

FIG 3 Knockdown of NOTCH1 abrogates the production of the smaller ROCK1 and MYPT1 phosphorylation with restoration of clonogenic growth potential in normal keratinocytes exposed to EDTA, BSA, or serum. (A) HCK1T cells stably expressing NOTCH1 shRNA (NOTCH1 RNAi) or control shRNA (EGFP RNAi) were treated with 2.5 mM EDTA, 5% bovine serum albumin, or 10% serum or left untreated. Cells were harvested at 3 days posttreatment, and cell extracts were subjected to immunoblotting analysis with the indicated antibodies. (B) Aliquots of 500 HCK1T cells stably expressing NOTCH1 shRNA (NOTCH1 RNAi) or control shRNA (EGFP RNAi) were seeded on 35-mm dishes under sparse conditions. Then cells were treated similarly to those for panel A. After being cultivated for 2 weeks, the cells were stained with Giemsa's dye, and colonies were counted. The photographs are of representative dishes, and the graph represents means \pm SDs. *, $P < 0.05$ according to Student's t tests.

(ab72479; Abcam, Paris, France), β -actin (sc-1616; Santa Cruz Biotechnology), α -tubulin (2144; Cell Signaling Technology), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (AM4300; Ambion, Inc., Austin, TX) were used as probes. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) and anti-goat (sc-2033; Santa Cruz Biotechnology) immunoglobulins were used as the secondary antibodies. The LAS3000 charge-coupled device (CCD) imaging system (Fujifilm Co. Ltd., Tokyo, Japan) was employed for detection of proteins visualized by Lumi-light Plus Western blotting substrate (Roche, Basel, Switzerland). The exposure time was adjusted to keep the signals within a linear and nonsaturated range, and the band signal intensities were quantified by densitometry using imaging software (Fujifilm Multi-Gauge) after normalization to that of internal controls, such as β -actin, α -tubulin, or GAPDH.

Fluorescence-activated cell sorting analysis. Cells were treated with a CycleTEST Plus DNA reagent kit (Becton Dickinson, Franklin Lakes, NJ) for propidium iodide staining and then analyzed with a Becton Dickinson FACSCalibur instrument.

Clonogenic assay. Aliquots of 500 cells were seeded on 35-mm dishes under sparse conditions. After cultivation for 2 weeks, the cells were stained with Giemsa's dye, and the colonies were counted.

Statistical analysis. Statistical analysis was carried out using Microsoft Excel software. Unless stated otherwise, all data are presented as means \pm standard deviations (SD) from at least three independent experiments; error bars represent SD in all figures. Intergroup comparisons were performed by the two-tailed Student's t test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Immediate activation of ROCK following expression of the NOTCH intracellular form. Previous work has shown stabilization of the noncovalent interaction between a ligand-binding extracellular domain and a transmembrane signaling subunit of

NOTCH by millimolar Ca^{2+} and transient activation of this heterodimeric NOTCH receptor by EDTA-mediated shedding of its extracellular domain, independent of cell-cell contact or binding of a ligand displayed on the surface of a neighboring cell (25). In line with this notion, somatic activating mutations of NOTCH1 within the heterodimerization domain are frequently found in human T cell acute lymphoblastic leukemia and are thought to increase the production of the intracellular form of NOTCH1 (53). To ascertain whether calcium depletion could induce activation of NOTCH1, normal human keratinocytes, which were maintained with serum-free, low-calcium medium, were subjected to EDTA treatment. We found that this chelator treatment elicited immediate and robust expression of the cleaved intracellular form of NOTCH1 in a time- and dose-dependent manner (Fig. 1A). Time course experiments revealed that this intracellular NOTCH1 arose transiently and that thereafter activation of the NOTCH target gene was induced, as well as upregulation of a differentiation marker and downregulation of a keratinocyte stemness marker, Δ Np63 α , in agreement with its proposed role in keratinocyte differentiation (Fig. 1B) (8, 9, 11). We also noted that the EDTA-treated cells underwent a drastic morphological change (Fig. 2A) with increased motility (see Movie S1 in the supplemental material), which was also seen in cells transduced with the NOTCH1 intracellular domain (ICN1) in much the same fashion (Fig. 2A). We have previously shown that these morphologically altered cells are differentiating cells strongly expressing K10 and involucrin (14).

ROCK inhibition with Y-27632 has been reported to inhibit differentiation and increase the proliferative capacity of primary human keratinocytes in culture, pointing to a role for ROCK

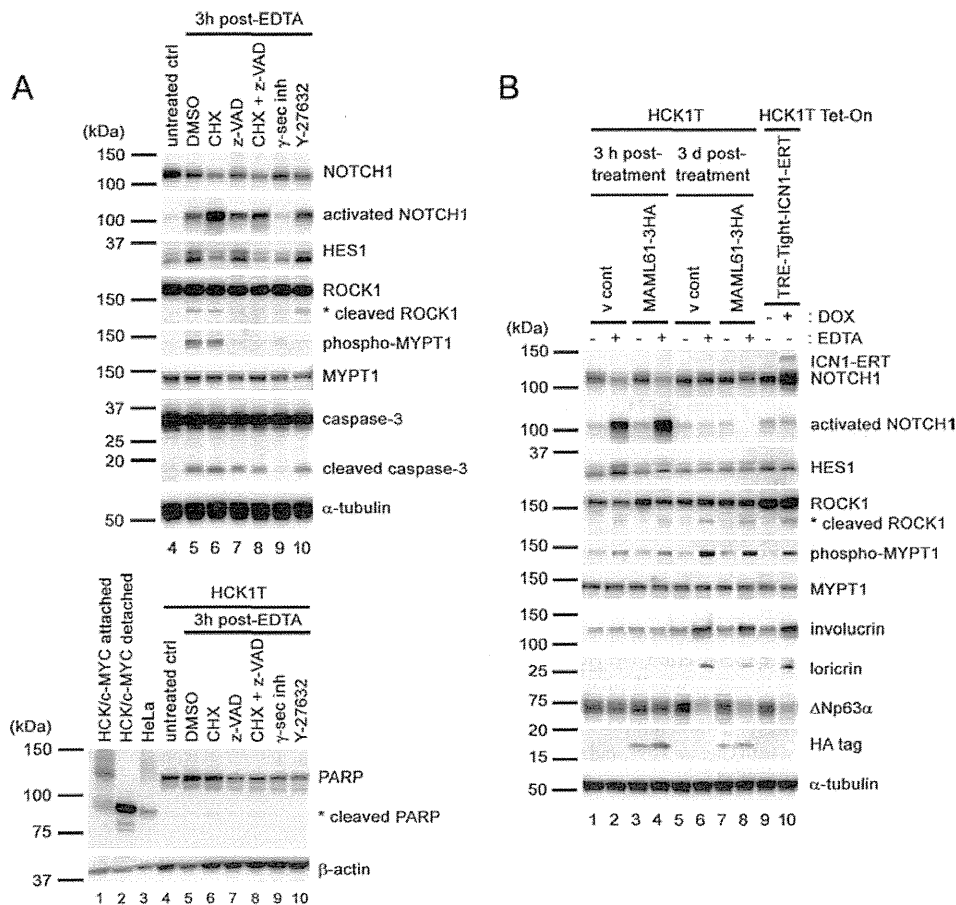


FIG 4 ROCK1 activation is independent of NOTCH1 transcriptional activity. (A) HCK1T cells, pretreated where indicated with 10 μ M cycloheximide (CHX) for 2.5 h, 20 μ M z-VAD-fmk for 2.5 h, 10 μ M γ -secretase inhibitor (DAPT) for 2.5 h, or 10 μ M Y-27632 for 2.5 h, were either left untreated or treated with 2.5 mM EDTA for 10 min and incubated with keratinocyte-SF medium for 3 h. Whole-cell extracts were subjected to immunoblotting analysis with the indicated antibodies. As a positive control for PARP cleavage, cell extracts from detached HCKs after c-MYC transduction were used. (B) HCK1T cells were stably transfected with dominant negative MAML1 (MAML61-3HA) or vector control (v cont). Cells were exposed to 2.5 mM EDTA (+) or left untreated (-), and cell lysates were prepared at 3 h and 72 h posttreatment. HCK1T Tet-On cells were then introduced with the TRE-Tight-ICN1-ERT tetracycline-inducible gene cassette. HCK1T Tet-On-ICN1-ERT cells were either left untreated (-) or treated (+) with 1 μ M/ml doxycycline (DOX) for 72 h.

in keratinocyte differentiation (35, 37). These observations prompted us to determine whether NOTCH activation by EDTA treatment during passage could induce differentiation or a decrease in proliferative potential, possibly through ROCK activation. As shown in Fig. 1B, immediately after production of the NOTCH1 intracellular form, additional ROCK1 polypeptides of smaller size emerged. The ROCK1 fragment with a relative molecular weight of \sim 130,000 was reminiscent of that observed in cells undergoing apoptosis, which has been identified as a caspase-3-cleaved, constitutively activated form (54, 55). Indeed, MYPT1, a downstream molecule of ROCK, was phosphorylated concomitantly with the appearance of the smaller ROCK1 proteins, indicating increased ROCK1 activity in cells with active NOTCH1 signaling.

In keratinocytes, NOTCH1 has been shown to be activated in response to differentiation stimuli such as suspension in methylcellulose, confluence-triggered cell-cell contact, and serum exposure, as well as with genotoxic stress (13, 14, 18, 49, 56). Therefore, we examined whether differentiation stimuli or expression of ICN1 could cause ROCK activation. The additional smaller proteins of ROCK1, but not ROCK2, and phosphorylation of MYPT1

were observed in response to serum exposure (Fig. 2B, left panel). Since albumin is the major protein in serum, to which approximately half of extracellular calcium is bound, we further tested the possibility that albumin exerts a differentiation-inducing effect through a mechanism similar to that for EDTA. As expected, albumin exposure also induced activation of NOTCH1, production of the smaller ROCK1, and upregulation of the differentiation markers (Fig. 3A, lane 3). Similarly, retroviral transduction of keratinocytes with ICN1 gave rise to the smaller ROCK1 in association with MYPT1 phosphorylation (Fig. 2B, left panel). Of importance, confluence-triggered cell-cell contact also induced smaller ROCK1 and MYPT1 phosphorylation, indicating that the ligand-mediated NOTCH activation is responsible for these events (Fig. 2B, right panel). Assuming that activated NOTCH1 evokes some apoptotic cascade, ROCK activation may occur in parallel in cells undergoing apoptosis. However, we failed to detect any indication of apoptotic cell death after ICN1 transduction (Fig. 2C). To further corroborate involvement of NOTCH1 in ROCK1 activation, we knocked down NOTCH1 expression. In contrast to the control case, NOTCH1 silencing abrogated the production of the smaller

