.* 2011). The compound identified as most potent, MPA, has been reported to potentiate β-cell differentiation through its IMPDH inhibitory effect in a zebra fish study (Rovira *et al.* 2011). Ribavirin, another IMPDH inhibitor (Hedstrom 2009), was also a hit candidate in our screening system. MPA supplement promoted the efficiency of β-cell differentiation and yielded β-cells capable of GSIS. Although the ES-derived MPA-treated β-cells showed comparable insulin secretion ability with that of the adult islets, they showed much lower c-peptide content. The low c-peptide content of the ES cell-derived cells might due to the 12-day rapid differentiation protocol, which is too short for the ES-derived β-cells to synthesize and accumulate sufficient insulin contents.

Inductive signals derived from the mesoderm and ectoderm participate in embryonic pancreatic development. Mesodermal signals from the notochord and the dorsal aorta were shown to promote Pdx1 and insulin expression (Kim *et al.* 1997; Hebrok *et al.*

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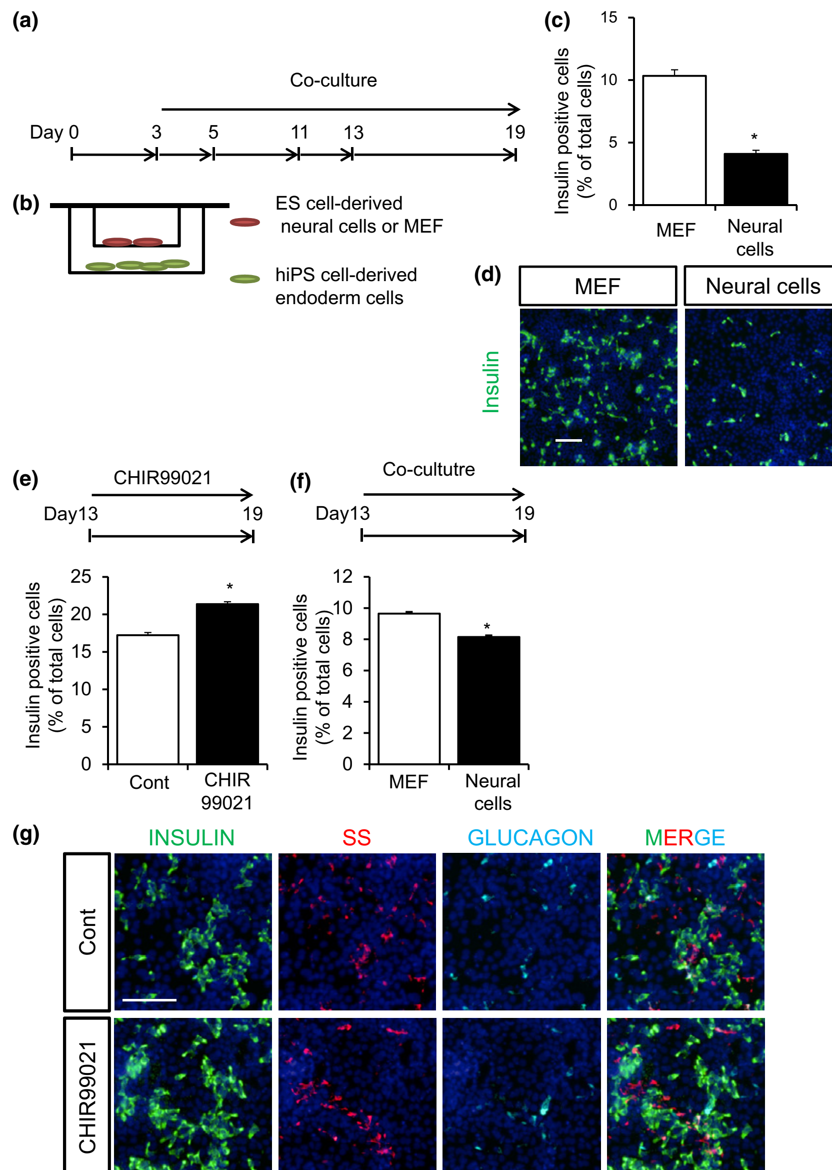

