Overexpression of Na\(^+\)/H\(^+\) exchanger 1 specifically induces cell death in human iPSCs via sustained activation of the Rho kinase ROCK

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Understanding the specific properties of human induced pluripotent stem cells (iPSCs) is important for quality control of iPSCs. Having incidentally discovered that overexpression of plasma membrane Na\(^+\)/H\(^+\) exchanger 1 (NHE1) induces cell death in iPSCs, we investigated the mechanism of NHE1-induced cell death. Doxycycline-induced NHE1 overexpression arrested cell growth, and nearly all cells were killed by a necrotic process within 72 h. NHE1 overexpression led to sustained activation of Rho-associated coiled-coil kinase (ROCK), accompanied by dramatic changes in cell shape, cell elongation, and swelling of peripheral cells in iPSC colonies, as well as marked stress fiber formation. The ROCK inhibitor Y27632 reduced NHE1-induced cell death. ROCK-dependent phenotypes were suppressed by a loss-of-function mutation of NHE1 and inhibited by an inhibitor of NHE1 activity, indicating that NHE1-mediated transport activity is required. Moreover, ROCK was activated by trimethylamine treatment–mediated cytosolic alkalization and accumulated in the plasma membrane near NHE1 in peripheral iPSCs of cell colonies. By contrast, cell death did not occur in mesendoderm-like cells that had differentiated from iPSCs, indicating that the NHE1-mediated effects were specific for iPSCs. These results suggest that NHE1 overexpression specifically induces death of iPSCs via sustained ROCK activation, probably caused by an increase in local pH near NHE1. Finally, monensin, a Na\(^+\)/H\(^+\) exchange ionophore, selectively killed iPSCs, suggesting that monensin could help eliminate iPSCs that remain after differentiation, a strategy that might be useful for improving regenerative medicine.

Successful generation of induced pluripotent stem cells (iPSCs)\(^2\) (1–3) provides great promise in the development of regenerative medicine and drug discovery. In addition to such clinical interests, iPSCs also provide a powerful experimental tool for basic research such as studying the function of unknown genes in nonproliferative terminally differentiated cells like cardiomyocytes, because iPSCs can differentiate into virtually any kind of cell in the body (3) and can easily acquire genetic manipulation because of their infinite proliferating ability. To better manipulate iPSCs in culture for various purposes, it would be important to know iPSC-specific physiological properties. For example, the passage of iPSCs has been hampered by their cell death upon dissociation (4). This technical problem was solved by discovery of the Rho-associated coiled-coil containing protein kinase (ROCK) inhibition, which efficiently reduces the dissociation-induced cell death of iPSCs (5, 6). Another serious problem in the clinical field was how to eliminate the undifferentiated iPSCs remaining after differentiation, which have the risk of teratoma formation (7). Thus, understanding the physiological properties specific to iPSCs is crucial for the quality control of iPSCs in culture and for the selective elimination of iPSCs.

During our trials of the knockdown or overexpression of various genes in human iPSCs, we accidentally discovered that the overexpression of Na\(^+\)/H\(^+\) exchanger 1 (NHE1) induced the cell death of iPSCs. NHE1 is a ubiquitous plasma membrane transporter that catalyzes an electroneutral Na\(^+\)/H\(^+\) exchange and thereby regulates the intracellular pH, Na\(^+\) concentration, and cell volume (8, 9). NHE1 is known to be activated in response to various stimuli, including growth factors and mechanical stress, leading to cytoplasmic alkalination (9). Bioactive lipids, ATP, or protein kinases such as ROCK were reported to activate NHE1 via direct or indirect interaction (9, 10). However, conversely, there are only a few reports to show that the pH gradient produced by NHE1 across the plasma membranes selectively amplifies certain downstream signaling pathways.

Our preliminary experiment showed that NHE1–induced cell death occurs only in the undifferentiated pluripotent state, and this event does not occur in differentiated mesendoderm-like cells produced by exposure to the glycerol synthase kinase
3β (GSK3β) inhibitor for only 2 days. This iPSC-specific phenomenon greatly encouraged us to study the mechanism for NHE1-induced cell death because such a study may develop a novel strategy to improve the manipulation of iPSCs, as well as to draw out a new function of NHE1. In this study, we report the molecular mechanism of NHE1-induced cell death of iPSCs and show that the sustained activation of ROCK is a key event.