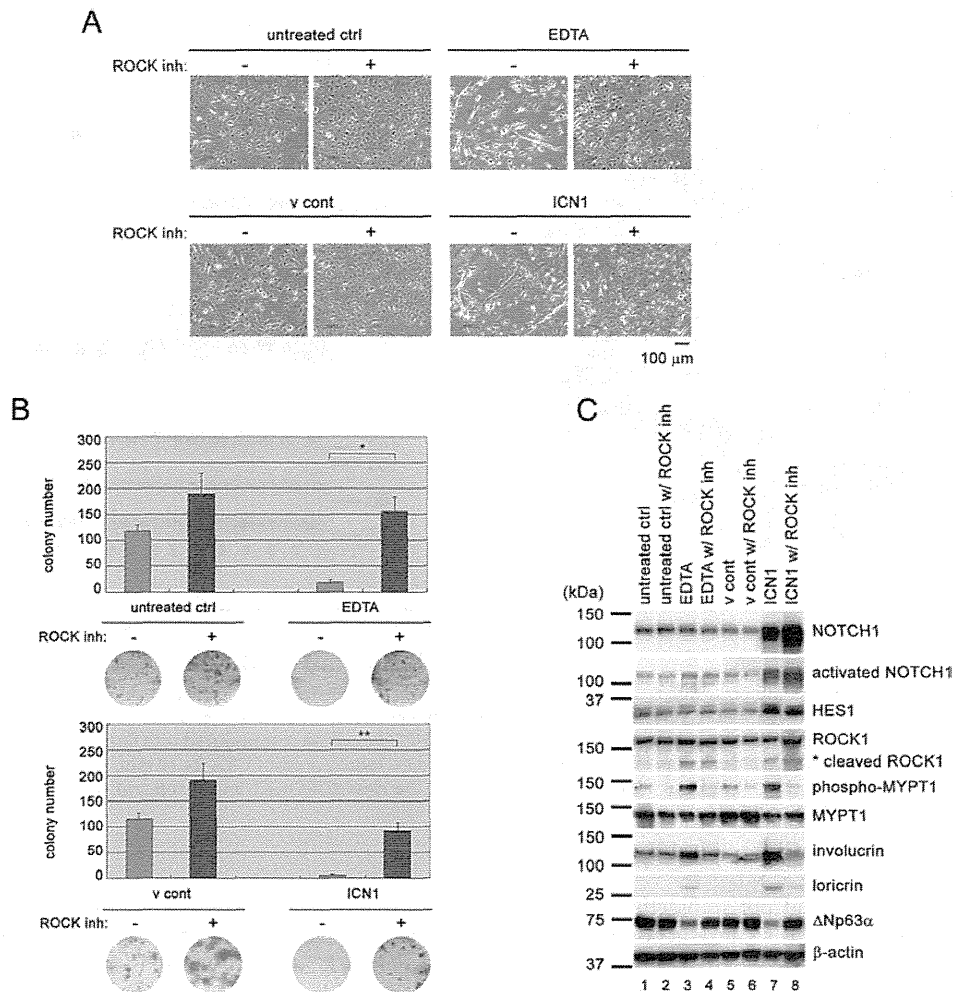


FIG 5 ROCK inhibition alleviates the growth-suppressive and differentiation-inducing effects of NOTCH1. (A) HCK1T cells pretreated with or without Y-27632 were either left untreated, treated with 2.5 mM EDTA, or transduced with ICN1 or vector control (v cont). Thereafter, cells were incubated in the presence or absence of Y-27632. Typical areas were photographed at 72 h after EDTA treatment. (B) Aliquots of 500 HCK1T cells were seeded on 35-mm dishes under sparse conditions. Cells pretreated with or without Y-27632 were then either left untreated, treated with 2.5 mM EDTA for 10 min, or transduced with ICN1 or vector control (v cont). After being cultivated for 2 weeks, the cells were stained with Giemsa's dye, and colonies were counted. The photographs are of representative dishes, and the graph represents means \pm SDs. *, $P < 0.05$; **, $P < 0.01$ (according to Student's t tests). (C) Cells were treated similarly to those for panel A and incubated for 72 h. Cell extracts were subjected to immunoblotting analysis with the indicated antibodies.

ROCK1 and MYPT1 phosphorylation after differentiation stimuli (Fig. 3A) with restoration of clonogenic growth potential (Fig. 3B). Treatment with a γ -secretase inhibitor had the same effects as NOTCH1 knockdown (Fig. 4A and unpublished observations). These results suggest that the observed ROCK activation is not an outcome of apoptosis but is positively regulated by NOTCH1 signaling in differentiating cells.

The intracellular form of NOTCH is responsible for ROCK activation independently of its transcriptional activity. Given the immediate appearance of the smaller ROCK1 after expression of the intracellular NOTCH1, it can be hypothesized that NOTCH1 activation of ROCK1 does not occur through *de novo* protein expression. In an effort to clarify this, we treated cells with cycloheximide (CHX), an inhibitor of translation. While CHX treatment completely inhibited induction of the NOTCH1 transcriptional target, HES1, it did not affect the generation of the smaller ROCK1 and MYPT1 phosphorylation (Fig. 4A, upper

panel, compare lanes 5 and 6). Since NOTCH1 requires the transcriptional coactivator, MAML1, in its transactivation complex, we also assessed the effect of dominant negative MAML1, MAML61-3HA. While exogenous expression of MAML61-3HA blocked the induction of HES1, it exerted no effect on the smaller ROCK1 production, MYPT1 phosphorylation, and expression of a differentiation marker, involucrin (Fig. 4B). It is still possible that the canonical NOTCH1 signaling plays a role in differentiation at a later stage, as the level of a terminal differentiation marker, loricrin, appeared to be attenuated to some extent in MAML61-3HA-expressing cells. To further explore the transcription-independent function of NOTCH1 in ROCK1 activation, we established tetracycline-inducible ICN1-ERT keratinocytes, expressing a chimeric protein consisting of the intracellular domain of NOTCH1 fused at the amino terminus to a mutated ligand-binding domain of the estrogen receptor (ERT) (57). In the presence of doxycycline (DOX) for 3 days and without addition of the

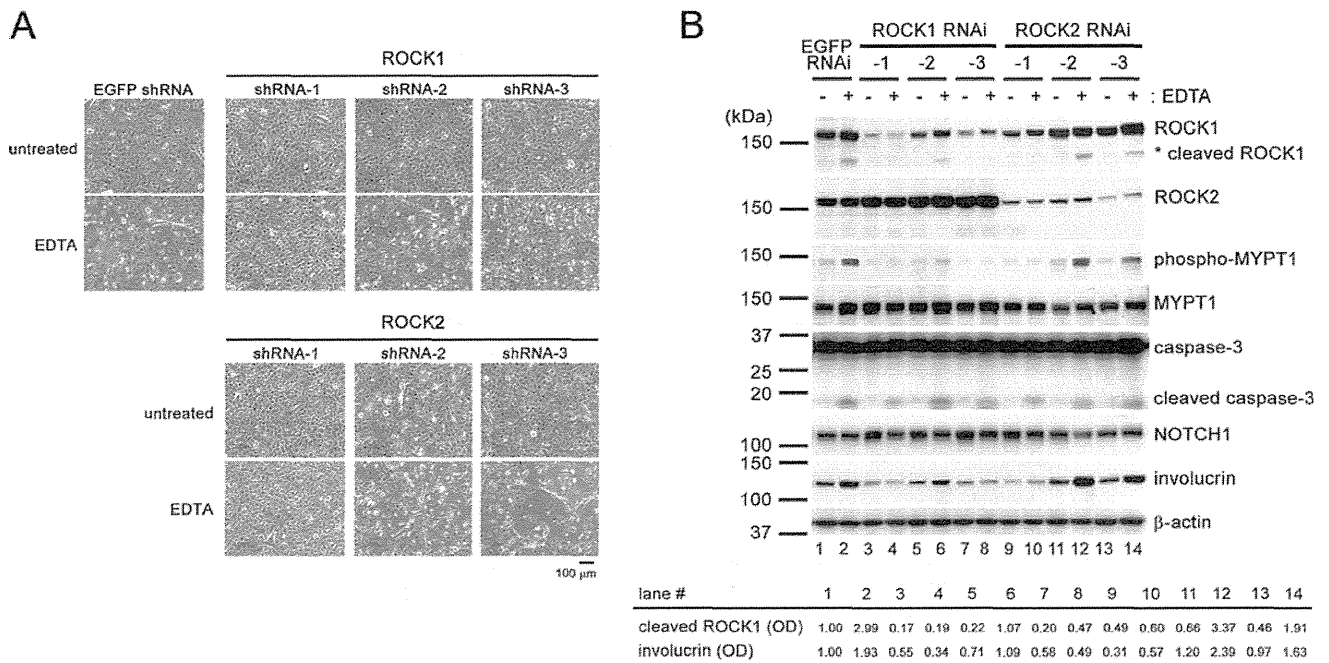


FIG 6 Knockdown of ROCK1 inhibits keratinocyte differentiation after EDTA treatment. (A) HCK1T cells stably transduced with shRNAs for ROCK1 and -2 were either left untreated or treated with 2.5 mM EDTA and then incubated with keratinocyte-SF medium. Typical areas were photographed at 3 days after EDTA treatment. Note that ROCK2 shRNA-2 knocked down ROCK1 expression as well. (B) HCK1T cells were transduced with three different shRNAs for ROCK1 and -2 or with control shRNA for EGFP. Cells were then either left untreated (–) or treated (+) with 2.5 mM EDTA for 10 min. After cultivation for 72 h, cells were harvested and analyzed by immunoblotting. The band optical densities (OD) for the cleaved ROCK1 and involucrin were quantified densitometrically, normalized to that of β -actin with the value for untreated control defined as 1.

estrogen homolog, ICN1-ERT did not transactivate the target gene but triggered the smaller ROCK1 production and MYPT1 phosphorylation (Fig. 4B, compare lanes 9 and 10). These results indicate a transcription-independent or cytoplasmic function of NOTCH1 in control of ROCK1 activity and its roles in cellular differentiation.

To elucidate whether the additional smaller fragment of ROCK1 is a constitutively active form generated by caspase-3-mediated cleavage (54, 55), cells were treated with the caspase inhibitor z-VAD prior to EDTA exposure. In the presence of active forms of NOTCH1, z-VAD markedly blocked generation of the smaller ROCK1 as well as MYPT1 phosphorylation (Fig. 4A, upper panel, compare lanes 5 and 7), indicating that the NOTCH intracellular form could induce immediate and constitutive activation of ROCK1 through caspase. This activation of caspase is much weaker than that observed in apoptotic cells, since cleavage of the well-known caspase substrate PARP was marginally detected only when pretreated with CHX (Fig. 4A lower panel), suggesting a nonapoptotic function of caspase activation in keratinocyte differentiation.

ROCK inhibition rescues the phenotype induced by NOTCH activation. Next, to address whether ROCK activation is the critical downstream event in NOTCH signaling, we tested whether the NOTCH gain-of-function phenotype could be reversed by ROCK inhibition. To this end, we assessed the differentiation status and clonogenic growth potential after EDTA treatment or ICN1 transduction in the presence of a ROCK inhibitor. ROCK inhibition significantly restored the morphology and clonogenicity of keratinocytes upon EDTA treatment or ICN1 transduction

(Fig. 5A and B), and suppressed both induction of differentiation markers and reduction of Δ Np63 α (Fig. 5C). Silencing of ROCK1 but not ROCK2 also blocked differentiation after EDTA treatment (Fig. 6A and B). Intriguingly, continuous ROCK inhibition rendered cells permissive to constitutive ICN1 expression as well as HES1 induction without indication of differentiation and maintained growth potential during at least three passages, while only cells expressing a small amount of ICN1 survived without the ROCK inhibitor (Fig. 7A). ROCK inhibition may allow increased NOTCH1 levels, possibly due to counterbalancing its biological effects. ROCK inhibitor withdrawal resulted in a prompt resumption of differentiation following MYPT1 phosphorylation in ICN1-expressing cells (Fig. 7B). These results suggest that ROCK is the critical downstream effector of NOTCH signaling in loss of stemness and differentiation of keratinocytes.

Ectopic expression of constitutively active ROCK1 induces keratinocyte differentiation. We next asked whether activated ROCK1 itself could initiate differentiation of normal human keratinocytes. For this purpose, we established several lines of keratinocytes expressing various exogenous ROCK1 mutants in a doxycycline-dependent manner. Upon induced expression of ROCK1 Δ C241, a truncated mutant corresponding to a caspase-cleaved constitutively active form, downregulation of Δ Np63 α and upregulation of a differentiation marker were evident to a level comparable to that observed in ICN1-ERT cells (Fig. 8A, compare lanes 2 and 8). Expression of wild-type ROCK1 exhibited only marginal effects (Fig. 8A, lane 6), while caspase-cleavage resistant ROCK1-D1113A and kinase-inactive ROCK1-K105A mutants failed to induce differentiation (Fig. 8A, lanes 10 and 12).