Figure 9 β-Cell differentiation of human induced pluripotent stem (iPS) cells is inhibited by co-culture with neural cells and promoted by Wnt pathway activation. (a–d) Toe human iPS cell differentiation into β-cells was inhibited by co-culture with mouse embryonic stem-derived neural cells from stage 2 (differentiation days 3–19). A schematic diagram of human iPS cell differentiation with the neural cell co-culture (a), and a diagram showing the transwell culture (b). The proportion of insulin-positive β-cells on day 19 (n = 6–9) (c). Immunostaining of insulin on day 19 (d). (e–g) Toe human iPS cell differentiation into β-cells was enhanced when added with CHIR99021, but inhibited when co-cultured with the neural cells, during stage 5 (differentiation days 13–19). The effect of adding CHIR99021 on the proportion of insulin-positive β-cells on day 19 (n = 3–6) (e). The effect of neural cell co-culture on the proportion of insulin-positive β-cells on day 19 (n = 8) (f). Immunostaining of insulin (green), somatostatin (SS, red), and glucagon (cyan) on day 19 (g). Data represent mean ± SEM. *$P < 0.05$, by Student's t-test. Scale bar indicates 100 μm.

1998; Lammert *et al.* 2001). The factors necessary for the survival and differentiation of the developing pancreatic epithelium are provided by the pancreatic mesenchyme (Scharfmann 2000; Gittes 2009). Ectoderm-derived neural crest cells migrate to the rostral foregut at approximately the same time as the pancre-

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atic cells start to bud from the dorsal gut endoderm (Young & Newgreen 2001). Connections between the neural cells and β-cells have been reported in both adult (Gilon & Henquin 2001; Imai *et al.* 2008; Grouwels *et al.* 2012) and embryo (Nekrep *et al.* 2008; Plank *et al.* 2011).

The most effective time window for MPA treatment to promote β-cell differentiation was in a rank order of days 5–6 > days 6–8 > days 8–10 > days 10–12. In our differentiation system, the pancreatic progenitor cells arise approximately on day 7 judging from the expression of *Pdx1* and *Neurog3*. Therefore, MPA affected Sox17-positive definitive endoderm cells, which exist at a large proportion on days 5–6 and are retained on days 6–8. Several reports have showed the promoting effects of MPA on cell differentiation (Gortz *et al.* 1997; Floryk & Huberman 2006; Moosavi *et al.* 2006). Nevertheless, both the target cells and the mechanism of MPA-mediated β-cell development are yet unknown. Our results show that MPA treatment significantly reduced neuronal cells from 5 h after MPA treatment, before the increase in Sox17-positive cells at 24 h after MPA treatment. The neuronal cell reducing effect of MPA was reversed by addition of guanosine, thereby indicating that MPA-mediated neuronal cell reduction is caused by the inhibition of *de novo* synthesis of guanosine. The enzyme IMPDH is rate-limiting in guanosine biosynthesis from the metabolites of the pentose phosphate pathway. This pathway plays a crucial role for cell viability not only in undifferentiated cells but also in neural progenitor/stem cells (Candelario *et al.* 2013). These facts underline the importance of guanosine in neuronal cell survival and differentiation. Our results are supported by previous reports that IMPDH inhibitors hamper neural cell differentiation (Messina *et al.* 2005; Skardelly *et al.* 2013). We therefore further examined the molecular mechanism of action of MPA on neuronal cells. Using Rho/Rac/Cdc42 activator I, we discovered that the activity of the small G proteins Rho, Rac and Cdc42, promotes neuronal differentiation; promoting effects of this agent on neural development have been described *in vivo* (Diana *et al.* 2007; De Viti *et al.* 2010). MPA has been previously reported to significantly increase apoptotic cell death of the neural progenitor cells (Skardelly *et al.* 2013). Therefore, small G proteins are involved at least in some aspect of the MPA-mediated neuronal cell reduction. We observed a negative correlation between neuronal cell development and β-cell differentiation on Rho/Rac/Cdc42 activation. Accordingly, MPA-mediated potentiation of β-cell differentiation is caused by neuronal cell reduction via inhibition of the IMPDH/guanosine synthesis/small G protein activation axis. In adults, neural input positively regulates β-cell mass and function (Gilon & Henquin 2001; Imai *et al.* 2008; Grouwels *et al.* 2012). In contrast, genetically reduced neural crest cells were shown to increase the β-cell number in mouse embryos (Nekrep *et al.* 2008; Plank *et al.* 2011). Nascent neural cells therefore negatively regulate the β-cell number, and these observations support our results.

