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A ROCK inhibitor permits survival of dissociated human embryonic stem cells

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Poor survival of human embryonic stem (hES) cells after cell dissociation is an obstacle to research, hindering manipulations such as subcloning. Here we show that application of a selective Rho-associated kinase (ROCK) inhibitor[1,2], Y-27632, to hES cells markedly diminishes dissociation-induced apoptosis, increases cloning efficiency (from ~1% to ~27%) and facilitates subcloning after gene transfer. Furthermore, dissociated hES cells treated with Y-27632 are protected from apoptosis even in serum-free suspension (SFEB) culture[3] and form floating aggregates. We demonstrate that the protective ability of Y-27632 enables SFEB-cultured hES cells to survive and differentiate into Bf1$^+$ cortical and basal telencephalic progenitors, as do SFEB-cultured mouse ES cells.

Differentiated cells produced from hES cells may be useful for treating degenerative diseases whose symptoms are caused by loss of a few particular cell types. With regard to neurological therapeutic research, specific types of neurons have been generated from mouse ES (mES) cells[3–7], and similar selective differentiation methods have been applied to hES cells[8–12]. However, hES cells have been technically much harder to culture than mES cells, showing problematic properties such as slow growth and insensitivity to leukemia inhibitory factor (LIF)[13,14]. In addition, hES cells are vulnerable to apoptosis upon cellular detachment and dissociation. They undergo massive cell death particularly after complete dissociation, and the cloning efficiency of dissociated hES cells is generally $\leq$1%[13–16]. Thus, hES cells are difficult, if not impossible, to use in dissociation culture, which is important for such procedures as clonal isolation following gene transfer[17] and differentiation induction.

In an effort to circumvent the problem of apoptosis (or anoikis[18]) in hES cell culture, we examined the effects of several caspase inhibitors, growth factors, trophic factors and kinase inhibitors. Of the compounds tested, Y-27632, a selective inhibitor of p160-Rho-associated coiled-coil kinase (ROCK)[2,19], was the most potent inhibitor of apoptosis. The chemical structure and pharmacological properties of Y-27632 are described in ref. 1. Although the role of ROCK in apoptosis is not well understood[19], recent reports indicate its possible involvement in certain cases of apoptosis, including chemically induced anoikis[20] and neuronal death in the embryonic motor column[21].

After a 1-h pretreatment with Y-27632 (10 μM, a commonly used working concentration[22]) and complete dissociation, hES cells were plated on a mouse embryonic fibroblast (MEF) feeder layer at low density (500 cells/well, 96-well plates) in maintenance medium containing Y-27632. By day 6, untreated dissociated hES cells had generated very few colonies (**Fig. 1a**). In contrast, Y-27632-treated dissociated hES cells produced many large colonies, which were almost all positive for alkaline phosphatase (ALP; **Fig. 1b**). The cloning efficiency (based on the ratio of ALP$^+$ colonies formed per initially seeded hES cells) was 26.6 ± 2.4% and 1.0 ± 0.4% in the presence and absence of Y-27632, respectively (**Fig. 1c**). Y-27632-treated hES cells grown at low density were also positive for the undifferentiated-state markers E-cadherin, Oct3/4 and SSEA4 (**Fig. 1d–f**).

After five low-density passages, Y-27632-treated hES cells retained the competence to differentiate into neural cells (see below), mesodermal cells and endodermal precursors *in vitro* (**Fig. 1g–k**). Even after 30 passages with low-density plating in the presence of Y-27632, hES cells continued to express the undifferentiated-state makers (E-cadherin, Oct3/4 and SSEA4; **Supplementary Fig. 1a–c** online) and formed teratomas after being grafted into the testes of severe combined immunodeficient mice (SCID; **Fig. 1l–n** and **Supplementary Fig. 1d–g**). At 30 passages the ability of the dissociated cells to form colonies was still dependent on Y-27632, indicating that Y-27632 treatment was not simply selecting altered hES cells that have irreversibly acquired autonomous proliferation ability[23,24] in low-density culture (**Supplementary Fig. 1h,i**).

Treatment with another selective ROCK inhibitor (10 μM Fasudil/HA1077) promoted colony formation by undifferentiated hES cells in a manner similar to that of Y-27632 (**Supplementary Fig. 1j**). In contrast, no substantial increase in colony formation was observed with inhibitors of unrelated kinases at various concentrations: protein kinase A (cAMP-Rp, 1–100 (μM; KT5720, 5–500 nM), protein