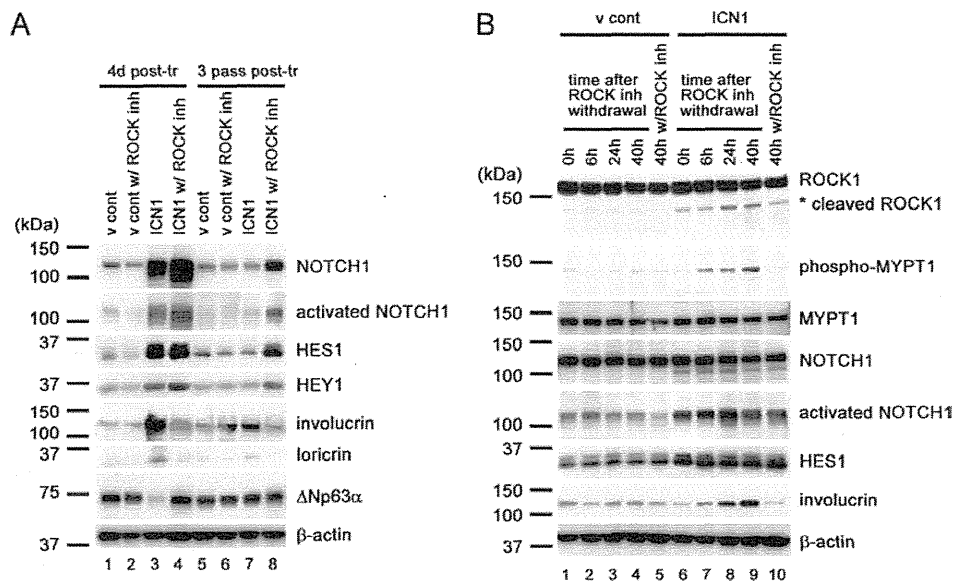


FIG 7 ROCK inhibition allows propagation of keratinocytes in the presence of constitutively active NOTCH1. (A) HCK1T cells pretreated with or without Y-27632 were transduced with the constitutively active form of NOTCH1 (ICN1) or vector control (v cont). Cells were cultivated until 22 days posttransduction (three passages) in the presence or absence of Y-27632. Cell extracts were prepared at 4 and 22 days posttransduction and subjected to immunoblotting analysis with the indicated antibodies. Note that the ROCK inhibitor inhibits differentiation and permits proliferation of ICN1-expressing cells. In contrast, control cells which have escaped after ICN1 transduction showed decreased levels of activated NOTCH1. (B) Cells were treated as for panel A. Cell lysates were prepared at the indicated time points after withdrawal of Y-27632 and subjected to immunoblotting analysis with the indicated antibodies.

It has been shown that the Rho inhibitor C3 as well as Y-27632 inhibited differentiation induced by single-cell suspension, although RhoA activity is unvaried during differentiation (35). In our monolayer culture model, conditional overexpression of a constitutively active form of RhoA was able to promote differentiation to some extent (Fig. 8B, lane 2). We then examined C3's effect on the process of keratinocyte differentiation. C3 only partially inhibited overall ROCK activation, judging from the phosphorylation levels of MYPT1 (Fig. 8C, compare lanes 2 and 7). Furthermore, this inhibition seemed to be closely associated with unexpected inhibition of the NOTCH1-caspase-ROCK1 axis through unknown mechanisms. Therefore, unlike keratinocyte differentiation induced by single-cell suspension assays, ROCK1 activation in monolayer culture essentially depends on its cleavage by caspase but not much on Rho activation, though we have not confirmed Rho activity during keratinocyte differentiation. These data support the conclusion that the NOTCH1-caspase-ROCK1 axis plays a major role in keratinocyte differentiation at least in monolayer culture. It is noteworthy that ROCK1 acts downstream of NOTCH1 but not NOTCH2, as ICN2 (a NOTCH2 intracellular form) did not induce ROCK1 cleavage and keratinocyte differentiation (Fig. 8A, compare lanes 2 and 4).

Proliferative defects upon dissociation in hiPSCs are partly attributable to NOTCH activity. It is well-known that human pluripotent stem cells such as hESCs undergo massive apoptosis when dissociated and that ROCK inhibitor treatment efficiently ameliorates such vulnerability to permit survival in clonal culture (39, 58). It is tempting to speculate that the NOTCH-ROCK pathway may also be activated in human pluripotent stem cells during the dissociation step with EDTA treatment and that this could be one mechanism underlying dissociation-induced loss of clonal

growth potential. In an attempt to assess this possibility, we employed two lines of human induced pluripotent stem cells (hiPSCs). As shown in Fig. 9A and B, virtually no hiPSC colonies were formed from cells dissociated by trypsin-EDTA treatment, whereas NOTCH inhibition by a γ -secretase inhibitor partially restored the efficiency of colony formation to a degree comparable to that with Rho inhibitor C3-treated cells. Combination inhibition of γ -secretase and Rho had an additive effect on the clonogenic growth capacity to the same extent as ROCK inhibition, suggesting that the NOTCH-ROCK and the Rho-ROCK axis act in parallel in this cellular context.

In line with a previous study showing ROCK-dependent myosin activation as the direct cause of dissociation-induced cell death in hESCs (41), we also found that treatment with the myosin inhibitor blebbistatin showed equal efficacy as ROCK inhibition (Fig. 9C and D).

To substantiate further the link between NOTCH and ROCK in hiPSCs, we next assessed the status of NOTCH and ROCK in adherent small clumps of cells treated with EDTA. In accordance with the data with keratinocytes, EDTA treatment triggered immediate NOTCH1 activation accompanied by ROCK1 cleavage and MYPT1 phosphorylation (Fig. 10A, lanes 2 and 6). Importantly, treatment with a γ -secretase inhibitor resulted in abrogation of ROCK1 activation (Fig. 10A, lanes 4 and 8), suggesting that regulation of ROCK1 activity by NOTCH1 is also conserved in hiPSCs. Because EDTA treatment instigated drastic morphological change which was blocked by NOTCH or ROCK inhibition (Fig. 10B), we speculated that this stimulus may have an impact on the cell fate. Indeed, 3 days after EDTA treatment, expression of the stemness marker OCT3/4 was substantially downregulated in still-attached control cells (Fig. 10C, compare lanes 7 and 8). In-

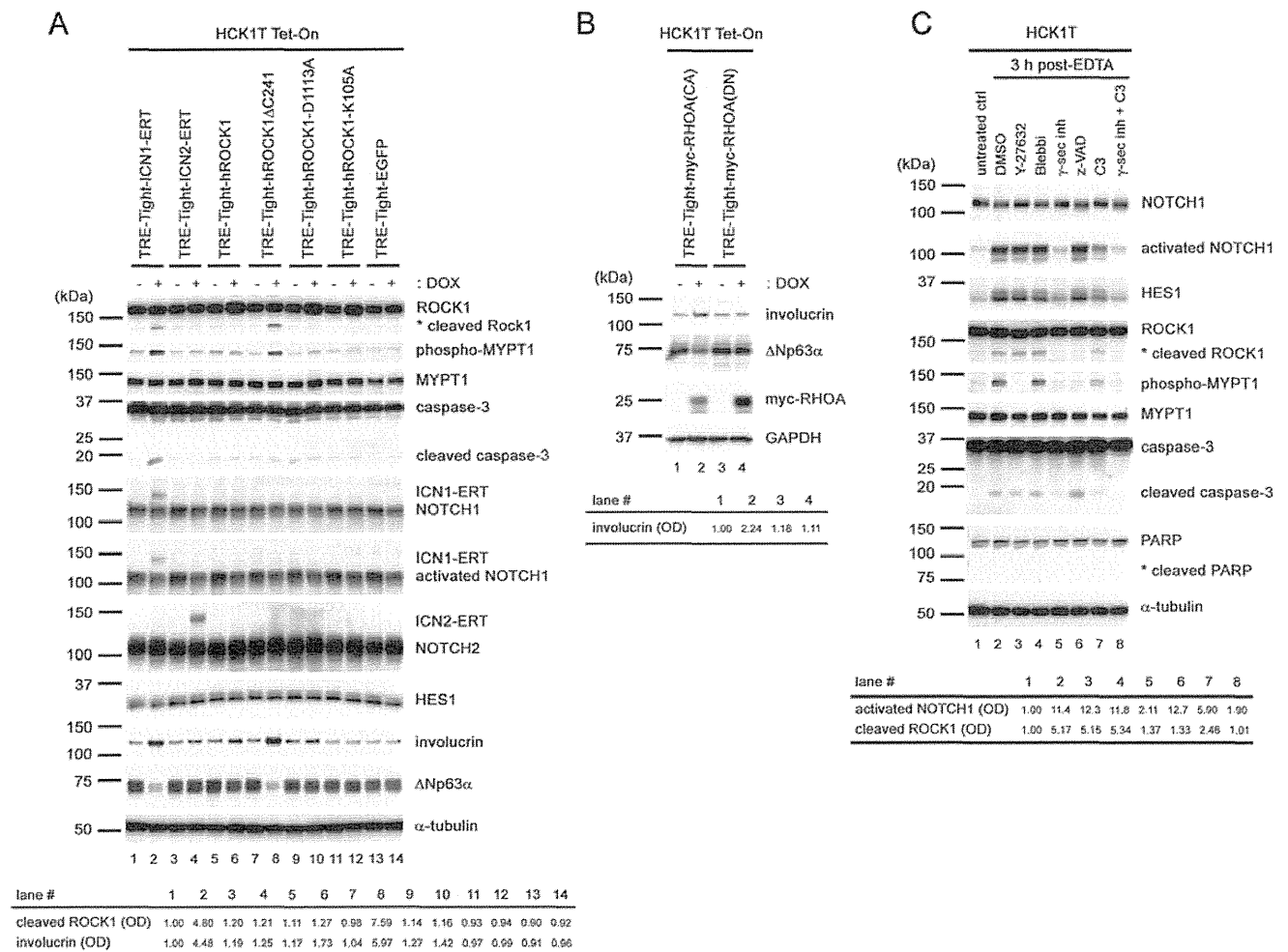


FIG 8 Ectopic expression of active ROCK1 induces keratinocyte differentiation. (A and B) HCK1T Tet-On cells were stably transduced with the indicated tetracycline-inducible gene constructs by retroviral gene transfer. Cells were either left untreated (–) or treated (+) with 1 μ g/ml doxycycline (DOX) for 72 h. Cell extracts were analyzed by immunoblotting with the indicated antibodies. A long-exposed image for activated NOTCH1 is shown. Quantification of relative optical densities (OD) for the cleaved ROCK1 and involucrin bands, normalized to the value for the α -tubulin or GAPDH control, are indicated. (C) HCK1T cells, pretreated where indicated with 10 μ M Y-27632, 10 μ M blebbistatin, 10 μ M γ -secretase inhibitor (DAPT), 20 μ M z-VAD-fmk, or 1.0 μ g/ml C3 for 3 h, were either left untreated or treated with 2.5 mM EDTA for 10 min and incubated with keratinocyte-SF medium for 3 h. Cell extracts were subjected to immunoblotting analysis with the indicated antibodies. Quantification of relative optical densities (OD) for the activated NOTCH1 and cleaved ROCK1 bands, normalized to the value for α -tubulin with the untreated control defined as 1, are indicated.

hibition of either NOTCH or Rho significantly prevented this transition (Fig. 10C). Of note, the inhibitor-treated colonies showed normal morphology in a mass, whereas the EDTA-treated controls exhibited heterogeneous and differentiated traits at this time point. These observations indicate that EDTA treatment induces acute ROCK1 activation and consequent loss of stemness at least partly in a NOTCH-dependent manner in hiPSCs.