Our transwell co-culture results imply the secretion of humoral inhibitory factors for β-cell differentiation secreted from the neural cells. Judging from the IPA, Wnt/β-catenin pathway activation was considered as one of the downstream pathways after MPA treatment. We paid particular attention to the involvement of the Wnt pathway in β-cell differentiation, because a role of Wnt in pancreas development and its action on exocrine cell development have been reported (Murtaugh *et al.* 2005; Heiser *et al.* 2006). Other studies have implicated the Wnt signaling in islet cell proliferation (Rulifson *et al.* 2007; Schinner *et al.* 2008). The canonical Wnt signaling component β catenin has been described as a requirement for not only exocrine but also endocrine cell development (Baumgartner *et al.* 2014). XAV939, a Wnt pathway inhibitor, completely reversed the effect of MPA on the β-cell differentiation, indicating that the neuronal cell-mediated inhibitory signal could be explained by Wnt signaling in β-cell differentiation. We show here that GSK3β inhibition by CHIR99021, a downstream event of Wnt activation, rescued the neural cell-mediated inhibition and promoted β-cell differentiation in the mouse ES cell differentiation system. Neural cell-mediated inhibitory effects on β-cell differentiation were also observed in human iPS cells. We confirmed that CHIR99021-treatment from days 5 to 12 potentiated β-cell differentiation from mouse ES cells. Previously, it was reported that Wnt signal activation during primitive gut tube stage enhanced β-cell differentiation (Nostro *et al.* 2011), Wnt signal increased Sox17 expression (Engert *et al.* 2013). The reduction in neural cells by MPA is an early event before endoderm differentiation. The removal of the inhibitory effects against Wnt signal emitted from the neural cells resulted in enhanced endodermal cell differentiation and/or proliferation. Our results also showed that CHIR99021 addition (Wnt signal activation) at endocrine progenitor stage in human iPS cells pro-

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moted β-cell differentiation, whereas neural co-culture at this stage inhibited differentiation. Taken together, these results confirm the importance of Wnt activation on β-cell differentiation in a multi-stage manner, which is conserved between mice and the humans.

In the present study, we established a 12-day rapid mouse ES cell differentiation system into β-cells and identified MPA as a potent compound for β-cell differentiation. We elucidated the involvement of neuronal cell reduction in MPA-mediated β-cell differentiation as the downstream events of MPA-mediated guanosine depletion and small G protein inactivation via IMPDH inhibition. To our knowledge, this is the first report that identifies the existence of neuronal cells exerting negative effects on β-cell differentiation through secretion of Wnt antagonizing factor. Although further studies are needed to identify the nature of the Wnt antagonizing factors derived from neural cells, we show that Wnt signaling plays an important role in β-cell differentiation.

Experimental procedures

Mouse ES cell culture

The SK7 and ING112 ES cell line was maintained on mouse embryonic fibroblast (MEF) feeder cells in Glasgow minimum essential medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 1000 U/mL leukemia inhibitory factor (LIF; Wako Pure Chemical Industries, Ltd, Osaka, Japan), 15% knockout serum replacement (KSR; Life Technologies, Carlsbad, CA, USA), 1% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 μM nonessential amino acids (NEAA; Nacalai Tesque, Tokyo, Japan), 2 mM L-glutamine (Nacalai Tesque), 1 mM sodium pyruvate (Life Technologies), 100 U/mL penicillin–streptomycin (Nacalai Tesque) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich).