**Results**

**Overexpression of NHE1 induces cell death in iPSCs**

The doxycycline (Dox)-inducible vector carrying complementary DNA (cDNA) for NHE1 tandemly tagged with the two human influenza hemagglutinin (HA) epitopes in the extracellular loop was stably transfected into human iPSCs. The resulting NHE1 transfectants were well-stained with the verified stem cell markers, NANOG or TRA-1–60, in the absence of Dox (Fig. S1), suggesting that the vector transfection itself does not affect the pluripotency of this iPSC cell line.

The expression of NHE1 was induced by the addition of Dox. The NHE1 proteins were highly expressed 24 h after Dox inclusion, as shown in the immunoblot with anti-NHE1 and anti-HA antibodies (Fig. 1A). Plasma membrane expression of NHE1 with extracellular HA epitope was confirmed only in Dox-treated cells. Staining was performed in cells without permeabilization after fixation. DAPI was used for nuclear staining.

**Figure 1. Overexpression of NHE1 induces the growth arrest and cell death of human-induced pluripotent stem cells (hiPSC).** The overexpression of NHE1 was performed by exposing the NHE1 transfectants of iPSCs to Dox for 24 h. A, proteins from Dox-treated and untreated cells were subjected to the immunoblot analysis with anti-NHE1 and anti-HA antibodies. The blot of α-tubulin was used as an internal reference. B, confocal microscopy observation. Of note, endogenous NHE1 mRNA is expressed in iPSCs as evidenced by qPCR (Fig. S7A), although it was barely detected by immunoblot with an anti-NHE1 anti-
body. Immunostaining of nonpermeabilized cells with anti-HA revealed that NHE1 was highly expressed in the plasma membranes only under Dox induction (Fig. 1B). Interestingly, 72 h after Dox addition, nearly all iPSCs were dead (Fig. 1C). The cell viability assay revealed that the overexpression of NHE1 almost completely arrested cell growth and induced cell death (Fig. 1D). Fig. 1E shows the concentration dependence of Dox on the cell viability of nontransfected or NHE1-transfected cells. Inclusion of Dox of more than 0.1 μg/ml markedly reduced the cell viability (Fig. 1E) and at the same time induced overexpression of NHE1 (Fig. 1F) only in NHE1 transfectants, indicating that NHE1 overexpression, but not Dox itself, is toxic to cells. Because of the overexpression of NHE1, the number of cells stained with NANOG or TRA-1-60 decreased (Fig. S1), suggesting that the pluripotency may be partially lost upon NHE1 overexpression.

To examine the mechanism of NHE1-induced cell death, we used apoptosis/necrosis detection reagents. Necrosis was monitored by profluorescent dye entering cells with loss of membrane integrity, whereas apoptosis was detected by luminescence for annexin V binding to the plasma membranes. Apoptosis is known to occur in a population of growing iPSCs in culture, but necrosis was not detected in the absence of Dox (Fig. 2A). In contrast, necrosis occurred under Dox inclusion of more than 24 h (Fig. 2B), in a similar time course to toxic compound Taxol (500 nm), which was used as a positive control experiment (Fig. 2C). These data suggest that NHE1-induced cell death is due to necrosis.

**NHE1-induced cell shape change and ROCK activation in iPSCs**

We compared the cell shape of control and NHE1-transfected iPSCs at 24 h after Dox addition. Surprisingly, overexpression of NHE1 resulted in abnormal cell elongation and expansion in the peripheral region of iPSC colonies (Fig. 3A). The cell area was measured in cells located in the center and peripheral regions of colonies. The cell area of peripheral cells under NHE1 overexpression was 10-fold larger than that of cells in the center or control cells without Dox (Fig. 3B). We found that NHE1-induced cell death first starts to occur in this peripheral region (Movie S1). Interestingly, NHE1 overexpression promoted an outstanding actin stress fiber formation (Fig. 3C). These data provide evidence that cell movements, such as increased contraction, cell elongation, and swelling, induced cell death via necrotic process.