DISCUSSION

Identification of NOTCH1 as a novel upstream regulator for ROCK1. The best-known upstream regulator for ROCK activity is Rho GTPase (32). In the normal cellular context, ROCK becomes an active kinase by conformational change through interaction with the GTP-bound form of Rho, which is thought to release the kinase domain from the carboxy-terminal autoinhibitory domain. In apoptotic cells, removal of the autoinhibitory domain of ROCK1 by caspase-3-mediated cleavage renders this protein con-

stitutively active (54, 55, 59). In the present study, we first found that the cleaved ROCK1 is also generated immediately after emergence of the NOTCH1 intracellular form in the context of keratinocyte differentiation and revealed that activated NOTCH1 causes constitutive activation of ROCK1 through caspase cleavage upon EDTA treatment or exposure to differentiation-inducing stimuli such as serum as well as cell-cell contact (Fig. 11). We further demonstrated that this type of ROCK1 activation is mediated by a transcription-independent function of NOTCH1. Thus, we conclude that noncanonical NOTCH1 signaling triggers differentiation and a consequential decrease in clonogenicity mostly if not completely through ROCK1 activation in keratinocytes. To our knowledge, this is the first demonstration of NOTCH1 function as a novel and critical upstream regulator for ROCK1 activity. The Rho inhibitor C3 inhibited MYPT1 phosphorylation to some extent in concert with reduction of activated NOTCH1 and

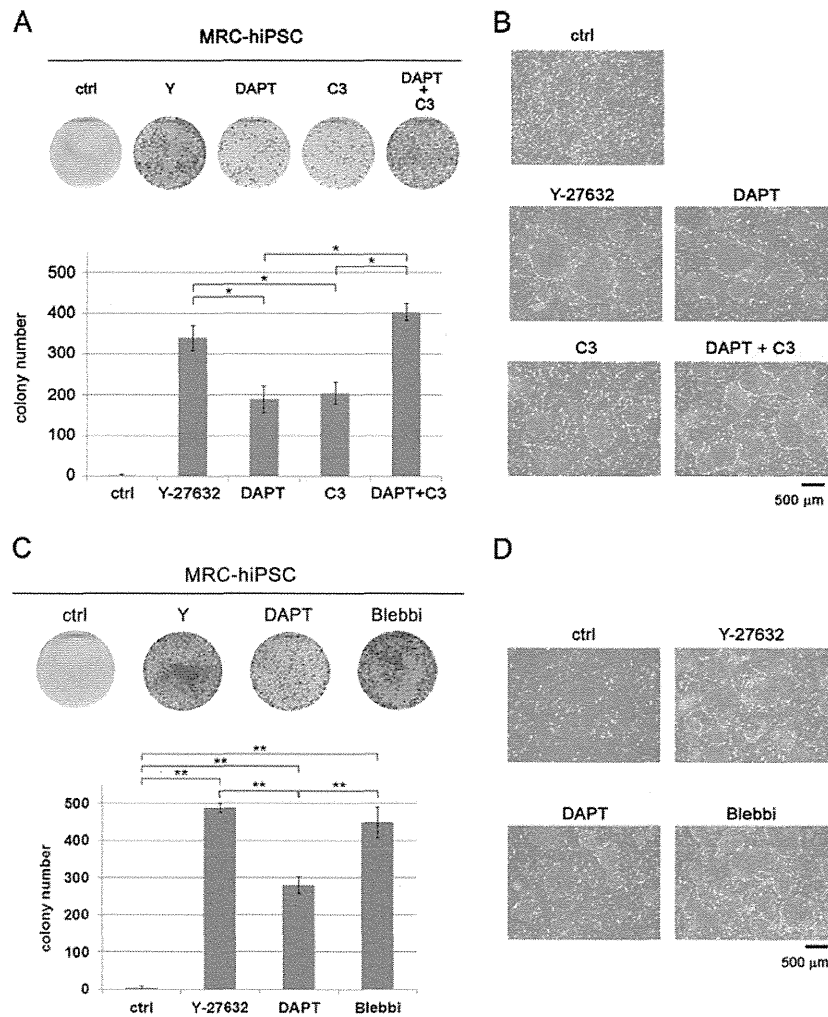


FIG 9 Activation of NOTCH and ROCK upon dissociation instigates loss of clonogenic growth capacity in hiPSCs. (A) MRC-hiPSCs, pretreated where indicated with 10 μ M Y-27632 (Y) for 3 h, 10 μ M DAPT for 3 h, 2.0 μ g/ml C3 for 3 h, and 10 μ M DAPT and 2.0 μ g/ml C3 for 3 h, were completely dissociated by trypsin-EDTA treatment and pipetting. The dissociated cells were counted with Vi-CELL to confirm that the viabilities of each samples were greater than 90%, and aliquots of 2,000 cells were seeded on 60-mm dishes in the presence of MEF feeders. After 10 days of cultivation, the cells were stained with Giemsa's dye, and colonies were counted. The photographs are of representative dishes, and the graph illustrates means \pm SDs. Treatment with any of these inhibitors significantly restored the clonal growth potential after dissociation. *, $P < 0.05$ according to Student's t tests. (B) Typical areas were photographed at 6 days postreplating. (C) MRC-hiPSCs, pretreated where indicated with 10 μ M Y-27632 for 3 h, 10 μ M DAPT for 3 h, and 10 μ M blebbistatin for 3 h, were completely dissociated. The dissociated cells were seeded as for panel A, and colonies were counted. The photographs are of representative dishes, and the graph represents means \pm SDs. **, $P < 0.01$ according to Student's t tests. (D) Typical areas were photographed at 6 days postreplating.

caspase-3 (Fig. 8C), implying a novel mechanism of ROCK inhibition by C3 independent of Rho inhibition. However, the mechanism underlying the inhibitory effect of C3 on NOTCH1 activation is currently unknown and awaits further investigation.

A recent report on the NOTCH1 nuclear interactome reveals ROCK1 as one of its interacting partners, raising the possibility of cross talk between these proteins (60). However, we have no evidence for interaction of the intracellular NOTCH1 proteins with caspase-3 and ROCK1 at present. Certainly, identification of a signaling mediator between NOTCH1 and ROCK1 warrants more research to provide a better understanding of this novel signaling pathway in cell biology. Interestingly, we failed to detect the intracellular form of NOTCH2 after differentiation induction, and ectopic expression of the NOTCH2 intracellular forms had a limited effect, if any, on ROCK1 cleavage and differentiation

(Fig. 8A, lane 4), suggesting different regulatory mechanisms for activation and downstream signaling among NOTCH family members.

Functional diversity of caspases in cellular processes other than apoptosis has been described (61). In embryonic mouse keratinocytes, caspase-3 has been shown to be a transcriptional target of Notch1 and to have a role in high commitment to terminal differentiation (62). It is likely that ROCK1 activity is fostered by canonical NOTCH1 signaling through upregulation of caspase-3 gene expression. Because there is also a report showing that caspase-8 functions in epidermal homeostasis and regeneration through regulation of proliferation and inflammatory responses (63), we tested the possible involvement of caspase-8 in ROCK1 cleavage in differentiated keratinocytes. However, caspase-8 inhibition demonstrated little influence on activation of the

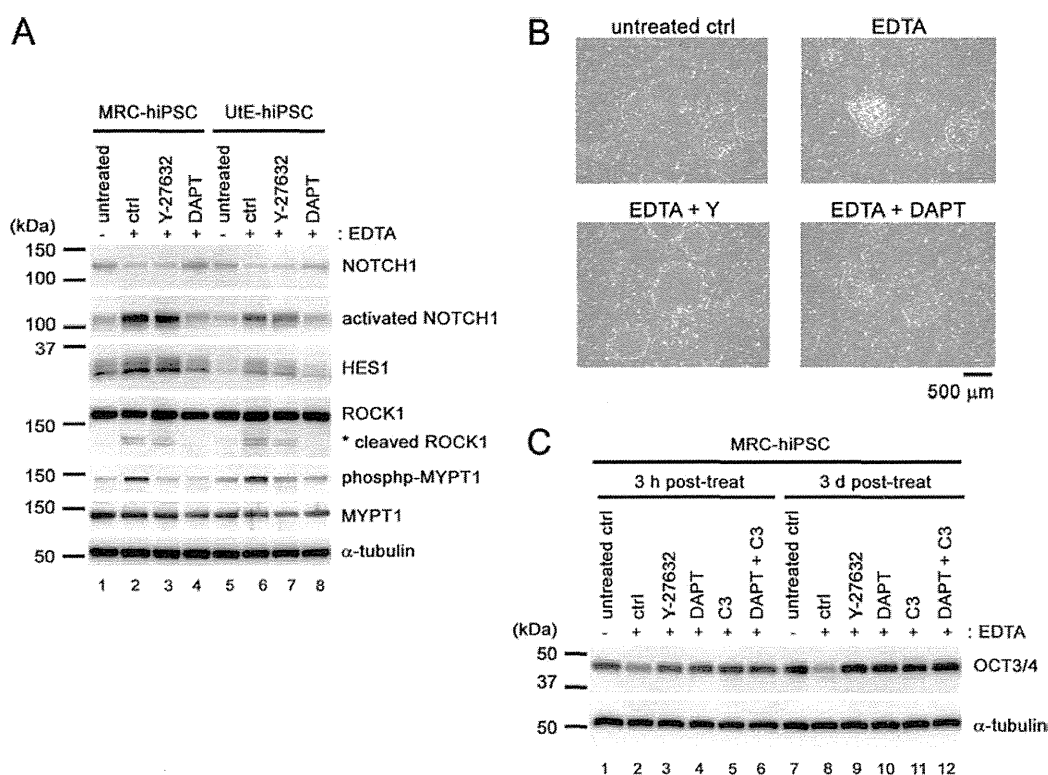


FIG 10 The defect in clonogenic growth upon dissociation in hiPSCs is partly attributable to NOTCH-dependent ROCK activation. (A) Adherent small clumps of MRC-hiPSCs and Ute-hiPSCs were pretreated with 10 μ M Y-27632 for 3 h or 10 μ M DAPT for 3 h and then either left untreated or treated with 2.5 mM EDTA in Hanks balanced salt solution without Ca^{2+} and Mg^{2+} [HBSS(-)] for 10 min at 37°C. After 3 h of incubation, hiPSC cell extracts were prepared and subjected to immunoblotting analysis with the indicated antibodies. (B) Typical MRC-hiPSC colonies were photographed at 3 days after EDTA treatment. (C) Adherent small clumps of MRC-hiPSCs, pretreated where indicated with 10 μ M Y-27632 for 3 h, 10 μ M DAPT for 3 h, 2.0 μ g/ml C3 for 3 h, and 10 μ M DAPT and 2.0 μ g/ml C3 for 3 h, were then either left untreated or treated with 2.5 mM EDTA. After 3 h and 3 days of cultivation, hiPSC cell extracts were prepared and subjected to immunoblotting analysis.

NOTCH1-ROCK1 pathway and keratinocyte differentiation (unpublished observations), suggesting that activation of caspase-3 is unlikely to be mediated by caspase-8 downstream of NOTCH1.

Biological relevance of the NOTCH-ROCK pathway. In stratified epithelia such as the epidermis, cells in the basal layer constitute a proliferating population including stem cells and transit-amplifying cells. In the upper layer, differentiation-committed daughter cells are thought to arise by asymmetric cell division and then move toward the surface to undergo terminal differentiation (64). During the transition from the basal to the suprabasal layer, downregulation of p63, particularly its predominant isoform $\Delta\text{Np63}\alpha$, results in detachment from the basement membrane, at least partly via decreased expression of cell adhesion molecules (65). Downregulation of $\Delta\text{Np63}\alpha$ also leads to upregulation of NOTCH1 gene expression and activity (9, 18). Having established that activated NOTCH1 triggers constitutive activation of ROCK1, we speculate that activated NOTCH1 may drive active movement of daughter cells toward the surface. This idea is supported by experiments showing that active ROCK1 induces formation of thick stress fibers and a cell contraction force which pushes cells upwards (54) and by the fact that Y-27632 inhibits differentiation as well as stratification in organotypic raft culture (37). Indeed, we observed that keratinocytes treated with EDTA exhibited increased cell motility and clambering movement, which were inhibited by Y-27632

(see Movies S1 and S2 in the supplemental material). We previously reported that $\Delta\text{Np63}\alpha$ represses both p53-dependent and -independent expression of the Notch1 gene to support the proliferative capacity of normal human keratinocytes as well as a subset of cervical cancer cell lines (18). Thus, we propose that the p63-NOTCH1-ROCK1 axis plays an essential role in establishment of stratified epithelia through actomyosin-driven cell movement, though we cannot exclude the possible involvement of the Rho-ROCK pathway in this process. In terms of cancer biology, loss of p63 function is paradoxically associated with metastatic progression (66), implying another face of p63 as a tumor suppressor through the NOTCH1-ROCK1 pathway.