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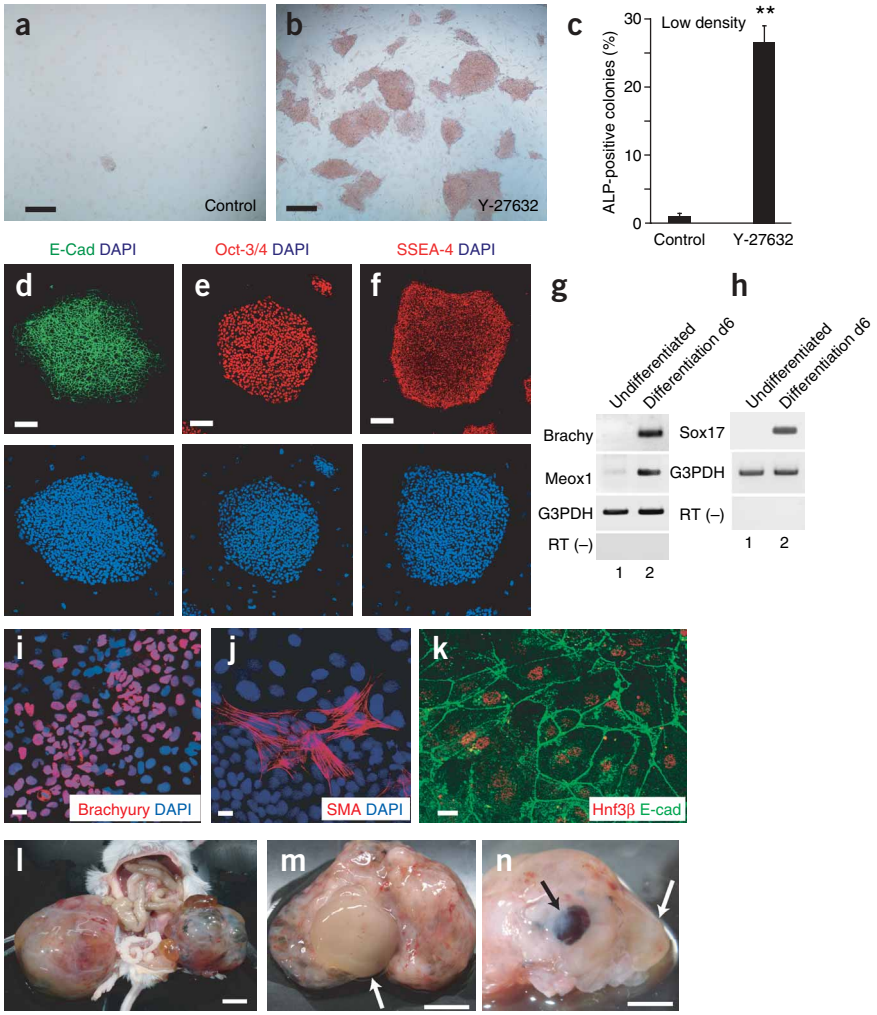

Figure 1 The ROCK inhibitor Y-27632 markedly increases the cloning efficiency of hES cells (KhES-1) without affecting their pluripotency. (**a**,**b**) Low-density culture of dissociated hES cells in the absence (**a**) and presence (**b**) of 10 μM Y-27632 on MEF cells for 7 d. Almost all colonies were positive for ALP. Bars, 500 μm. (**c**) Ratios of ALP$^+$ colonies to the number of initially seeded hES cells (**, $P < 0.01$ versus control, $n = 3$). (**d**–**f**) Immunostaining of Y-27632-treated hES cell colonies with anti-E-cadherin (**d**), anti-Oct3/4 (**e**) and anti-SSEA-4 (**f**) antibodies. Bottom panels are nuclear DAPI staining. Bars, 100 μm. Y-27632 treatment did not cause a drastic change in actin-bundle formation of hES cells (not shown). (**g**) RT-PCR analysis of the early mesodermal markers *T* and *Meox1* in differentiating hES cells. RT(–), GAPDH PCR without reverse transcription. (**h**) RT-PCR analysis of the early endodermal marker *Sox17* in differentiating ES cells. (**i**–**k**) Immunostaining for the mesodermal and endodermal markers in differentiating hES cells on an 8-well chamber slide coated with collagen I and IV. Expression of the mesodermal marker Brachyury (red) in a number of differentiating cells (**i**). DAPI was used for nuclear staining (blue; **c**). Bar, 10 μm. (**j**) Immunostaining of smooth muscle actin (SMA; red) in hES cell (Y-27632-treated)-derived cells cultured on OP9 cells for 12 d. Nuclei were stained with DAPI (blue). Bar, 5 μm. (**k**) Immunostaining of Hnf3β and E-cadherin in an hES cell-derived epithelial sheet on day 6. Bar, 5 μm. (**l**–**n**) Teratoma formation (100%, $n = 20$) from hES cells maintained at low density in the presence of Y-27632 (30 passages). Bars, 1 cm. The cells were bilaterally injected into the SCID mouse testes (**l**). After 9 weeks, the teratomas contained a mixture of well-differentiated tissues including macroscopic cartilages (white arrows; **m**,**n**) and pigment epithelium (black arrow; **n**).

kinase C (bisindolylmaleimide, 0.01–5 (μM; staurosporine, 1–50 nM), MAPK (PD98059, 0.5–50 (μM), PI3K (LY294002, 1–50 (μM) or MLCK (ML-7, 0.3–30 μM) (**Supplementary Fig. 1j** and data not shown; in all cases, the cloning efficiencies were <2%).

Y-27632 treatment increased colony formation of dissociated hES cells when they were cultured on MEF cells but not on collagen-coated dishes (data not shown). It is unlikely that the effect of Y-27632 was mediated by the MEF cells, as cloning efficiency was increased even in feeder-free culture[25] using Matrigel and MEF-conditioned medium (**Fig. 2a**,**b**).