NHE1-induced cell shape change was inhibited by the ROCK inhibitor Y27632 (Fig. 3A, far right panel). Therefore, we predicted that cell death may be due to the activation of ROCK. Indeed, Dox-induced cell death was perfectly blocked by the high concentration (>100 μM) of Y27632 (Fig. 4A). We measured ROCK activity by monitoring the phosphorylation of the downstream protein substrate myosin phosphatase target 1 (MYPT1). As expected, NHE1 overexpression increased phosphorylation of MYPT1 (Fig. 4B and C) in the Y27632-inhibitable manner. The phosphorylation of MYPT1 increased almost 6-fold upon NHE1 overexpression (Fig. 4D). High concentration (100 μM) of Y27632 was required for complete inhibition of ROCK. Essentially, phosphorylation of MYPT1 at 10 μM was still significantly higher than that at the 100 μM inhibitor (Fig. 4, E and F). These data suggest that less than 10 μM of the inhibitor is not sufficient for inhibition of cell death because of highly enhanced ROCK activity.

**Effect of NHE1 loss-of-function mutation**

Next, we examined the effect of loss-of-function mutation (E262I) of NHE1, which lacks the Na\(^+/\)H\(^+\) exchange activity (11). Dox treatment induced overexpression of E262I in the plasma membrane (Fig. 5A and Fig. S2). However, NHE1-induced ROCK activation did not occur in the case of E262I (Fig. 5A and B). This loss-of-function mutation greatly suppressed growth arrest and cell death under Dox treatment for 72 h (Fig. 5C). Dox induced a slight decrease in cell viability (Fig. 5C), which may be because of NHE1 overexpression, such as ER stress. In addition to a minimum effect on cell survival, the mutation blocked NHE1-induced cell shape change (Fig. 5D) and stress fiber formation (Fig. 5E). Thus, NHE1-induced ROCK activation, growth arrest, cell death, cell shape change, and stress fiber formation were all suppressed by loss-of-function mutation, suggesting that the transport activity of NHE1 is required for these all events.
ROCK activity is pH-dependent

Given the data on the requirements of the NHE1 activity, we measured the intracellular pH as described under “Experimental procedures.” In this experiment, to avoid the effect of bicarbonate, we switched the growth medium (Stem-fit) to HEPES-buffered Dulbecco’s modified Eagle’s medium (DMEM). Fluorescence ratio of carboxy-SNARF-1 was plotted against pH \( i \) clamped by high K\(^+\)/nigericin solutions with various pH \( \text{pH}_i \) (Fig. 6A). Based on this pH calibration curve, the data show that overexpression of the WT NHE1, but not E262I, significantly elevated \( \Delta \text{pH}_i \) (0.3 pH unit) the resting pH \( \text{pH}_i \) and that the NHE1 inhibitor cariporide (50 \( \mu \text{M} \)) abrogated this pH \( \text{pH}_i \) elevation (Fig. 6B), suggesting that pH \( \text{pH}_i \) was increased by NHE1-dependent H\(^+\) extrusion across the plasma membrane of iPSCs.

When Dox-treated (24 h) NHE1 transfectants were exposed to cariporide for up to 60 min, NHE1-induced ROCK activation was canceled (Fig. 6, C and D). We next examined the effect of trimethylamine (TMA), which is known to artificially induce the sustained pH \( \text{pH}_i \) elevation by absorbing H\(^+\) in the cytosol (13). Indeed, pH \( r \) was greatly (~0.5 pH unit) increased by a 30-min incubation with TMA (Fig. 6B). Interestingly, TMA markedly enhanced ROCK activity in HEPES-buffered DMEM (Fig. 6, E and F). Such activation also occurred in the growth medium (Fig. S3), suggesting that a pH \( r \) increase is itself capable of activating ROCK. To determine the pH dependence of ROCK activity, we performed \textit{in vitro} kinase reaction. Indeed, \textit{in vitro} ROCK activity increased more than 2-fold by increasing the medium pH from 7.0 to 8.2 (Fig. S4).