Two isoforms of ROCK, ROCK1 (ROK β , p160ROCK) and ROCK2 (ROK α), have been identified in mammals; they share 65% overall identity and 92% identity in their kinase domains, and a number of lines of investigation have suggested nonoverlapping functions for these two isoforms (67–69). In this regard, ROCK1, but not ROCK2, has been shown to be constitutively activated by caspase-3 and to play roles in membrane blebbing during apoptosis (54, 55). We observed that knockdown of ROCK1 but not ROCK2 resulted in a reduced propensity for differentiation (Fig. 6B) and that a constitutively active form of ROCK1 induced differentiation (Fig. 8A). In parallel with our data, previous work has shown that the conditional expression of the activated form of ROCK2 also results in induction of differen-

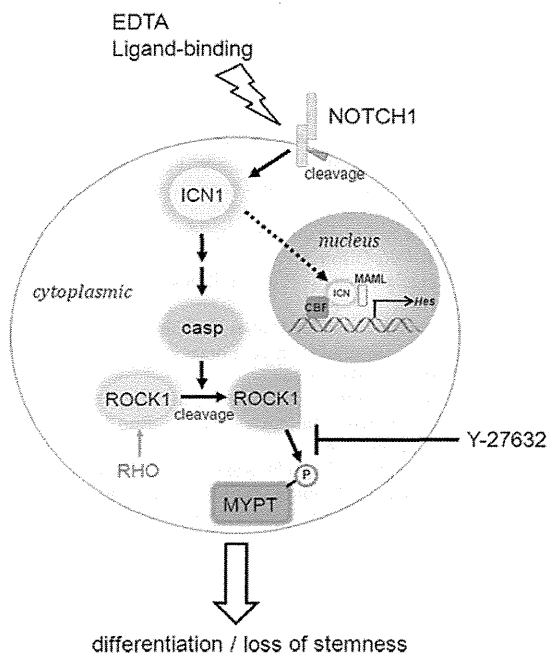


FIG 11 Proposed model for the NOTCH-ROCK pathway and its biological significance. NOTCH1 can be activated by dissociation of cells with EDTA as well as physiological ligand binding. The cytoplasmic form of NOTCH1, independent of its transcriptional activity, triggers caspase-mediated cleavage (activation) of ROCK1 by an as-yet-unknown mechanism. ROCK1 activation drives actomyosin reorganization through MYPT phosphorylation which results in cell differentiation or loss of stemness. The ROCK inhibitor Y-27632 blocks this pathway so as to inhibit differentiation of keratinocytes and maintain stemness of hiPSCs, as shown in this study.

tiation markers in keratinocytes (35). In contrast, it was reported that a ROCK inhibitor accelerated calcium- or suspension-induced keratinocyte differentiation (70). More recently, Notch1 was reported to be a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1,2 and MRCKalpha kinases (56). Furthermore, an oncogenic aspect of ROCK2 has also been reported (52, 71, 72). Apparent anomalies between these studies and our results imply complexity of the signals downstream of ROCK1 and ROCK2. However, our results clearly demonstrate that prompt activation of ROCK1 by non-canonical NOTCH1 signaling promotes keratinocyte differentiation and limits clonogenic cell growth. Part of the discrepancies may be explained by the use of different experimental systems. Our experimental system focused on early stages of keratinocyte differentiation in monolayer culture. However, our results are consistent with a more recent report that ROCK inhibition blocks differentiation as well as stratification in organotypic raft culture (37). It is possible that the canonical NOTCH1 signal and transcriptional repression of ROCK1,2 plays different roles in later stages of differentiation or tumor formation.

In spite of these important functions of ROCK1,2 in keratinocyte differentiation, no abnormalities in the epidermis of either Rock1- or Rock2-null mice have been described (67, 68, 73, 74). This suggests functional redundancy and compensatory mechanisms for these two proteins, though they show apparently quite different functions in culture. Synthetic effects in mice with compound heterozygous and/or homozygous disruptions in the Rock1 and Rock2 genes need to be delineated in detail.

Technical implications of NOTCH-ROCK dysregulation during cell passage. Our data show that keratinocytes of different tissue origins as well as hiPSCs exhibiting epiblast-like cell states have a conserved NOTCH1-ROCK1 pathway and that NOTCH activation is at least partly responsible for impaired clonogenicity upon dissociation with EDTA (Fig. 1C, 9, and 10). ROCK inhibition could robustly restore the proliferation capacity and contribute to the maintenance of stemness through blockade of cellular differentiation and/or apoptosis, unscheduled biological outcomes imposed by the EDTA treatment in cell culture. A recent groundbreaking report provided evidence that the combination of a ROCK inhibitor and fibroblast feeders indefinitely extends the life spans of many different types of human epithelial cells (38). Thus, it is highly likely that dissociation of cells with EDTA activates the NOTCH1-ROCK1 pathway and adversely affects the proliferative capacity of a broad spectrum of normal epithelial cells. Furthermore, most cell lines established through such procedures may represent a selected population resistant to the activation of the NOTCH-ROCK pathway.

In conclusion, our present study unveiled a link between non-canonical NOTCH signaling and ROCK activation and revealed a previously unrecognized function of NOTCH1 as a critical regulator of ROCK1 in dictating the cell fate. Our findings also imply a possible pitfall in cell culture and delineate a molecular rationale for the beneficial effects of the ROCK inhibitor Y-27632 in cultivating various cell types.

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T.Y. conceived the project, designed, performed, and analyzed experiments, and wrote the manuscript. K.N. performed the hiPSC clonogenic assay. N.G. and S.-I.O. provided cDNA clones and performed plasmid construction, respectively. M.F., A.U., and T.N. discussed and gave important advice on some experiments. T.K. participated in the experimental design and performed experiments.

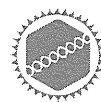
We declare no conflict of interest related to this work.

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RESEARCH

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Human cytomegalovirus induces apoptosis in neural stem/progenitor cells derived from induced pluripotent stem cells by generating mitochondrial dysfunction and endoplasmic reticulum stress

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Abstract

Background: Congenital human cytomegalovirus (HCMV) infection, a leading cause of birth defects, is most often manifested as neurological disorders. The pathogenesis of HCMV-induced neurological disorders is, however, largely unresolved, primarily because of limited availability of model systems to analyze the effects of HCMV infection on neural cells.

Methods: An induced pluripotent stem cell (iPSC) line was established from the human fibroblast line MRC5 by introducing the Yamanaka's four factors and then induced to differentiate into neural stem/progenitor cells (NSPCs) by dual inhibition of the SMAD signaling pathway using Noggin and SB-431542.

Results: iPSC-derived NSPCs (NSPC/iPSCs) were susceptible to HCMV infection and allowed the expression of both early and late viral gene products. HCMV-infected NSPC/iPSCs underwent apoptosis with the activation of caspase-3 and -9 as well as positive staining by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Cytochrome c release from mitochondria to cytosol was observed in these cells, indicating the involvement of mitochondrial dysfunction in their apoptosis. In addition, phosphorylation of proteins involved in the unfolded protein response (UPR), such as PKR-like eukaryotic initiation factor 2a kinase (PERK), c-Jun NH2-terminal kinase (JNK), inositol-requiring enzyme 1 (IRE1), and the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) was observed in HCMV-infected NSPC/iPSCs. These results, coupled with the finding of increased expression of mRNA encoding the C/EBP-homologous protein (CHOP) and the detection of a spliced form of X-box binding protein 1 (XBP1) mRNA, suggest that endoplasmic reticulum (ER) stress is also involved in HCMV-induced apoptosis of these cells.

Conclusions: iPSC-derived NSPCs are thought to be a useful model to study HCMV neuropathogenesis and to analyze the mechanisms of HCMV-induced apoptosis in neural cells.

Keywords: Human cytomegalovirus, iPSC cells, Neural stem/progenitor cells, Apoptosis, ER stress

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Background

Congenital cytomegalovirus (CMV) infection is a major cause of birth defects resulting mainly from primary CMV infection during pregnancy. At birth, approximately 5 to 10% of congenitally infected newborns are estimated to be symptomatic exhibiting multi-organ disorders including neurological defects such as mental retardation, sensorineural hearing loss, and microencephaly [1,2]. A latest study showed that if laboratory findings including those from magnetic resonance imaging (MRI) images of the brain are considered, up to 30% of congenitally infected newborns exhibit some abnormal signs [3]. Sixty to 90% of congenitally infected children who are symptomatic at birth, and 10 to 15% of those who are asymptomatic at birth develop one or more long-term sequelae. Although CMV infects a wide variety of cell types, infection of the nervous system gives most serious and long-lasting damages to the host.

As a part of understanding the HCMV neuropathogenesis, it is important to scrutinize the cellular response to CMV infection in neural cells. Some human neural cell lines can be infected with HCMV with different permissiveness to HCMV gene expression and replication [4-7]. A recent study has shown that neural progenitor cells isolated from developing human brain tissues are susceptible to CMV infection and undergo apoptosis following infection [8,9]. However, the amount of neural cells obtainable from human brain tissues is limited.

Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are characterized by the ability to differentiate into tissues derived from any of the three embryonic germ layers. Recent advances in the method to induce efficient differentiation of either ESCs or iPSCs into specific cell lineages offer an opportunity to establish model systems for viral infections of various cell types, including neural cells. Furthermore, differentiated cells derived from pluripotent stem cells are obtainable in potentially unlimited amounts. Previous works revealed that while mouse ESCs are not susceptible to murine CMV (MCMV), NSPCs that are differentiated from them are susceptible and their proliferation and differentiation are suppressed by MCMV [10-13]. Experiments with human ESCs are, however, complicated with ethical problems.

In this study, to analyze the pathological effects of HCMV on neural cells, we prepared NSPCs from human iPSCs and examined whether NSPCs are susceptible to HCMV infection. The results indicated that NSPCs are susceptible to HCMV infection and undergo apoptosis caused by mitochondrial dysfunction and endoplasmic reticulum (ER) stress.

Methods

Cells and viruses

The human fetal lung fibroblast MRC5 was grown in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The human foreskin fibroblast cell line hTERT-BJ1 immortalized with the human telomerase reverse transcriptase (Clontech, Palo Alto, CA) was grown in a medium consisting of 4 parts of DMEM and 1 part of medium 199 (Sigma) supplemented with 10% FBS, 1 mM sodium pyruvate (Sigma), and 2 mM glutamine (Invitrogen). HCMV laboratory strain Towne (ATCC VR-977) was propagated in hTERT-BJ1 cells. The human iPSC line MRC-iPS-25 that was established from MRC5 by retroviral vector-mediated transduction of the *c-Myc*, *Oct-4*, *Klf4*, and *Sox2* genes [14,15] were cultured on mitomycin C-treated mouse embryonic fibroblasts (MEFs) in an iPSC medium consisting of Knockout DMEM/F12 (Invitrogen) supplemented with non-essential amino acids (0.1 mM, Invitrogen), glutamax I (1 mM, Invitrogen), 20% Knockout Serum Replacement (Invitrogen), β -mercaptoethanol (55 μ M, Invitrogen) and basic fibroblast growth factor (10 ng/mL; Peprotech, Rocky Hill, NJ).

Induced differentiation on iPSCs into neural stem cells

MRC-iPSC-25 cells cultured under feeder-free conditions were induced to differentiate into neural stem/progenitor cells (NSPCs) by the method of dual inhibition of the SMAD signaling pathway described previously [16]. In brief, feeder-free iPSCs were treated with the mTeSR1 medium (StemCell Technologies, Vancouver, BC, Canada) containing Y27632 (Wako Pure Chemicals, Osaka, Japan) and maintained with a daily medium change for 4 days. Then the medium was replaced with iPSC medium supplemented with SB431542 (10 nM, Wako Pure Chemicals) and Noggin (500 ng/ml, Wako Pure Chemicals). This date was designated day 0. On day 2, culture medium was replaced with a medium consisting of 3 parts of iPSC medium and 1 part of N2 medium (Knockout DMEM/F12 containing $1\times$ N2 supplement) supplemented with SB431542 (10 nM) and Noggin (500 ng/ml). On day 4, culture medium was replaced with a medium consisting of 1 part of iPSC medium and 1 part of N2 medium supplemented with SB431542 (10 nM) and Noggin (500 ng/ml). On day 6, cells were expanded in StemPro NSC SFM (Invitrogen). MRC-iPSC-25 cells cultured under feeder-free conditions and NSPC/iPSCs were infected with the Towne strain HCMV at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU) per cell. To detect infectious virions produced from HCMV-infected NSPC/iPSCs, supernatant was collected and replaced with fresh medium every two days after infection. hTERT-BJ1 cells were inoculated with the supernatant and examined by IFA for expression of IE1/IE2.