Differentiation of mouse ES cells into pancreatic β-cells

For the differentiation studies, ES cells were dissociated and plated on gelatin-coated 96-well plates at a density of 5000 cells/well in Dulbecco's modified eagle medium (DMEM: Life Technologies) containing 4500 mg/L glucose supplemented with 10% FBS, 100 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin and 0.1 mM β-mercaptoethanol on day 0. On day 1, the culture medium was replaced with Medium 1, which was composed of DMEM containing 4500 mg/L glucose supplemented with 10 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin, 0.1 mM β-mercaptoethanol, 1% Insulin–Transferrin–Selenium Supplement (ITS; Life Technologies), 0.25% Albumax and 10 ng/mL recombinant human Activin-A (R&D Systems, Minneapolis, MN, USA). Media were replaced every other day. On day 5, the cells were cultured in Medium 2, consisting of Medium 1 supplemented with 10 μM retinoic acid (Sigma-Aldrich). Finally, from days 6 to 12, cells were cultured in Medium 3, composed of DMEM containing 1000 mg/L glucose supplemented with 100 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin, 0.1 mM β-mercaptoethanol, 1% ITS, 0.25% Albumax and 3 mM nicotinamide (Sigma-Aldrich). The media were replaced every other day. Embryoid bodies (EBs) were formed in liquid suspension culture with DMEM containing 4500 mg/L glucose supplemented with 10% FBS, 100 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin and 0.1 mM β-mercaptoethanol for 2 days followed by plating into gelatin-coated 96-well plates for further differentiation. The medium used for β-cell differentiation was the same as above mentioned.

Differentiation of mouse ES cells into neural cells

Embryonic stem cells were dissociated and plated on gelatin-coated 96-well plates at a density of 50 000 cells/well in Dulbecco's modified eagle medium (DMEM: Life Technologies) containing 4500 mg/L glucose supplemented with 10% FBS, 100 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin and 0.1 mM β-mercaptoethanol on day 0. On day 1, the culture medium was replaced with N2B27 medium supplemented with 10 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin, 0.1 mM β-mercaptoethanol, 1% ITS (Life Technologies) and 10 μM SB431542 (Wako Pure Chemical Industries, Ltd). Media were replaced every other day. On day 5, the cells were cultured in MPA-containing medium and then fixed on day 6.

Co-culture of endodermal cells with neural cells

For the generation of ES-derived neural cells for co-culture, embryoid (EB) was used. EBs were formed by seeding ES cells on a 60 mm ultra-low attached culture dish (Corning, NY) at a density of 1 000 000 cells/dish in DMEM containing 4500 mg/L glucose supplemented with 10% FBS, 100 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin and 0.1 mM β-mercaptoethanol. The medium was replaced on day 2. On day 4, EBs were dissociated and seeded on the gelatin-coated upper chamber of transwells. The next day, media were changed to N2B27 media supplemented with 10 μM NEAA, 2 mM L-glutamine, 100 U/mL penicillin–streptomycin, 0.1 mM β-mercaptoethanol and 10 μM SB431542 (Wako Pure Chemical Industries, Ltd). The co-culture was started by adding the upper chamber containing ES-derived neural cells to the ES cell-derived endodermal cells in the lower chamber on differentiation day 6. The co-culture was maintained by day 12. The same media as the β-cell differentiation method mentioned above was used from days 6 to 12.