NHE1 promotes translocation of ROCK to the plasma membrane

To determine how ROCK is related to NHE1, we examined the subcellular localization of ROCK1. Interestingly, ROCK (ROCK1) partially accumulated in the plasma membrane of particularly peripheral cells in cell colonies and co-localized with NHE1 under the overexpression of NHE1 (Dox\(^+\)), whereas ROCK1 is diffusely localized in the cytosol in the control condition (Dox\(^-\); Fig. 7, A and B, see allow positions). As expected, part of ROCK1 was enriched in the membrane fraction after cell fractionation (Fig. 7C). However, we did not detect the co-immunoprecipitation of ROCK1 and its upstream factor Rho with NHE1 in the detergent-solubilized protein samples (Fig. 7D). These data suggest that ROCK1 is partially localized at the plasma membrane near NHE1 under overexpression of NHE1, although it may not directly interact with NHE1. Interestingly, a part of phosphorylated MYPT1 also accumulated in the plasma membrane near NHE1, particularly in the peripheral regions of cell colonies (Fig. S5), suggesting that NHE1-induced ROCK activation and phosphorylation of its downstream targets predominantly occur near the plasma membrane.
Differentiated mesendoderm-like cells are resistant to NHE1 and monensin toxicities

To test whether NHE1-induced cell death also occurs in differentiated cells, we produced mesendoderm-like cells by treating the NHE1 transfectants with the GSK3β/H9252 inhibitor (Fig. 8A for experimental design). As shown in Fig. 8B, 2 days after the start of differentiation, the expression of the MESP1 (mesoderm posterior protein 1) gene was up-regulated (Fig. 8B), indicating that the treatment of cells with the GSK3β inhibitor promoted the differentiation of iPSCs. After replating, Dox inclusion for 3 days resulted in the overexpression of NHE1 (Fig. 8C). However, in contrast to iPSCs, we did not detect signs of cell death (Fig. 8D). Rather, the cell viability was even increased by Dox treatment (Fig. 8E), which may be due to the well-known growth-promoting effect of NHE1 (8). In contrast to iPSCs, ROCK activity was relatively high in the differentiated cells under control conditions; however, overexpression of NHE1 did not further activate ROCK (Fig. 8, F and G). Moreover, unlike iPSCs, ROCK did not appear to accumulate in the plasma membrane under overexpression of NHE1 (Fig. S6A) and was not enriched in the membrane fraction (Fig. S6B).

Based on the findings that NHE1 is selectively toxic to iPSCs, we examined whether Na+/H+ ionophore monensin can selectively kill the iPSCs. The antibiotic monensin is known to behave as a Na+/H+ antiporter in the plasma membrane. As predicted, monensin was very toxic to iPSCs, but not to differentiated cells. Exposure of cells to 1 μM monensin for 24 h resulted in the intense cell death of iPSCs (Fig. 9A) but had nearly no effect on the differentiated cells (Fig. 9B). Such toxicity to monensin was also observed in another iPS cell line prepared from a patient in our laboratory (data not shown). A cell viability assay revealed that monensin was 100-fold more toxic to iPSCs than the differentiated cells (Fig. 9C; IC50 = 0.05 μM versus ~5 μM). Indeed, like the overexpression of NHE1, treatment with 1 μM monensin increased the level of phosphorylated myosin phosphatase targeting subunit 1 (pMYPT1) (Fig. 9, D and E), suggesting that monensin can enhance ROCK activity. However, monensin-induced cell death was not observed in the differentiated cells (Fig. 9D).
The death of iPSCs was not significantly suppressed by Y27632 (Fig. 9F), suggesting that this was not merely due to ROCK activation.

**Discussion**

In this study, we discovered that overexpression of NHE1 induces the cell death of iPSCs. Interestingly, overexpression of NHE1 induced a tremendous elongation and swelling of cells in the peripheral regions of colonies, together with intense stress fiber formation. The time-lapse recording revealed that cell death occurs in such elongated cells at colony edges. NHE1 promoted profluorescent DNA dye entering cells that had lost membrane integrity without affecting annexin V binding to cell membranes (Fig. 2B). These observations suggest that overexpression of NHE1 induces cell death by necrosis rather than apoptosis via abnormal cell elongation, swelling, and the resultant cell rupture. We examined the effect of knockdown (Fig. S7A, >95% knockdown efficiency) of endogenous NHE1 on cell death using CRISPRi. NHE1 knockdown did not influence dissociation-induced cell death (Fig. S7B), apoptosis/necrosis during culture in growth medium (Fig. S7C), or cell growth rate (Fig. S7E). Thus, at least endogenous NHE1 does not contribute to such events, probably because of its low expression level.