Antibodies

Antibodies used were as follows: rabbit anti-Sox2, rabbit anti-Nanog, rabbit anti-Oct-4, rabbit anti-cleaved caspase-

3, rabbit anti-cleaved caspase-9, rabbit anti-phospho-IEF2 α (Ser51), rabbit anti-phospho-PERK (Thr980), and rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185)(Cell Signaling Technology, Beverly, MA); mouse anti-CMV IE1/IE2, rabbit anti-Musashi-1, and rabbit anti-cytochrome c (Millipore, Temecula, CA); rabbit anti-Nestin and mouse anti- α -tubulin (Sigma-Aldrich, St. Louis, MO); rabbit anti-Pax6 (Covance, Princeton, NJ), mouse anti-CMV gB (Abcam, Cambridge, MA); mouse anti-pp65 (Virusys Corporation, Sykesville, MD); rabbit anti-phosphorylated IRE1 α (Pierce/Thermo Scientific, Rockford, IL); Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR); horseradish peroxidase-conjugated donkey anti-rabbit IgG and horseradish peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare, UK).

Immunofluorescence microscopy and immunoblot analysis

Cells were fixed with 4% paraformaldehyde in PBS (Wako chemicals) at room temperature (RT) for 15 min. After fixation, cells were treated with 1.0% Triton X-100 in PBS for 15 min at RT and blocked with 10% goat serum in PBS for 30 min. Cells were incubated with the primary antibody at 4°C overnight, followed by washing in PBS and incubation with the corresponding secondary antibody at 37°C for 1 h. Nuclei were stained with DAPI. For the assessment of cell death, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed according to the manufacturer's instructions (Roche). Immunoblot analyses were performed as described previously [17].

Reverse transcriptase (RT)-PCR and real-time quantitative RT-PCR

Total RNA was isolated from mock- or HCMV-infected cells using TRIzol reagent (Invitrogen). Reverse transcription was performed on each RNA sample (5 μ g) using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Primer sequences are shown in Table 1. RT-PCR products were resolved by electrophoresis on 2% agarose gel and then visualized by ultraviolet illumination after ethidium bromide staining. Real-time quantitative RT-PCR was performed using TaqManTM Universal Master Mix II with UNG (Applied Biosystems) on a StepOne Plus PCR System (Applied Biosystems). Amplifications were achieved in a final volume of 25 μ l containing TaqMan probes labeled with FAM on the 5'-end and MGB on the 3'-end. The primers and probes for *UL136* were: forward primer, 5'-GGCCGTTGAACGGAGCTAT-3' and reverse primer, 5'-CCATTTCCACCGTGTCGAA-3'; and TaqMan probe, 5'-FAM-TACTACGGCAGCGGCT-MGB-3'. The forward and reverse primers and reporter probes for HCMV *IE1*, *UL89*, and Human *G6PD* were described previously [18].

Table 1 List of primer sequences for RT-PCR

Gene	Forward primer	Reverse primer
IE1*	ATGGAGTCCTCTGCCAAGAG	ATTCTATGCCGCACCATGTCC
IE2*	ATGGAGTCCTCTGCCAAGAG	CTGAGACTTGTCTCCTCAGGTCCTG
vL-10*	ATGCTGTCCGGTATGGTCTCTTC	CTTTCTCAGTGCAGATACTCTTCG
UL36*	GACCTACGGGACACGCTGATG	TGTGGAAGTGGTCGCAAGTGC
UL38	GACTACGACCACGCATAGCA	GGGAACAGAGCGTTCCAATA
pp65	CGCAACCTGGTGCCCATGG	CGTTTGGGTTGCGCAGCGGG
Nanog*	GCTTGCCTTGCTTTGAAGCA	TTCTTACCAGGGACCTTGTC
Oct-4	GAGCAAACCCGGAGGAGT	TTCTCTTCCGGCCTGTCAC
Sox1	GCGGAAAGCGTTTCTTTG	TAATCTGACTTCTCTCCC
Sox2	ATGCACCCTACGACGTGA	CTTTTGACCCCTCCCATTT
Pax6*	AACAGACACGCCCTCACAACA	CGGGAACCTGAACTGGAAGTGC
Nestin*	CAGCGTTGGAACAGAGGTTGG	TGGCACAGGTGTCTCAAGGGTAG
MAP2*	CCACCTGAGATTAAGGATCA	GGCTTACTTTGCTTCTCTGA
GFAP*	GTACCAGGACCTGCTCAAT	CAACTATCCTGCTTCTGCTC
OSP*	ACTGCTGCTGACTGTTCTTC	GTAGAAACGGTTTTACCAA
XBP1*	CCTTGTAGTTGAGAACCAGG	GGGGCTTGGTATATATGTGG
CHOP*	TGGAAGCCTGGTATGAGGAC	TCACCATTCCGGTCAATCAGA
β -actin*	ACCATGGATGATGATATCCG	TCATTGTAGAAGGTGTGGTG
GAPDH*	CCACCCATGGCAAATCCATGGCA	TCTAGACGGCAGGTCAAGTCCACC

Asterisks (*) indicate that amplified fragments contain splicing junctions. Amplified fragments for UL38, pp65, Oct-4, Sox1, and Sox2 did not contain splicing junctions, and therefore control experiments without reverse transcriptase confirmed the RNA origin of the PCR products.

Results

Preparation of human iPSC-derived neural stem/progenitor cells

Figure 1A demonstrates that MRC-iPS-25 cells have a typical iPSC colony morphology. The expression of pluripotency markers of iPSCs such as Nanog and Oct-4 in MRC-iPS-25 cells was confirmed by indirect immunofluorescence assay (IFA) (Figure 1B). The HCMV-encoded proteins IE1/IE2 were not detected in MRC-iPS-25 cells following inoculation with the virus, indicating that MRC-iPS-25 cells are either not susceptible to HCMV infection or do not support expression of the IE genes (Figure 1B).

NSPC/iPSCs prepared by induced differentiation of MRC-iPS-25 cells proliferated in a monolayer and displayed a rounded, immature neural morphology (Figure 1A). IFA (Figure 1C) showed that NSPC/iPSCs expressed the NSC markers Nestin, Sox2, and Pax6, indicating that NSPC/iPSCs have the authentic NSPC phenotype.

In vitro HCMV infection of iPSC-derived NSPCs

To examine the susceptibility of NSPC/iPSCs to HCMV infection, these cells were inoculated in vitro with the HCMV Towne strain at an MOI of 1 PFU per cell (Figure 2A). On the second day post-infection (dpi), NSPC/iPSCs started to show morphological changes including increased cell volume and cell fusion, and the number of cells with these

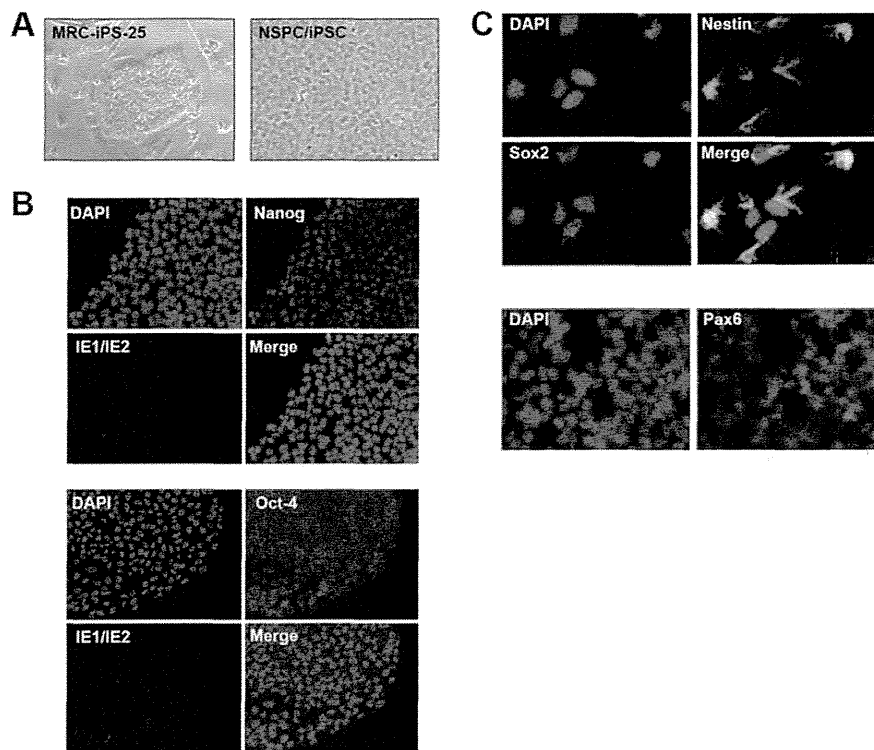


Figure 1 Differentiation of MRC-iPS-25 cells to neural stem/progenitor cells. **(A)** Phase-contrast images of MRC-iPS-25 cells cultured on a feeder layer of MEFs (left) and NSPC/iPSCs (right). **(B)** Immunofluorescence analysis of Towne-infected MRC-iPS-25 cells cultured under feeder-free conditions at 2 dpi stained with primary antibodies for pluripotent markers (Nanog or Oct-4) and HCMV IE1/IE2 proteins. Antigen proteins were detected with Alexa Fluor 488-conjugated goat anti-mouse IgG or Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody. Nuclei were stained with DAPI. **(C)** Immunofluorescence analysis of NSPC markers Nestin, Sox2, and Pax6 in NSPC/iPSCs. NSPC/iPSCs were fixed and reacted with anti-Nestin (green), anti-Sox2 (red), and anti-Pax6 (red) antibodies, followed by detection with secondary antibodies. Immunofluorescence signals were obtained using a fluorescence microscope IX71. Representative results from three independent experiments are shown.

changes increased until 7 dpi (Figure 2A). To examine whether NSPC/iPSCs were capable of supporting HCMV gene expression, total RNA extracted from the infected NSPC/iPSCs was analyzed by RT-PCR. As shown in Figure 2B, mRNAs encoding IE1, IE2, vIL-10, and pp65 as well as those encoding HCMV anti-apoptotic proteins, such as UL36 and UL38, were detected. The kinetics of HCMV gene expression was analyzed by quantitative real-time RT-PCR (Figure 2C). IE1 mRNA was detected first on 1 dpi and increased steadily until 5 dpi. mRNAs for UL89 and UL136 were detected somewhat later and increased gradually until 7 dpi. The results showed the NSPC/iPSCs are susceptible to HCMV infection and allow the expression of several viral genes of both early and late functions.

Expression of HCMV genes in NSPC/iPSCs was evaluated at the protein level by immunoblot analysis on day 1, 2, 5, and 7 following HCMV infection. As shown in Figure 2D, the immediate-early protein IE1 was first detected at 1 dpi and its level increased until 5 dpi. Another immediate-early protein IE2 was detected a little later, becoming visible at 5 dpi. The expression of the HCMV lower matrix protein

pp65 (ppUL83), already visible at 1 dpi, was markedly elevated at 5 and 7 dpi. The HCMV envelope glycoprotein B (gB; UL55) was detected at 5 to 7 dpi. Thus the expression of HCMV proteins of both immediate-early and late functions was demonstrated in NSPC/iPSCs.