Next we tested the protective effect of Y-27632 on single hES cells seeded into each well of a 96-well plate. Treatment with Y-27632 substantially increased ALP$^+$ colony formation (from 1.3 ± 0.6% to 24.7 ± 6.5%; **Fig. 2c**,**d**). Thus, Y-27632 treatment supports the maintenance of and colony formation by hES cells grown in single-cell culture. Y-27632 promoted colony formation by dissociated hES cells at various cell densities (**Supplementary Fig. 1k**). Y-27632 treatment did not enhance chromosomal anomaly after long-term culture (**Supplementary Fig. 1l**).

Y-27632 treatment also facilitated the selective subcloning of hES cells after gene transfer. In low-density dissociation culture, hES cells transfected with the drug-selectable vector (*pCAGGS-Venus-Hygro*) formed hygromycin-resistant colonies at a reasonable frequency ($4.4 \pm 3.2 \times 10^{-5}$ colonies/initially seeded cell, five experiments) with Y-27632 treatment (**Fig. 2e**; strong expression of Venus-GFP, **Fig. 2f**), but rarely without it ($<1 \times 10^{-6}$).

We next studied the effects of Y-27632 on cell growth. Dissociated hES cells were plated on MEF cells and treated with Y-27632 either for the first 12 h after dissociation only (group 1, blue; **Fig. 2g**) or for the entire culture period continuously (group 2, green). In both groups, a large number of cells survived and grew into colonies, whereas little survival was seen in cultures of dissociated hES cells grown without Y-27632 treatment (no Y-27632, purple). These observations suggest that the survival-promoting effect of Y-27632 on dissociated hES cells in adhesion culture is largely attributable to the increase in cell survival during the first half-day of the dissociation/replating procedure.

After the first half-day of culture with Y-27632, no significant difference in the number of surviving cells was observed between groups 1 and 2 for the first 3 d (**Fig. 2g**). Interestingly, after day 3, the cell number in group 2 cells was slightly (but significantly) larger than in group 1 (**Fig. 2g**; the population doubling time during days 2–6 was 49.0 ± 2.0 h and 41.5 ± 1.4 h for groups 1 and 2, respectively; $P < 0.01$). No substantial increase in the number of apoptotic cells was found in either groups on days 3 or 5 (in all cases, < 1% of cells were positive for active Caspase 3). More than 96% of the hES cells in both groups were positive for the cycling population marker Ki67 on days 3 and 5 (**Fig. 2h**). Flow cytometric analysis of cell-cycle phases (**Fig. 2i**–**n**) also showed a slight but significant ($P < 0.01$) increase in

the S-phase population ratio and a marginal decrease in the G0/G1-phase population ratio in group 2 as compared with group 1 on days 3 and 5 (**Fig. 2k,n**), suggesting that Y-27632 also has a moderate growth-promoting effect.

To determine whether Y-27632 would block apoptosis under even more severe conditions, we subjected dissociated hES cells (2.5×10^5 cells/2ml on a nonadhesive 35-mm culture plate) to serum-free suspension culture and evaluated apoptosis. The majority (80.1 ± 4.9%, $n = 3$) of untreated dissociated hES cells became terminal dUTP nick-end labeling $(TUNEL)^+$ on day 2 (**Fig. 3a**). In contrast, only a small portion (9.1 ± 7.1%) of hES cells treated with Y-27632 was $TUNEL^+$ (**Fig. 3b,c**). In comparison, only weak protection against apoptosis was observed when the cells were treated with the potent pan-caspase inhibitor I (Z-VAD-fmk[26], 10 μM) or a cocktail of neurotrophins (BDNF/NT-3/NT-4, 50 ng each)[15] in this suspension culture ($TUNEL^+$ in 50.0 ± 13.4% and 71.7 ± 7.0% cells, respectively; **Fig. 3c**).

Consistent with these observations, Y-27632 treatment significantly ($P < 0.01$) increased the numbers of surviving cells on day 2 of suspension culture (25.2 ± 6.8% and 11.2 ± 5.6% with and without Y-27632, respectively, of the initial number of seeded hES cells) and day 6 (68 ± 3.2% and 7.7 ± 1.6% with and without Y-27632, respectively; $P < 0.01$) (**Fig. 3d**). Y-27632-treated hES cells formed aggregates in suspension by day 2 and continued growing thereafter (**Fig. 3e,f**). In contrast, treatment with the pan-caspase inhibitor increased the number of surviving hES cells on day 2, but not on day 6 (**Fig. 3d**). Z-VAD-fmk did not substantially support cell survival or aggregate formation on day 6 even at a high dose (100 μM; data not shown). The neurotrophin treatment induced no obvious increase in cell survival on either day (**Fig. 3d**), or of aggregate formation on day 6 (data not shown).