NHE1-induced cell death was suppressed by the ROCK inhibitor. Overexpression of NHE1 resulted in the remarkable and sustained activation of ROCK, which requires a high concentration (>100 μM) of inhibitor for complete inhibition. ROCK is known to activate various downstream targets such as myosin light chain phosphatase and its subunit (MYPT1), ERM (ezrin/radixin/moesin) family proteins, adducin and Lin-11, Isl-1, and Mec-3 (LIM) kinase, thereby enhancing cellular contraction, stress fiber formation, and cell migration (14–16), which would result in the gross cell shape change observed in our cell system. Activation of ROCK did not occur in the overexpression of the loss-of-function mutant for NHE1 and was inhibited by the NHE1 inhibitor, strongly suggesting that NHE1 transport activity is required for ROCK-dependent phenomena. Interestingly, artificial cytosolic alkalinization with TMA increased ROCK activity, and high pH elevated in vitro ROCK activity. These results suggest that the elevated cytosolic pH via the H+ extrusion driven by NHE1 results in ROCK activation. Many publications reported that NHE1 is activated by ROCK as a downstream target (17–22). This ROCK-induced NHE1 activation may further enhance ROCK activity as a positive feedback loop.

According to a classical model, ROCK is maintained in an inactive state in the cytosol by autoinhibitory interactions between the carboxyl-terminal regulatory Rho-binding domain and two split PH and C1 domains (PH-C1 module) (14). Upon activation, this autoinhibition is relieved by Rho binding to the Rho-binding domain and by the binding of the PH-C1 module...
to phosphoinositide-containing membranes. Earlier studies reported that ROCK translocated to the plasma membrane upon activation by interaction with Rho-GTPase (23). Indeed, we observed that a part of ROCK is translocated to the plasma membrane upon overexpression of the WT NHE1, but not E262I, although direct interaction with NHE1 was not detected. Furthermore, pMYPT1 was also partly detected in the membranes. Thus, we conclude that NHE1-dependent activation of ROCK occurs near the plasma membrane (see Fig. S8 for a possible mechanism).

We previously reported that NHE1 exists in the lipid raft, which would produce a high pH microdomain (11). Other studies also supported such a restricted membrane localization of NHE1 (17, 24, 25). We showed that the enzymatic activity of ROCK itself is increased by high pH in vitro, but upstream activators for ROCK may be also pH-dependent. For example, the cytosolic alkalinization by NHE1 was reported to activate the phospholipase A2 (26), thereby producing arachidonic acid, leading to the activation of ROCK (27). Additionally, Rho-GTPase and Rho activator guanine nucleotide exchange factors may also be pH-dependent. However, we could not identify the pathways that were NHE1-dependently amplified through experiments using inhibitors (data not shown). Against the Rho-dependent ROCK activation mechanism, recent studies (28, 29) argue that ROCK may function as a molecular ruler, in which the kinase domain is positioned near the substrate by a long coiled-coil region, at a proper distance from the plasma membrane, and that the tethering of the PH-C1 module to the membranes is a critical determinant for ROCK activity. The PH-C1 module of ROCK preferentially binds to phosphoinositides with high negative charge density like phosphatidylinositol triphosphate via interaction with positively charged residues of PH-C1 module (30). Therefore, another possible explanation for the pH-dependent change of ROCK activity is that a pH microenvironment may modulate the tethering of the PH-C1 module to membrane phosphoinositides by affecting the electrostatic charge interaction (Fig. S8).