We next examined the expression of cellular mRNAs encoding the pluripotency and neural differentiation markers (Figure 2E). Expression of the iPSC markers Nanog and Oct-4 remained at low levels following infection with HCMV, although that of Nanog tapered. While expression of the NSPC markers Sox2 and Pax6 were kept at high levels following HCMV infection, that of another NSPC marker Nestin was markedly suppressed at 7 dpi. In addition, expression of the neuronal marker microtubule-associated protein 2 (MAP2), the astrocyte marker glial fibrillary acidic protein (GFAP), and the oligodendrocyte marker oligodendrocyte-specific protein (OSP) was detected at low levels. Interestingly, Sox1, a marker specific to the neuroectodermal lineages [19], was markedly upregulated following infection with HCMV. Expression of the NSPC markers was evaluated

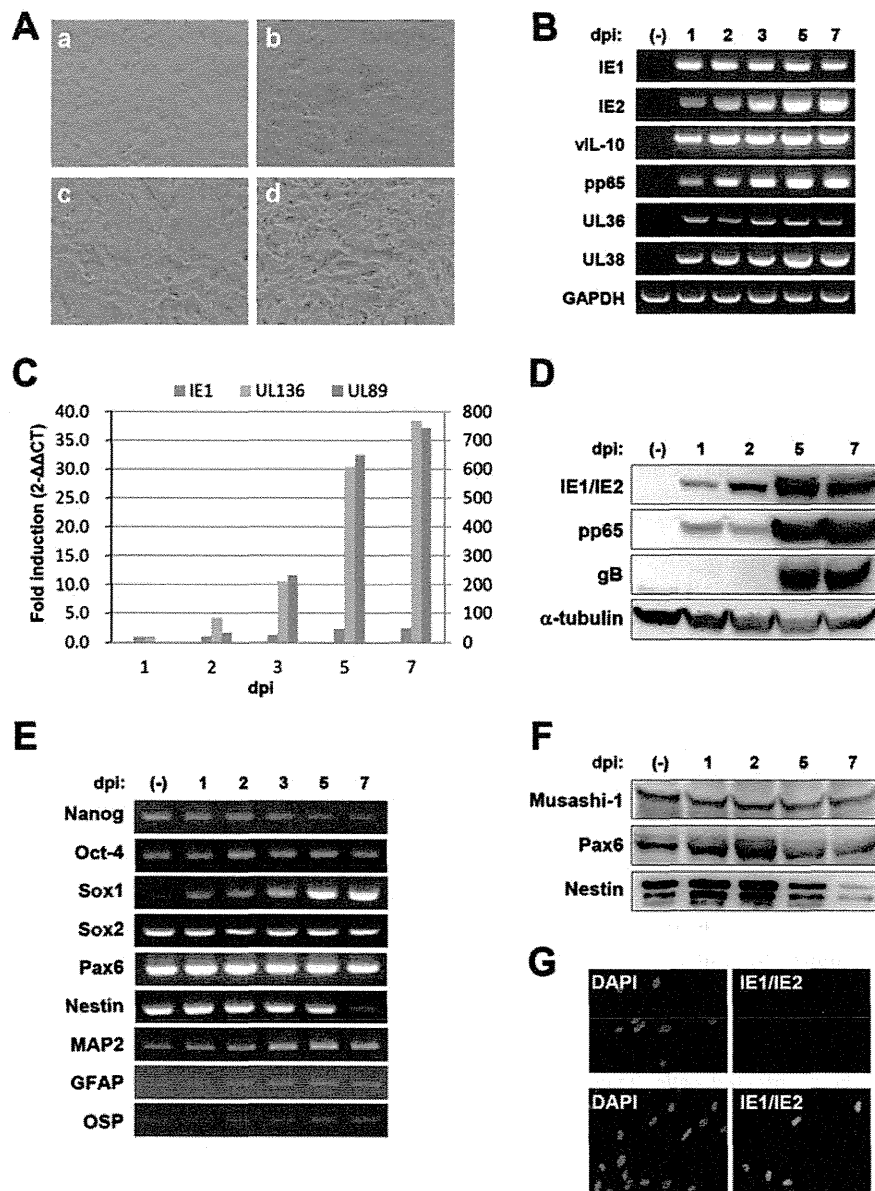


Figure 2 Analysis on the expression of viral and cellular gene products in NSPC/iPSCs. (A) Morphological changes of Towne-infected NSPC/iPSCs were observed under the inverted microscope before infection (a), 2 dpi (b), 5 dpi (c), and 7 dpi (d). (B) RT-PCR analysis of HCMV-encoding gene expression. Total RNAs isolated from NSPC/iPSCs harvested before (–) HCMV infection or at 1, 2, 3, 5, and 7 dpi with HCMV Towne strain were subjected to RT-PCR assays. GAPDH gene expression was assayed for the control. (C) The kinetics of mRNA expression for IE1, UL89, and UL136 in Towne-infected NSPC/iPSCs was examined by real-time quantitative RT-PCR assay. The mRNA expression was normalized to that of GAPDH gene. Real-time PCR data was analyzed by the $2^{-\Delta\Delta CT}$ method. The fold induction was calculated as the ratio of mRNA levels detected at each time point to that detected at 1 dpi. The y-axis represents fold induction of IE1 and UL136 mRNA (left y-axis) and UL89 mRNA (right y-axis). (D) Immunoblot analysis of HCMV protein expression in HCMV-infected NSPC/iPSCs. Whole-cell lysates of NSPC/iPSCs harvested before (–) HCMV infection or at 1, 2, 5, and 7 dpi with HCMV Towne strain were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against IE1/IE2, pp65, gB, and α -tubulin. (E) RT-PCR analysis of pluripotency and neural differentiation marker gene expression in HCMV-infected NSPC/iPSCs. (F) Immunoblot analysis of neural differentiation marker protein expression in HCMV-infected NSPC/iPSCs. Whole-cell lysates of NSPC/iPSCs were analyzed by immunoblotting with antibodies against Musashi-1, Pax6, and Nestin. (G) hTERT-BJ1 cells inoculated with culture supernatant collected from mock-infected NSPC/iPSCs (upper panel) or Towne HCMV-infected NSPC/iPSCs (lower panel) at 8 dpi were subjected to immunofluorescence test with anti-IE1/IE2 antibody (green). Nuclei were stained with DAPI. Representative results from two independent experiments are shown.

also at the protein level by immunoblot analysis on 1, 2, 5, and 7 dpi (Figure 2F). In accordance with the results with RT-PCR, expression of Pax6 and Nestin was confirmed, and that of Nestin was found markedly decreased 7 dpi. Another NSPC marker Musashi-1 was also detected. To examine whether HCMV-infected NSPC/iPSCs produce infectious virions, culture supernatants collected from Towne HCMV-infected NSPC/iPSCs were inoculated to hTERT-BJ1 cells. Inoculated cells expressed IE1/IE2 indicating that infectious virions were produced from HCMV-infected NSPC/iPSCs (Figure 2G). The supernatant contained 30 PFU/mL of HCMV at 4, 6, 8 dpi, while no plaque forming virus was detected at 2 dpi.

HCMV infection induces apoptosis in iPSC-derived NSPCs

To examine whether HCMV infection in NSPC/iPSCs induces apoptotic responses, we performed the TUNEL assay combined with IFA using an antibody specific to HCMV gB. As shown in Figure 3A, NSPC/iPSCs expressing gB was positive for TUNEL staining and those without gB expression was consistently negative. We also performed IFA to analyze the activation status of caspases using antibodies specific to the activated forms of caspase-3 and caspase-9. The results show that the activated forms of caspase-3 and caspase-9 were specifically detected in more than 80% of HCMV-infected NSPC/iPSCs expressing IE1/IE2 proteins (Figure 3B and 3C), but not in mock-infected NSPC/iPSCs (Figure 3E). To see whether mitochondrial dysfunction is involved in the activation of caspase 9, intracellular distribution of cytochrome c was analyzed in HCMV-infected cells by IFA. As shown in Figure 3D and 3E, strong signals of cytochrome c were detected in the cytosol of cells expressing IE1/IE2 proteins, while only faint signals of cytochrome c were detected in cells not expressing IE1/IE2 proteins or in mock-infected cells. These results indicate that HCMV infection of NSPC/iPSCs activated apoptotic responses involving release of mitochondrial cytochrome c and serial activation of caspases.

Unfolded protein response in HCMV-infected NSPC/iPSCs

The unfolded protein response (UPR), induced by the accumulation of improperly folded proteins within the ER lumen (ER stress), is associated with multiple cellular responses such as neurodegeneration and apoptosis. ER stress sensor molecules, such as PKR-like eukaryotic initiation factor 2 α kinase (PERK) and inositol-requiring enzyme 1 (IRE1), are activated on UPR and engage downstream signaling pathways. To examine whether the caspase-9 activation in HCMV-infected NSPC/iPSCs (Figure 3C) is associated with UPR, we analyzed phosphorylation status of IRE1 α and its downstream target c-Jun NH2-terminal kinase (JNK) in immunofluorescence assays. Both IRE1 α and JNK were specifically phosphorylated in

HCMV-infected NSPC/iPSCs (Figure 4A and 4B), but not in mock-infected NSPC/iPSCs (Figure 4C). In concordance with the previous reports that activated IRE1 α catalyzes the non-conventional splicing of the mRNA encoding X-box binding protein 1 (XBP1) [20,21], the spliced XBP1 mRNA increased gradually after HCMV infection in NSPC/iPSCs (Figure 4D). We also analyzed phosphorylation status of another sensor molecule PERK, an ER-associated serine/threonine protein kinase, and its downstream target the alpha subunit of eukaryotic initiation factor 2 (eIF2 α). Phosphorylated forms of PERK and eIF2 α were specifically detected in HCMV-infected NSPC/iPSCs (Figure 4E and 4F), but not in mock-infected NSPC/iPSCs (Figure 4G). The transcription factor activating transcription factor 4 (ATF4), that is preferentially translated on activation of PERK, induces the expression of C/EBP-homologous protein (CHOP/GADD153), a transcription factor with proapoptotic functions [22]. In accordance with these previous findings, the mRNA level of CHOP increased gradually after HCMV infection in NSPC/iPSCs (Figure 4H). These results suggest that UPR is involved in the activation of caspase cascade leading to apoptosis in HCMV-infected NSPC/iPSCs.

Discussion

Important findings in this study are as follows: i) NSPC/iPSCs derived from MRC-iPS-25 cells were susceptible to HCMV infection and allow the expression of viral gene products of both early and late functions and production of infectious virions. In contrast, MRC-iPS-25 cells before induction of differentiation was either resistant to HCMV or did not support the expression of HCMV immediate-early genes; ii) the HCMV-infected NSPCs undergo apoptosis; and iii) the mechanism of the apoptosis included cytochrome c release from mitochondria to cytosol and activation of UPR-related signaling pathways.

Neuropathogenesis of HCMV infection has been studied mainly with neural cells isolated from human brain. These studies demonstrated that HCMV can infect human neural precursor cells (NPCs) isolated from fetal brains and interfere with their differentiation. Luo *et al.* [23] showed that HCMV infection in primary NPCs reduced the expression of Nestin, suggesting that HCMV affects the differentiation potential of NPCs. Similar results were also obtained from experiments with mouse NSCs infected with MCMV [10,13,24]. Those previous findings obtained from experiments with primary cultures of brain-derived neural cells were thus mostly reproduced in our experiments using NSPC/iPSCs. In addition, similar to the results of Odeberg *et al.* [8] that used NPCs derived from human brain, we also demonstrated that HCMV infection induced apoptosis in NSPC/iPSCs obtained from iPSCs. It is thus supposed that neural cells differentiated from iPSCs are a useful