Because cell dissociation and suspension culture are commonly used to induce *in vitro* differentiation of mES cells, Y-27632 treatment

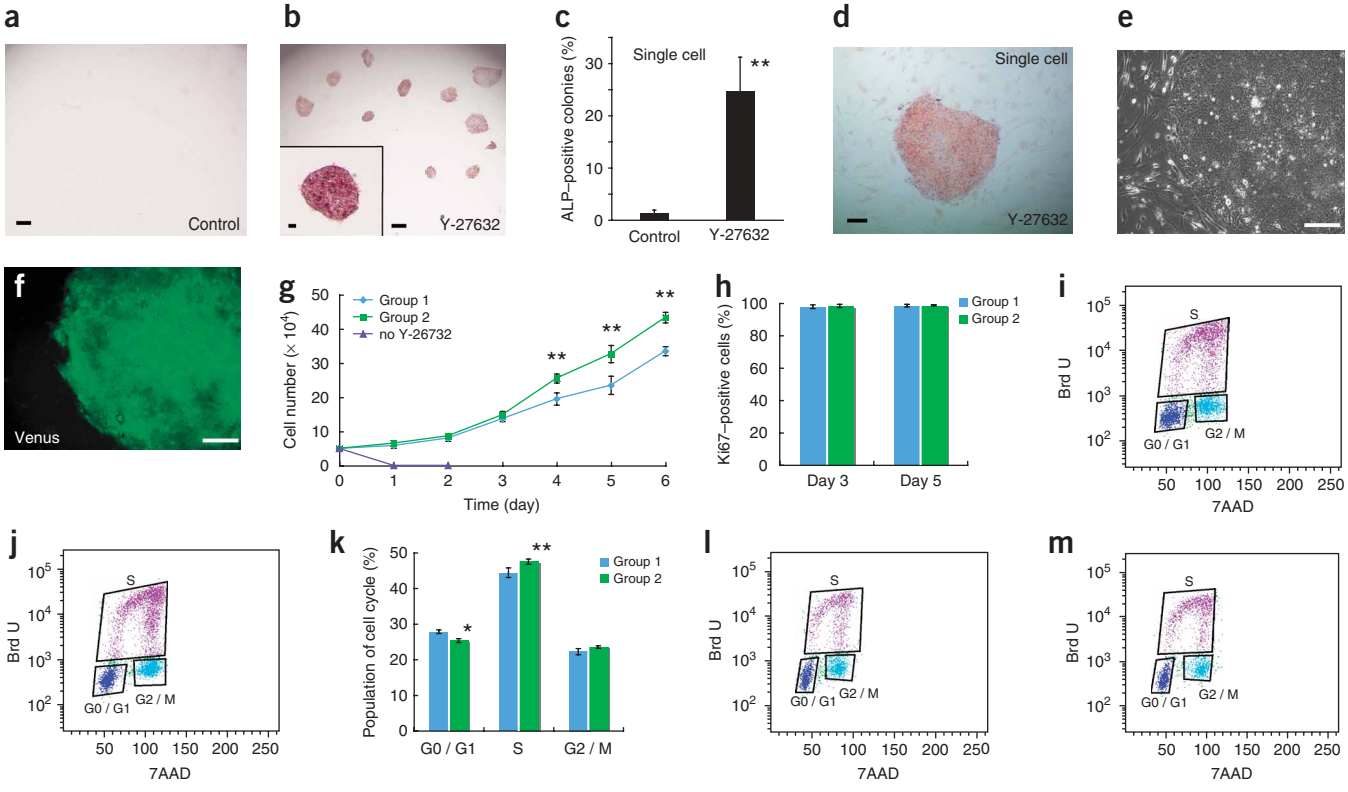

Figure 2 Y-27632 directly enhances the cloning efficiency of hES cells (KhES-1). (**a**,**b**) Feeder cell–free culture of hES cells on Matrigel-coated plates in MEF-conditioned medium. Bars, 500 μm. Colony formation from dissociated hES cells was clearly enhanced by Y-27632 (**b**; inset, a high magnification view of a typical colony; bar, 100 μm) whereas few colonies formed in its absence (**a**; < 0.2% and 10.2 ± 1.2% without and with Y-27632, respectively; $P < 0.001$, $n = 3$). (**c**,**d**) Culture of a single hES cell on MEF cells in each well of a 96-well plate in the presence of 10 μM Y-27632 for 7 d. (**c**) Percentages of the presence of an ALP^+ colony (**d**) in each well (**, $P < 0.01$ versus control, $n = 3$ studies). Control, untreated cells. Bar, 100 μm. (**e**,**f**) Formation of hygromyin-resistant colonies from Y-27632-treated hES cells in low-density dissociation culture on MEF cells 12 d after transfection. Bars, 100 μm. (**e**) Phase-contrast view. (**f**) Venus-GFP expression. (**g**) Growth curve of hES cells cultured on MEF cells with different time courses of Y-27632 treatment. Group 1 (blue), Y-27632 treatment during the first 12 h only (with 1-h pretreatment); group 2 (green), continuous Y-27632 treatment during the entire culture period; No Y-27632, no Y-27632 treatment at all (purple). For each condition, 5×10^4 dissociated cells/well (6-well plate) were plated on MEF cells. **, $P < 0.01$, group 2 versus group 1 ($n = 3$ studies). (**h**) Percentages of $Ki67^+$ (mitotic) cells in $Nanog^+$ hES cells in groups 1 (blue) and 2 (green) on days 3 and 5. (**i**-**n**) Flow-cytometric analysis of cell-cycle phase-specific populations. (**i**,**j**,**l**,**m**) Flow-cytometry patterns. *X*-axis, DNA content shown by 7-AAD-binding; *y*-axis, Brd U uptake after a 1-h exposure. (**k**,**n**) Relative percentages of phase-specific populations among the hES cells in groups 1 (blue) and 2 (green). (**i**-**k**) day 3. (**l**-**n**) day 5. *, $P < 0.05$; **, $P < 0.01$, group 2 versus group 1 ($n = 3$ studies). The degree of increase in cell growth is not very large and cannot explain the robust increase of cloning efficiency (1% versus 27%).