An interesting observation was that NHE1-induced cell death did not occur in the mesendoderm-like cells differentiated from iPSCs. Although here we reported data on mesendoderm-like cells as an example of differentiated cells, many researchers, including our group, have overexpressed NHE1 in various nonpluripotent differentiated cells, such as fibroblasts, HEK293 cells, and cardiomyocytes (9). However, NHE1-induced cell death was not observed in these differentiated cells. In contrast to iPSCs, in the differentiated cells like mesendo-
NHE1-induced cell death of iPS cells via ROCK activation

Figure 7. ROCK accumulates in the plasma membrane upon overexpression of NHE1. A and B, subcellular localization of ROCK1 in untreated or Dox-treated iPSCs, respectively. ROCK1 was stained with the anti-ROCK1 antibody followed by an Alexa 488–conjugated secondary antibody, whereas NHE1 was stained with anti-HA followed by an Alexa 546–conjugated secondary antibody under nonpermeabilized conditions before ROCK1 staining. The slight intracellular red staining is due to fluorescence from mKate provided by the vector. C, cell fractionation was performed in Dox-treated or untreated iPSCs. Proteins (10 μg) from cytosolic and membrane fractions were subjected to immunoblot analysis. Antibodies against E-cadherin and GAPDH were used as membrane and cytosol markers, respectively. D, NHE1 was immunoprecipitated using HA-agarose beads, and then proteins were analyzed by immunoblotting using 4–12% gradient gel.

In summary, iPSCs, but not differentiated cells, were selectively toxic to NHE1 overexpression. Our study would provide a new hypothesis for pH-dependent ROCK activation and dictate the outstanding difference of physiological states between iPSCs and differentiated cells, other than pluripotency. Furthermore, the discovery of monensin by analogy to NHE1 may provide a simple idea for the elimination of iPSCs.

Experimental procedures

Reagents

The following antibodies were used: rabbit anti-NHE1 (33), mouse anti–HA (Roche), mouse anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore), mouse anti–ROCK1 (Millipore), rabbit anti–pMYPT1 (Millipore), rabbit anti–Rho-GTPase (Proteintech), rabbit anti–E-cadherin (CST), and rabbit anti–α-tubulin (MBL). All other chemicals employed were of the highest available purity.

Molecular biology

A PCR-based strategy was performed to construct cDNA, as previously reported (34). For the construction of human NHE1 (NCBI accession number: NM_003047.5) incorporating extracellular HA tags, two tandem HA tags and glycine linkers consisting of 25 amino acids (GGYPYDVPDYAGGGYPYDVPDYAGG) were inserted between amino acids 281 and 282 of NHE1 (designated NHE1–2HA) (11). The cDNA was amplified by PCR and inserted into the expression vector KT2–014-PB-TW-KRAB-dCas9-CNKB by exchanging the dCas9 region with TW-KRAB-dCas9-CNKB by exchanging the dCas9 region with NHE1–2HA. The loss-of-function mutation (E262I) plasmid was also made using the PCR-based strategy. For CRISPRi, four different single guided RNAs (sgRNAs) were designed to target NHE1 near the transcription start site of genes of interest (200 bp upstream) (35). The location of the transcription start site was determined and retrieved using the UCSC genome browser (https://genome.ucsc.edu) (42). The sgRNA oligonucleotides were designed using the CRISPR design website, annealed, and cloned into the sgRNA expression vector PB-U6-CNKB, which is described (36).

Interestingly, an antibiotic monensin operating as a Na⁺/H⁺ antiporter induced the cell death of iPSCs at a lower concentration than mesendoderm-like cells. Although ROCK was activated, the monensin-induced cell death of iPSCs was not suppressed by the ROCK inhibitor, suggesting that it occurs via a ROCK-independent mechanism. A recent study (32) reported that monensin has been screened as a toxic compound selective to metastatic cancer cells and that the structure/function of the Golgi apparatus and mitochondria were largely damaged by monensin. Whatever the mechanism is for monensin-induced cell death, importantly, monensin can selectively kill iPSCs. The presence of a few remaining undifferentiated human iPSCs would cause undesirable teratomas in the clinical application of iPSC-based therapy. Monensin may be a potential novel tool to eliminate iPSCs selectively, although the effect of monensin should be widely examined in many other types of cells in the future.