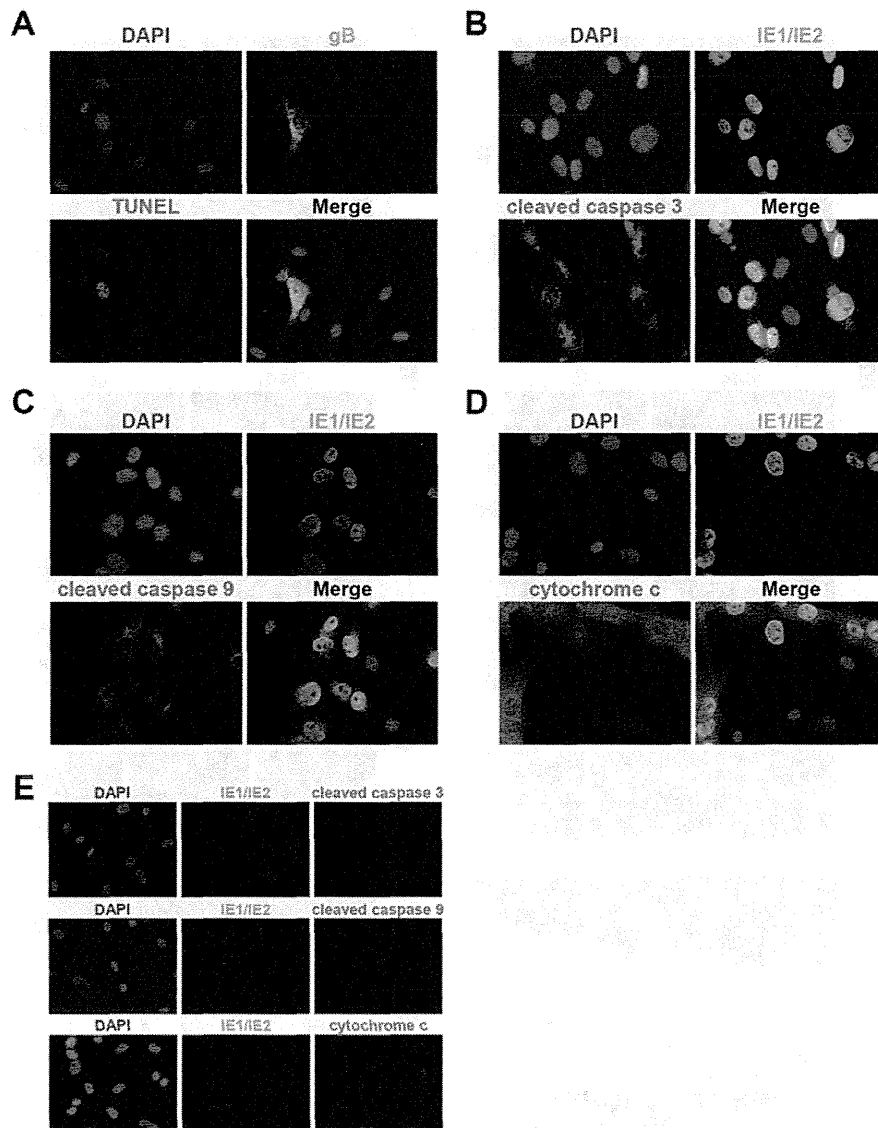


Figure 3 HCMV-induced apoptosis of NSPC/iPSCs. (A) Towne-infected NSPC/iPSCs at 6 dpi were subjected to TUNEL assay followed by immunofluorescence test with an anti-gB antibody. TUNEL-positive nuclei were stained in red. The anti-gB antibody was detected with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (green). Nuclei were stained with DAPI. (B-D) Towne-infected NSPC/iPSCs at 3 dpi were subjected to immunofluorescence test with anti-IE1/IE2 antibody in combination with anti-cleaved caspase 3 (B), anti-cleaved caspase 9 (C), or anti-cytochrome c (D) antibody. Alexa Fluor 488-conjugated goat anti-mouse IgG (green) or Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (red) was used as a secondary antibody. Nuclei were stained with DAPI. (E) Mock-infected NSPC/iPSCs were subjected to immunofluorescence test with anti-IE1/IE2 antibody in combination with anti-cleaved caspase 3 (upper panel), anti-cleaved caspase 9 (middle panel), or anti-cytochrome c (lower panel) antibody. Nuclei were stained with DAPI. Representative results from two independent experiments are shown.

model to investigate neural pathogenesis of HCMV. In the human brain, NSCs are predominantly found in the subventricular region where CMV infections preferentially occur [25,26]. Analysis on the effects of HCMV infection on NSPCs can be therefore particularly relevant.

In the regulation of cellular apoptotic responses, mitochondrial dysfunction and ER stress are involved in the activation of the initiator caspase caspase-9 that

functions as a trigger of cascade protease reactions leading to cell death. The finding of cytochrome c release from mitochondria to cytoplasm in HCMV-infected NSPC/iPSCs indicates that mitochondrial dysfunction is involved in the activation of caspase-9 in these cells. In addition, the demonstration of phosphorylated forms of proteins involved in UPR, including PERK, JNK, IRE1 α , eIF2 α , as well as that of unconventional splicing of

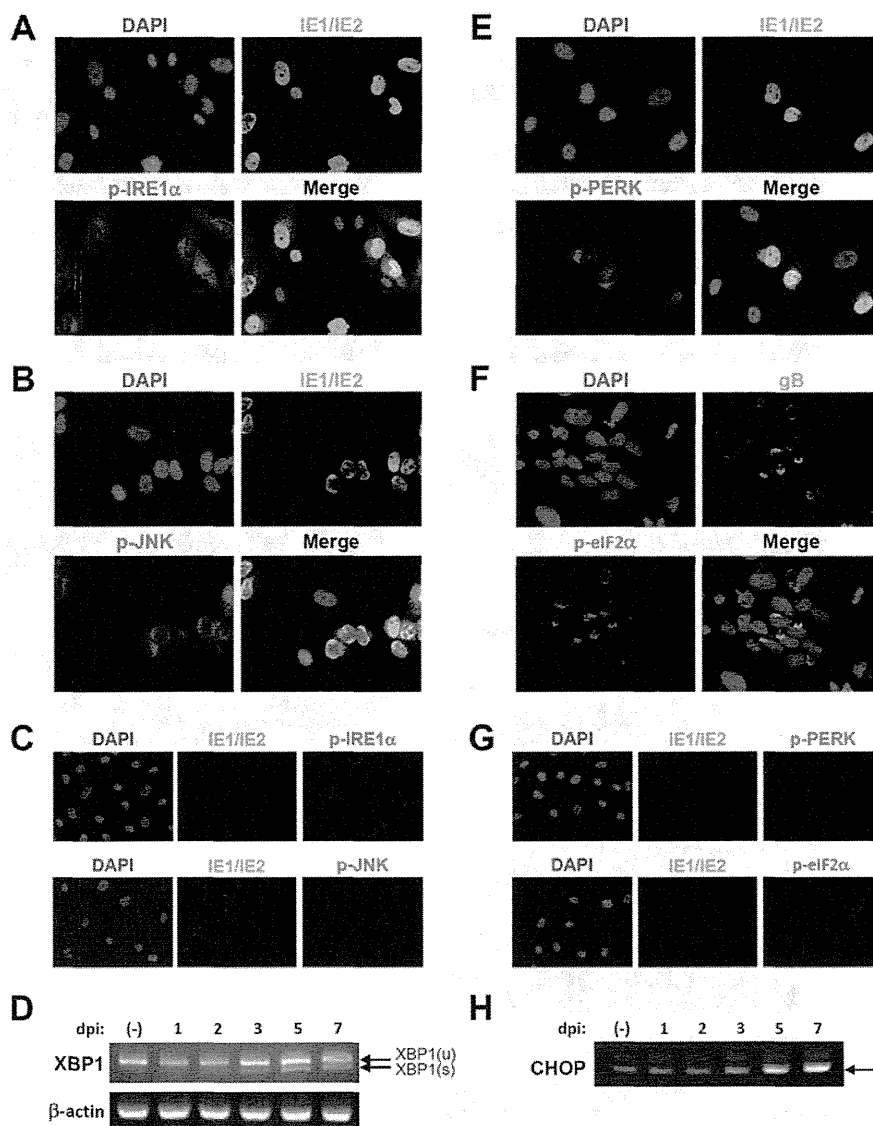


Figure 4 HCMV-induced UPR in NSPC/iPSCs. **(A and B)** Towne-infected NSPC/iPSCs at 3 dpi were subjected to immunofluorescence test with anti-IE1/IE2 antibody in combination with anti-phosphorylated IRE1 α **(A)** or anti-phosphorylated JNK **(B)** antibody. Nuclei were stained with DAPI. **(C)** Mock-infected NSPC/iPSCs were subjected to immunofluorescence test with anti-IE1/IE2 antibody in combination with anti-phosphorylated IRE1 α (upper panel) or anti-phosphorylated JNK (lower panel) antibody. Nuclei were stained with DAPI. **(D)** Detection of XBP1 (u, unsliced) and XBP1 (s, spliced) mRNAs in HCMV-infected NSPC/iPSCs. Total RNAs isolated from NSPC/iPSCs harvested before (-) HCMV infection or at 1, 2, 3, 5, and 7 dpi with HCMV Towne strain were subjected to RT-PCR assays. Amplified DNA fragments were separated in a 2% agarose gel and then photographed. Beta-actin gene expression was assayed for the control. **(E and F)** Towne-infected NSPC/iPSCs at 3 dpi were subjected to immunofluorescence test with anti-IE1/IE2 antibody in combination with anti-phosphorylated PERK **(E)** or anti-gB antibody in combination with anti-phosphorylated eIF2 α **(F)** antibody. **(G)** Mock-infected NSPC/iPSCs were subjected to immunofluorescence test with anti-IE1/IE2 antibody in combination with anti-phosphorylated PERK (upper panel) or anti-phosphorylated eIF2 α (lower panel) antibody. Nuclei were stained with DAPI. **(H)** Expression of CHOP mRNA in HCMV-infected NSPC/iPSCs. Total RNAs isolated from NSPC/iPSCs harvested before (-) HCMV infection or at 1, 2, 3, 5, and 7 dpi with HCMV Towne strain were subjected to RT-PCR assays. Representative results from three independent experiments are shown.

XBP1 mRNA and up-regulation of CHOP, indicate that ER stress also plays a role in HCMV-induced apoptosis of NSPC/iPSCs. These results are in accordance with the work reported by Isler *et al.* [27] who demonstrated that HCMV-induced UPR in human foreskin fibroblasts.

HCMV is known to encode anti-apoptotic proteins such as viral inhibitor of caspase-8-induced apoptosis (vICA) encoded by UL36 [28], and pUL38 which protects against ER stress-induced cell death by modulating the UPR pathway [29]. Our RT-PCR analysis demonstrated

that such viral anti-apoptotic genes were expressed at transcription level in NSPC/iPSCs following HCMV infection (Figure 2B). Although these viral anti-apoptotic proteins did not block apoptosis of NSPC/iPSCs, they might have contributed for efficient viral replication by delaying apoptosis.

iPSCs are expected to be an innovative tool for not only regenerative medicine but also for the elucidation of pathogenesis of various diseases. Recent studies have shown that human iPSCs can be used also for modeling viral infection. Hepatocyte-like cells derived from human iPSCs were shown to be susceptible to hepatitis virus C infection and supported its replication [30,31]. Sensory neurons derived from human iPSCs were reported to be susceptible to infection with both varicella-zoster virus and herpes simplex virus [32]. While the present work was in progress, D'Aiuto and others reported on the preparation of an iPSC-derived model of HCMV infection in neural precursor cells [33]. Whereas our data described in the present study is largely consistent with their results, we further analyzed the mechanisms of apoptosis induction and elucidated the involvement of mitochondrial dysfunction and ER stress.

In conclusion, human NSPCs derived from iPSCs can be a useful model to study HCMV neuropathogenesis associated with congenital HCMV infection. They can be particularly valuable in analyzing the mechanisms of HCMV-induced apoptosis in neural cells.

Abbreviations

HCMV: Human cytomegalovirus; iPSC: Induced pluripotent stem cell; ESC: Embryonic stem cell; NSPC: Neural stem/progenitor cell; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; UPR: Unfolded protein response; ER: Endoplasmic reticulum; PERK: PKR-like eukaryotic initiation factor 2a kinase; JNK: c-Jun NH2-terminal kinase; IRE1: Inositol-requiring enzyme 1; eIF2 α : Alpha subunit of eukaryotic initiation factor 2; CHOP: C/EBP-homologous protein; XBP1: X-box binding protein 1; IFA: Indirect immunofluorescence assay; Dpi: Days post-infection; MAP2: Microtubule-associated protein 2; GFAP: Glial fibrillary acidic protein; OSP: Oligodendrocyte-specific protein; ATF4: Activating transcription factor 4; MOI: Multiplicity of infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HN, HL, KM, and HA performed the experimental studies, and KI helped to analyze the data. KM, MT, HA, YM, HO, NK, and AU participated in the characterization of iPSCs and their derivatives. HN, HL, and SF wrote the manuscript. NI revised the manuscript. All authors read and approved the final manuscript.

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