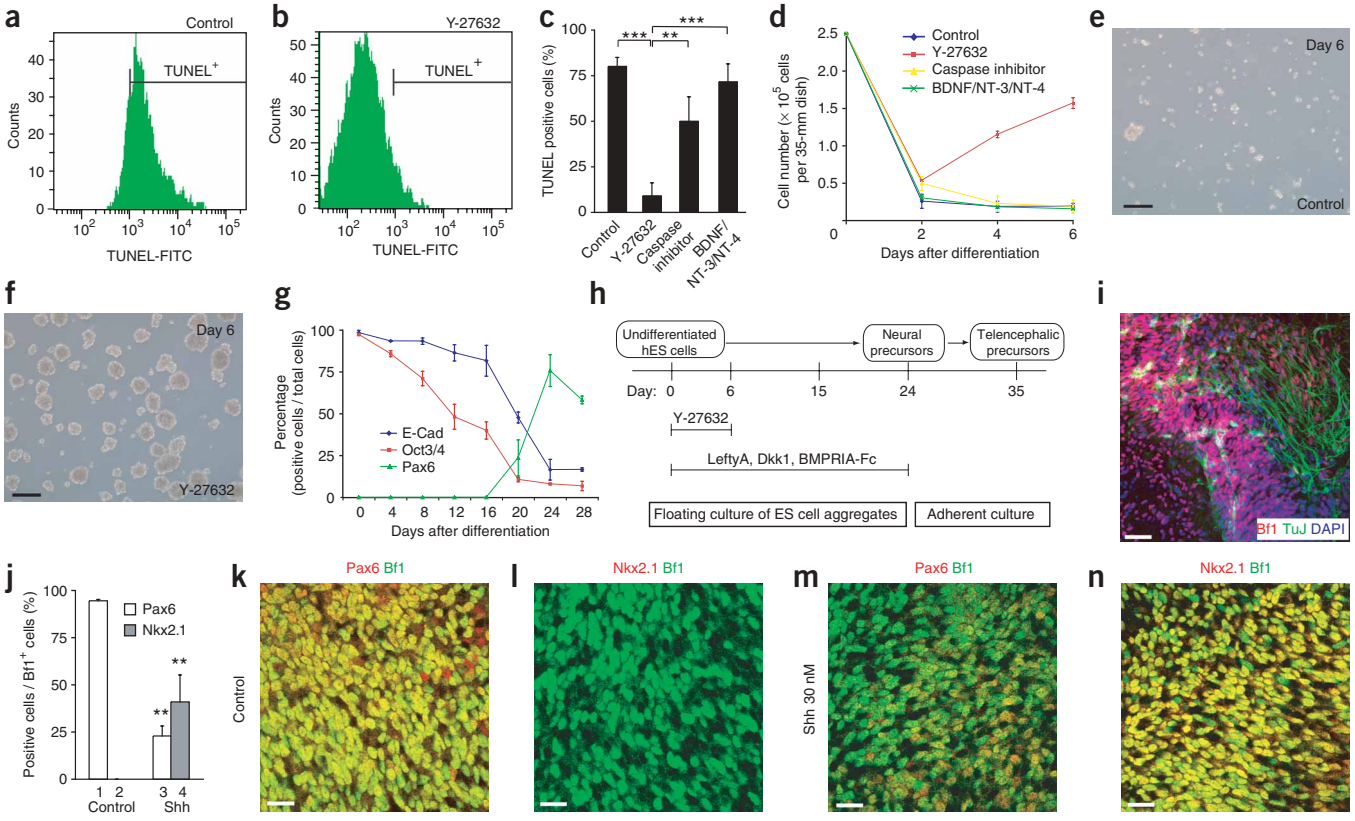

Figure 3 Y-27632 prevents apoptosis and promotes survival of dissociated hES cells (KhES-1) in suspension culture. (**a**–**c**) TUNEL assay. Dissociated hES cells were cultured in suspension for 2 d in the absence (**a**) or presence (**b**) of 10 μM Y-27632. TUNEL$^+$ cells were analyzed by FACS. (**c**) Effects of Y-27632, Caspase inhibitor I (Z-VAD-fmk) and a neurotrophin cocktail (BDNF, NT-3 and NT-4) on percentages of apoptotic cells (**, $P < 0.01$; ***, $P < 0.001$, between each pair; $n = 3$ studies). (**d**–**f**) Supportive effects of Y-27632 on hES cell survival/growth in suspension culture. (**d**) Cell numbers 2, 4 and 6 d after culturing 2×10^5 dissociated hES cells in 35-mm plates ($n = 3$). On day 6, efficient formation of cell aggregates was observed with the Y-27632-treated ES cells (**f**), but not with the control cells (**e**). Bars, 300 μm. (**g**) Time-course analysis of the expression of Pax6 (green), Oct3/4 (red) and E-cadherin (blue) in SFEB-h-cultured hES cells. (**h**) Schematic of the culture protocol. (**i**) Immunostaining of hES cell-derived neural cells induced in SFEB-h culture. Bf1 (red), TuJ1 (green), DAPI (blue). Bar, 50 μm. Note that some Bf1$^+$ cells were positive for the neuronal marker TuJ1. (**j**–**n**) Immunostaining analysis of SFEB-h-induced neural cells. Bars, 25 μm. (**j**) Percentages of Bf1$^+$ telencephalic cells that were positive for Pax6 and Nkx2.1 (**, $P < 0.01$ versus control; $n = 3$). Immunocytochemistry of SFEB-h-induced neural cells cultured without (**k**,**l**) or with (**m**,**n**) Shh (30 nM). Bf1 (green; **k**–**n**), Pax6 (red; **k**,**m**) and Nkx2.1 (red; **l**,**n**).

would be more useful if it were compatible with differentiation culture. SFEB (serum-free culture of embryoid body-like aggregates)[3] is a serum-free suspension culture method for mES cells, involving dissociation and reaggregation, that efficiently induces neural differentiation. We next applied Y-27632 to SFEB culture of hES cells (**Fig. 3e**,**f**).

Dissociated hES cells were cultured until day 24 in suspension (2×10^5 cells/ml, 4 ml/60-mm dish) using a serum-free differentiation medium to which Y-27632 was added for the first 6 d. To enhance neural differentiation, we added three kinds of inhibitors of anti-neuralizing signals (Wnt, Nodal and BMP)[3,27] to the culture medium (Dkk1, LeftyA and BMPRIA-Fc, respectively; **Supplementary Fig. 2a** online) during days 0–24. Under these conditions, the hES cells grew well as floating aggregates and the majority of cells expressed neural markers[9] such as Pax6 (**Fig. 3g** and **Supplementary Fig. 2b**) and Nestin (**Supplementary Fig. 2c**) on day 24. In contrast, the expression of the undifferentiated-state markers Oct3/4 and E-cadherin gradually decreased during this culture period. Thus, dissociated hES cells treated with Y-27632 efficiently differentiate into neural cells when cultured under the modified SFEB culture conditions (SFEB-h culture, hereafter).