In summary, iPSCs, but not differentiated cells, were selectively toxic to NHE1 overexpression. Our study would provide a new hypothesis for pH-dependent ROCK activation and dictate the outstanding difference of physiological states between iPSCs and differentiated cells, other than pluripotency. Furthermore, the discovery of monensin by analogy to NHE1 may provide a simple idea for the elimination of iPSCs.

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Cell culture, DNA transfection, and selection of stable clones

The human iPSC cell lines (WTC11 and C5) were previously described (37–39). These iPSCs were maintained in StemFit AK02N (Ajinomoto) medium on a plate coated with iMatrix-511 silk (Nippi) and passaged every 5–7 days with Accutase (Innovative Cell Technologies) (40). ROCK inhibitor Y-27632 (10 μM) (WAKO Pure Chemical Industries) was added to the medium for 24 h after each passage. For overexpression of NHE1, the expression vector was transfected into iPSCs (WTC11 cell line) by nucleofection (Nucleofector, LONZA, Japan). For nucleofection, 0.5–1.0 million CRISPRi iPSCs, the expression vector (7 μg), and piggyback transposase pCW-hyP-Base (3 μg) were mixed. After selection by blasticidin (10 μg/ml; Wako Pure Chemical Industries) and induction for 24 h by doxycycline (1.0 μg/ml; Sigma–Aldrich), the level of mRNA of NHE1 was measured by RT-PCR, and cell colonies expressing NHE1 were picked up. In some experiments, NHE1 transfectants were differentiated into the mesendoderm-like cells by treating cells for 1 day with GSK3β inhibitor CHIR99021 (10 μM) and then by removing the inhibitor for 1 day (41). For CRISPRi, each sgRNA-expression plasmid (7 μg) and pCW-hyPBase (3 μg) was transfected into CRISPRi; the iPSC line (C5) was established as described (37). After selection by blasticidin and induction by Dox, the best sgRNA for the knockdown of NHE1 was selected, and the cell colonies with the highest knockdown of NHE1 were picked up. The following oligonucleotides were used for the best sgRNA: forward, TTGGAGG-GAAAGAAGTATCGCGT; and reverse, AAACACGCGATA-CTTCTTTCCCT.

Measurement of cell viability, apoptosis, and necrosis

The cells (0.5–1.0 × 10^5/well) were plated in 96-well plates, cultured for 1–2 days, and treated with the indicated compounds for 24–72 h. Cell viability was measured using a Cell Counting kit 8 (Dojindo Chemicals). According to the protocol, a water-soluble tetrazolium solution (10 l) was added to the plates, and absorbance at 405 nm was measured after incubation for 1–4 h. Apoptosis and necrosis assays were performed with the GloMax plate reader (Promega) using the RealTime-Glo™ annexin V apoptosis and necrosis assay kit (Promega). For apoptosis, annexin V binding to exposed phosphatidylserine in the plasma membrane was monitored using luminescence, whereas for necrosis, profluorescent DNA dye entering cells that had a loss of membrane integrity was monitored using fluorescence.

Cell fractionation

Dox-treated (24 h) and untreated NHE1 transfectants of iPSCs cultured in 100-mm dishes were harvested, and the cells were suspended in 500 μl of fractionation buffer (a 20 mM HEPES, pH 7.4, 10 mM KCl, 2 mM MgCl2, 0.2 mM DTT, and...
protease inhibitor mixture). The cells were homogenized using Bioruptor II (BMBio) (25 cycles of a 10-s pulse with a 20-s interval) and centrifuged for 5 min at 10,000 × g. The supernatant was further centrifuged for 30 min at 100,000 × g. The resulting supernatant and precipitate after the second centrifugation were kept as the cytosolic and membrane fractions.