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Differentiation of human iPS cells into pancreatic β-cells

Differentiation studies followed the 5-stage β-cell differentiation protocol reported by Shahjalal *et al.* with minor modifications. The Toe human iPS cell line was maintained on Synthemax II (Corning)-coated 60 mm dishes in Essential 8 medium (Life Technologies). Undifferentiated Toe iPS cells were dissociated and plated on Synthemax II-coated 6-well plates at a density of 500 000 cells/well in Essential 8 medium on day -2. On day 0, differentiation was initiated by replacing the culture with Medium A, consisting of DMEM with 4500 mg/L glucose supplemented with 3 μM CHIR99021 (Merck KGaA, Darmstadt, Germany), 100 ng/mL recombinant human Activin-A and 2% B27 supplement (Life Technologies). On days 1 and 2, the media were replaced with Medium A without CHIR99021. On day 3, cells were passaged and seeded on Synthemax II-coated 96-well plates at 100 000 cells per well in Medium B: RPMI (Life Technologies) supplemented with 0.25 μM SANT-1 (Wako Pure Chemical Industries, Ltd), 50 ng/mL recombinant human FGF10 and 2% B27 supplement. From day 5, the cells were cultured in Medium C, consisting of DMEM with 4500 mg/L glucose supplemented with 0.25 μM SANT-1, 0.1 μM LDN193189 (Wako Pure Chemical Industries, Ltd), 10 μM SB431542, 2 μM retinoic acid and 1% B27 supplement. From day 11 on, the media were changed to Medium D, consisting of DMEM with 4500 mg/L glucose supplemented with 0.1 μM LDN193189, 5 μM TGF-β type I receptor kinase inhibitor II (Merck KGaA), 0.3 μM (−)-indolactam V and 1% B27 supplement. Finally, from days 13 to 19, cells were cultured in Medium E, which was composed of Knockout DMEM/F-20 (Life Technologies) supplemented with 50 ng/mL exendin-4 (Anaspec, Inc., Fremont, CA), 10 mM nicotinamide and 1% B27 supplement.

Screening

In our assay, 303 compounds (StemSelect small molecule regulators library, Merck KGaA), which comprised pharmacologically active, structurally diverse small molecules, including extracellular domain-targeting reagents and cell-permeable compounds, were screened for their ability to promote β-cell differentiation. Compounds were added to Medium 3 at a concentration of 0.625 or 2.5 μM. The final concentration of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was 0.1%. Insulin- and GFP double-positive cells were defined as β-cells. The cells were assayed by immunostaining, and imaging data were collected with a PerkinElmer Operetta high-content analysis system coupled to Harmony software.

Chemicals

Mycophenolic acid (Merck KGaA), XAV939 (Tocris bioscience, Ellisville, MO, USA), CHIR99021 (Wako Pure Chemical Industries, Ltd) and guanosine (Sigma-Aldrich) were dissolved and diluted in DMSO. Rho/Rac/Cdc42 activator I (Cytoskeleton, Denver, CO, USA) was dissolved and diluted in water.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min. The following antibodies were used: guinea pig anti-insulin (Dako Denmark A/S, Glostrup, Denmark, 1/500), mouse antiglucagon (Sigma-Aldrich, 1/500), rabbit anti-GFP (Medical & Biological Laboratories, 1/1000), mouse anti-Nkx6.1 (Developmental Studies Hybridoma Bank), goat anti-sox17 (R&D Systems, 1/100), mouse anti-neuron-specific β III tubulin (Tuj1, R&D Systems, 1/100), mouse anti-nestin (Millipore, 1/100), goat anti-Flk-1 (R&D Systems, 1/100), rat anti-Pdgf receptor a (Pdgfra, affymetrix, 1/100), rabbit anti-CD31 (abcam, 1/100), rabbit anti-p63 (abcam, 1/100), mouse anti-cytokeratin 14 (CK14, abcam, 1/100), mouse anti-cytokeratin 18 (CK18, Millipore, 1/100), rabbit anti-fatty acid binding protein 4 (FABP4, Novus, 1/100), Alexa488 goat anti-rabbit IgG (Life Technologies, 1/1000), Alexa488 goat anti-rat IgG (Life Technologies, 1/1000), Alexa568 goat anti-guinea pig IgG (Life Technologies, 1/1000), Alexa568 donkey anti-rat IgG (Life Technologies, 1/1000), Alexa647 goat anti-mouse IgG (Life Technologies, 1/1000), CF488 donkey anti-goat IgG (Biotium, 1/1000), CF568 donkey anti-goat IgG (Biotium, 1/1000), CF568 donkey anti-mouse IgG (Biotium, 1/1000), CF568 donkey anti-rabbit IgG (Biotium, 1/1000) and CF647 donkey anti-mouse IgG (Biotium, 1/1000). The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Roche Diagnotics, Basel, Switzerland). TUNEL assay was carried out using the *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics, Mannheim, Germany).