A unique characteristic of SFEB culture observed with mES cells is the efficient differentiation of telencephalic cells (30–40% of total cells)[3,12]. Bf1 (Foxg1) is an early bona-fide telencephalic marker and required for telencephalic development in the embryo[3,28,29]. Therefore, we next tested whether SFEB-h culture induced the differentiation of Bf1$^+$ cells from hES cells as SFEB culture does for mES cells. On day 24 of SFEB-h culture, hES cell aggregates were plated onto dishes coated with poly-D-lysine, laminin and fibronectin and cultured until day 35 (**Fig. 3h**). On day 35, hES cell-derived neural cells frequently expressed Bf1 (32.9 ± 2.6%, **Fig. 3i**), indicating their telencephalic differentiation.

The early embryonic telencephalon is subdivided into the pallial (Bf1$^+$/Pax6$^+$ cortical anlage) and basal (e.g., Nkx2.1$^+$) regions. The majority of Bf1$^+$ cells derived from Y-27632-treated hES cells coexpressed Pax6 (95.8 ± 0.7%; **Fig. 3j**,**k**), whereas Nkx2.1 was detected in only a few Bf1$^+$ cells (1% or less; **Fig. 3j**,**l**). Consistent with a previous report on mES cells[3], Shh treatment (30 nM; days 15–35) decreased the Pax6$^+$ population (23.2 ± 5.3%; **Fig. 3j**,**m**) and increased the proportion of Nkx2.1$^+$ cells among the Bf1$^+$ cells (41.5 ± 14.5%; **Fig. 3j**,**n**). Thus, using Y-27632 treatment, hES cells, just like mES cells, generate cells with pallial and basal telencephalic characteristics in this hSFEB culture.

In summary, the use of the ROCK inhibitor Y-27632 enables hES cells to grow and differentiate as mES cells do under unfavorable

culture conditions such as dissociation and suspension. The improvement in cloning efficiency conferred by Y-27632 may be particularly advantageous for isolating relatively rare clones (e.g., those for homologous recombination) and also for recloning hES cells to obtain a uniform cell quality. So far, we have not observed obvious adverse effects of continuous Y-27632 treatment on pluripotency (**Fig. 1l–n** and **Supplementary Fig. 1a–g**) or chromosomal stability (**Supplementary Fig. 1l**) in maintenance culture even after a substantial number of passages (although more extensive future studies would be beneficial). ROCK inhibitors such as Y-27632 and Fasudil, are already used clinically in cardiovascular therapies[2], suggesting that they are safe for use with hES cells.