**Immunoblotting and immunoprecipitation**

For the immunoblot analysis, cells in 24-well plates were solubilized using the SDS-PAGE sample buffer (Invitrogen) and boiled for 10 min at 100 °C. After SDS-PAGE 10% handmade gel or 4–12% NuPAGE gradient gels (Invitrogen), the proteins were transferred electrophoretically onto polyvinylidene fluoride membranes and subjected to immunoblotting with the indicated antibodies (1:500 to 1:1000 dilution). The immunoblotted proteins were visualized using an enhanced chemiluminescence detection system (Millipore). The immunoblot was performed for α-tubulin as an internal control. Immunoblots with commercially available antibodies (pMYPT1, α-tubulin, ROCK1, E-cadherin, ROCK1, RhoA, GAPDH) produced a single major band showing the calculated molecular weight. For co-immunoprecipitation, the cells were washed with ice-cold PBS and solubilized in a lysis buffer (150 mM sodium chloride, 20 mM Tris, pH 7.4, 0.5% nonyl phenoxypolyethoxylethanol, and protease inhibitor mixture) for 20 min on ice. After centrifugation for 10 min at 16,000 × g, the supernatant was incubated with anti-HA magnet beads (Thermo Fisher Scientific). Subsequently, the beads were washed five times with ice-cold lysis buffer, and the proteins eluted with SDS-PAGE sample buffer were subjected to SDS-PAGE.

**RT-PCR**

Total RNA was isolated using a standard procedure after solubilization with RNAzol (Mol. Res. Ctr.). RNA (1 µg) was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.). Expression levels of mRNA for targets relative to internal control GAPDH were measured using the StepOne Plus real-time PCR system (Thermo Fisher Scientific) using TaqMan probes purchased from Thermo Fisher Scientific.

**Confocal microscopy**

Dox-treated (24 h) or untreated cells on glass-bottomed dishes were fixed with formaldehyde for 10 min and permeabilized with PBS containing 0.1% Triton X-100 for 3 min. After blocking them in PBS with 5% BSA, the cells were treated for 60 min with the anti-ROCK1 antibody (1:100). To stain extracellular HA-tagged NHE1, the cells were treated with mouse anti-HA (1:100) after fixation (without permeabilization). Subsequently, the cells were fluorescently stained with Alexa Fluor 488– or Alexa Fluor 546–labeled secondary antibodies (1:400). For nuclear staining, permeabilized cells were then incubated for 10 min with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescently labeled cells were observed using a laser scanning confocal attachment (Leica SP8).

**Measurement of intracellular pH (pHi)**

The pHi was measured by monitoring the ratio of the pH-sensitive fluorescent dye. The cells were plated in 96-well plates, treated or untreated with Dox for 24 h in the growth medium, and then incubated for 30 min at 37 °C in HEPES-
buffered DMEM containing 10 µg/ml carboxy-SNARF-1-AM. The cells were further incubated for 30 min in HEPES-buffered DMEM containing various chemicals without dye. For pH calibration, the cells were incubated for 30 min in a high K⁺/nigericin buffer (120 mM KCl, 2 mM calcium chloride, 1 mM MgCl₂, and 5 µg/ml nigericin) adjusted to various pH using Mes for pH 5.5–6.5, Mops for pH 7.0–7.5, or Tris for pH 8.0–9.0 (20 mM each). The fluorescent intensities at emission wavelengths 580 and 620 nm (excitation, 480 nm) were calculated, and pH values were determined using pH calibration curves.

**Measurement of in vitro ROCK activity**

The recombinant MYPT1 (0.2 µg) was incubated at 37 °C with 5 ng of recombinant active ROCK1 (SignalChem) in 100 mM KCl, 10 mM MgCl₂, 0.2 mM DTT, and mixed buffers (Mes, Mops, and Tris; 10 mM each) adjusted to various pH with KOH/HCl. The kinase reaction was started by adding ATP (0.2 mM) and terminated 10 min later by adding the SDS-PAGE sample buffer. The proteins were then subjected to immunoblotting with the anti-pMYPT1 antibody.

**Statistical analysis**

The data are expressed as the means ± S.D. for more than three determinations. The data were statistically analyzed using JMP Pro 14.2.0 (SAS Institute Inc.). We used two-way analysis of variance followed by the Tukey–Kramer method (Figs. 3B, 4C, 5B, and 8G) or one-way analysis of variance followed by the Dunnett method (Figs. 4F, 6, B, D, and F; and 9E; and Fig. S3) for comparison of multiple data. Every statistical parameters (F and p values, and p values for the post hoc tests were listed in the Table S1. We also used the unpaired Student’s t test for comparisons between two groups (Fig. 9F). Values of p < 0.05 were considered statistically significant.

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