Real-time PCR analysis

Total RNA was extracted from the cells using RNeasy micro kits (Qiagen, Hilden, Germany). Complementary DNA was synthesized using SuperScript VILO (Life Technologies). Gene expression levels were quantified by real-time PCR assay with TaqMan probes and TaqMan universal PCR master mix (Life Technologies) on an ABI Prism 7900HT Sequence Detector System (Life Technologies). The level of each gene expression was normalized to that of β glucuronidase (GusB). All of the TaqMan probes for real-time PCR are listed in Table S5 in Supporting Information.

Glucose-stimulated c-peptide secretion and c-peptide content

The differentiated cells were precultured for 30 min at 37 °C with 5.5 mM glucose containing DMEM/1% FBS with minimal supplements. Cells were then incubated for 1 h at 37 °C with 5.5 mM glucose (low glucose) or 25 mM glucose (high glucose) contained in the media. After incubation, supernatants

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were obtained from each well and the cells were lysed with 1% Triton X-100/PBS supplemented with protease inhibitor. The c-peptide concentration of the supernatants and cell lysates were then measured using an enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi Co. Ltd, Gunma, Japan).

Microarray analysis

Affymetrix Gene Chip Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA, USA) was used and expression data analysis was carried out using SPOTFIRE software 3.3.2 (Spotfire Inc., Somerville, MA, USA) and IPA.

Statistics

Data are expressed as the mean ± standard error. Differences between the two groups were analyzed by Student's *t*-tests. Dunnett's tests were used for multiple comparisons. The respective statistical analysis applied is noted in each figure legend. A *P*-value below 0.05 was considered statistically significant. Statistical analyses were carried out using an SAS software package version 8.2 (SAS Institute Japan Ltd, Tokyo, Japan).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1 MPA potentiates β cell differentiation from ING112 mouse ES cell line. a) A schematic diagram of the time window for treatment with MPA (2 μM) in a β cell differentiation system. b) The proportions of GFP (Insulin)-double positive β cells on day 12 (n = 8). Data represent mean ± SEM. $^{\#}P < 0.05$, by Dunnett's test.

Figure S2 Immunochemistry of mesodermal and ectodermal markers. Cells on differentiation day 5 in the β differentiation were immunostained for mesodermal markers, Flk1, Pdgfra and CD31, and surface ectodermal markers, p63, CK14 and CK18. Scale bar indicates 100 μm.

Figure S3 MPA potentiates β cell differentiation from mouse ES cells via EB formation. a) A schematic diagram of the β differentiation via the EB formation protocol. b–d) The proportion of Tuj1-positive neuronal cells (b), Sox17-positive endodermal cells (c) on day 8 (n = 4), and insulin- and GFP (Pdx1)- double positive β cells on day 14 (n = 6) (d). Data represent mean ± SEM. $*P < 0.05$, by Student's *t*-test. Scale bar indicates 100 μm.

Figure S4 The mouse ES-derived neural cells a, b) Immunostaining of neural cell markers, Tuj1 and Nestin. Numbers indicate the proportions of positive cells. c) Almost no positive FABP4-positive adipocytes were detected. Scale bar indicates 100 μm.

Table S1 The top 12 hit compounds

Table S2 Top10 IPA canonical pathways for up- or down-regulated genes after MPA treatment

Table S3 Top 10 IPA biofunctions for up- or down-regulated genes following MPA treatment

Table S4 Genes related to nervous system development and function down-regulated by MPA treatment

Table S5 Gene assay list

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