The mechanism of Y-27632's action in blocking apoptosis is an intriguing question that awaits future investigation. Given that Y-27632 and another ROCK inhibitor (Fasudil), but not inhibitors of other kinases, had similar protective effects at commonly used concentrations[1,2] (**Supplementary Fig. 1j**), ROCK is a reasonable candidate target of the antiapoptotic activity of Y-27632. The upstream activation mechanism of ROCK is complex and involves both Rho-independent and dependent pathways (e.g., ROCK is also activated by Caspase-3 cleavage[19]). Future studies of the mode of action of Y-27632 may shed new light on why hES cells, unlike mES cells, are so prone to die upon dissociation.

METHODS

Maintenance culture of hES cells. The hES cells (KhES-1, 2 and 3) were used following the hES cell research guidelines of the Japanese government. Undifferentiated hES cells were maintained as described previously[12,30]. Cells were cultured on a feeder layer of MEF cells (Invitrogen; inactivated with 10 μg/ml mitomycin C and seeded at 1.5×10^5 per 10-cm plate) in DMEM/F12 (Sigma) supplemented with 20% (vol/vol) Knockout Serum Replacement (KSR, Invitrogen), 2 mM glutamine, 0.1 mM nonessential amino acids (Invitrogen), 5 ng/ml recombinant human bFGF (Upstate) and 0.1 mM 2-mercaptoethanol (2-ME) under 2% CO_2. For passaging, hES cell colonies were detached and recovered *en bloc* from the feeder layer by treating them with 0.25% trypsin and 0.1 mg/ml collagenase IV in PBS containing 20% KSR and 1 mM $CaCl_2$ at 37 °C for 7 min, followed by tapping the cultures and flushing them with a pipette. Two volumes of culture medium were added, and the detached ES cell clumps were broken into smaller pieces (10–20 cells) by gently pipetting them several times. The passages were done at a 1:4 split ratio. For storage, the ES cell colonies were recovered *en bloc* (without further dissociation) from a 6-cm culture dish, suspended in 1 ml of ice-cold culture medium supplemented with 2 M DMSO, 1 M acetamide and 3 M polypropylene glycol, and quickly frozen in a 2-ml cryogenic tube (BD Labware) by directly submerging the tube in liquid N_2. The day on which ES cells were seeded to start dissociation culture was defined as day 0.

ROCK inhibitor treatment and transfection. First, hES cells were detached from the feeder layer and partially dissociated as described for the maintenance passage procedure. Next, contaminating MEF cells were removed by incubating the cell suspension on a gelatin-coated plate at 37 °C for 2 h in the maintenance culture medium (in this procedure, MEF cells adhere to the dish bottom, but the ES cells do not[12]). The hES cell clumps were recovered from the suspension by centrifugation, washed with PBS, incubated in 0.25% (wt/vol) trypsin-EDTA (Invitrogen) at 37 °C for 5 min, dissociated into single cells by pipetting and then passed through Cell Strainer (BD Falcon). The dissociated cells were seeded onto an MEF feeder layer in flat-bottomed 96-well plates at low density (500 cells/well, 0.32 cm^2; three wells for one condition) or at clonal density (one cell/well).

For ROCK inhibitor treatment, Y-27632 (Calbiochem; water soluble) was added to culture medium at 10 μM 1 h before detaching the cells from the feeder layer and also upon seeding the cells onto a new MEF layer. A single half-day treatment of Y-27632 was sufficient for enhanced survival of dissociated hES cells in low-density adhesion culture on MEF cells. In contrast, suspension culture of hES cells required Y-27632 treatment for the first 4–6 d to obtain the maximal effect (Y-27632 treatment for the first 2 d only is less effective in promoting cell survival on day 6 and thereafter). Another ROCK inhibitor, Fasudil (HA1077; Calbiochem), also promoted colony formation by dissociated hES cells in low-density culture to a similar extent as Y-27632. We mainly used Y-27632 in this study because of its high specificity to ROCK[1]. Although the KhES-1 line was mainly used in this study, Y-27632 treatment also had similar promoting effects on the survival of three independent hES cell lines (KhES-1, 2 and 3)[30] in both maintenance and differentiation cultures with dissociated cells. The cloning efficiencies of KhES-1 are shown in **Figure 1**; those of KhES-2 were 18.8 ± 4.4% and 0.4 ± 0.2% and those of KhES-3 were 20.4 ± 2.6% and 4.1 ± 1.5%, with and without Y-27632, respectively. With respect to the cost, the use of the ROCK inhibitors at the indicated concentrations is comparable to or less expensive than the use of LIF at the working concentration for mES cell culture (1,000 U/ml). Furthermore, Y-27632 is useful in even wider applications including the feeder-dependent differentiation[12] of dissociated hES cells (unpublished observations).

Transfection of hES cells with a drug-selectable plasmid, *pCAGGS-Venus-Hygro*, (*Venus-GFP* and *Hygro* are driven by the *CAG* and *GK* promoters, respectively) was performed with the ExGen 500 system (Fermentas) as described[17] (Lipofectamine 2000, Invitrogen, also worked). The following day, after pretreatment with Y-27632, cells were dissociated and replated onto MEF cells (10-cm dish) at various densities as described above. One day after replating, hygromycin (final concentration 50 μg/ml) was added to the culture medium. Culture on Matrigel substrate (BD Biosciences) in MEF-conditioned medium was done as described previously[25].

TUNEL assay and FACS analyses. Dissociated hES cells (2.5×10^5 cells) prepared as above were seeded onto a 35-mm nonadhesive bacterial-grade dish, and cultured in DMEM/F12 supplemented with 20% KSR, 2 mM glutamine, 0.1 mM nonessential amino acids and 0.1 mM 2-ME. After 2 d of culture, the cells were dissociated to single cells by trypsin digestion, fixed in 4% (wt/vol) paraformaldehyde, and subjected to TUNEL assay using the MEBSTAIN Apoptosis Kit Direct (MBL) according to the manufacturer's instructions. Flow cytometrical analysis of $TUNEL^+$ cells was performed using BD fluorescence-activated cell sorting (FACS)Aria as described[3]. Z-VAD-fmk and the neurotrophins were purchased from Calbiochem and R&D, respectively. Flow-cytometrical profiling of DNA content with7-AAD (7-amino-actinomycin D; BD Bioscience) staining was analyzed using BD FACSCanto and FACSDiva. BrdU uptake (1 h) was analyzed using the FITC BrdU Flow Kit (BD Pharmingen). Before the single-cell dissociation for flow-cytometric analysis, hES cell colonies were detached *en bloc* from feeder cells, and contaminating MEF cells were removed by incubating on a plastic dish in culture medium for 2 h.

SFEB culture with Y-27632. SFEB culture was carried out as described previously[3] with a minor modification. Dissociated hES cells (prepared as above; 2×10^5 cells/ml) were seeded onto a nonadhesive bacterial-grade dish and cultured in DMEM/F12 supplemented with 20% KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-ME, 100 U/ml penicillin and 100 μg/ml streptomycin. Y-27632 was added to the culture medium at 10 μM for the first 6 d (in addition to the 1-h pretreatment) in this study, although Y-27632 did not interfere with neural differentiation even when it was added continuously during days 0–24 (data not shown). The medium was changed every other day. Dkk1 (500 ng/ml), LeftyA (5 μg/ml), and soluble BMPRIA-Fc (1.5 μg/ml; all from R&D) were added to the culture from day 0 to day 24. For long-term culture to induce telencephalic differentiation, floating SFEB aggregates were replated on day 25 onto a culture slide (8-well CultureSlide, BD) that was precoated with poly-D-lysine, laminin and fibronectin, and cultured until day 35 in Neurobasal + B27 supplement (without vitamin A) + 2 mM L-glutamine. For ventralization experiments, Shh (30 nM, R&D) was added during days 24–35. Medium was changed every third day for the first 6 d and every other day for the rest of the differentiation culture. Although neural differentiation ($Nestin^+$, $Pax6^+$) could be also induced by suspension culture of large hES cell clumps (detached *en bloc* from the feeder layer) in the same differentiation culture medium without Y-27632, these aggregates survived only at a low frequency (<3%) and, in these few aggregates, the number of $Bf1^+$ cells was generally low (<10% of surviving cells on day 35).

Statistical analysis. The statistical significance (*P* values) in mean values of two-sample comparison was determined with Student's *t*-test (Microsoft

Excel). The statistical significance in the comparison of multiple sample sets versus control (**Supplementary Fig. 2a**) was analyzed with Dunnett's multiple comparisons test using the Instat program (GraphPad). The statistical significance in mean values among multiple sample groups was examined with Bonferroni's (**Fig. 3c**) and Tukey-Kramer's (**Supplementary Fig. 1k**) multiple comparisons test after one-way ANOVA test, or with two-way ANOVA and Bonferroni's *post-hoc* test (**Figs. 2g** and **3d**) using the Prism 4 program (GraphPad). Values shown on graphs represent the mean ± s.d.

Alkaline phosphatase staining, immunostaining and chromosomal analysis. Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase Kit (Sigma). Immunostaining was performed as described previously[3]. Briefly, cells were fixed with 4% paraformaldehyde at 4 °C for 15 min, and the staining was visualized using secondary antibodies conjugated with FITC, cy3 or cy5. Floating aggregates were fixed in 4% paraformaldehyde, embedded in OCT, and sectioned at 10 μm on a cryostat. For immunostaining cells in large colonies in adhesion culture, confocal microscopy (Zeiss LSM 510) was used to observe the cells inside the colony with good resolution. The total number of cells was counted by staining the nuclei with DAPI. For statistical analyses, 50–100 colonies were examined in each experiment. Experiments were performed at least three times. Commercial antibodies used for immunostaining were as follows: BD Bioscience Pharmingen (Oct-3/mouse monoclonal/611202/1:200), Covance (human Nestin/rabbit polyclonal/PRB-570C/1:1,000, neuronal class III β-tubulin/rabbit polyclonal/PRB-435P/1:600, neuronal class III β-tubulin/mouse monoclonal/MMS-435P/1:300), R&D systems (Pax6/mouse monoclonal/MAB1260/1:500; Hnf3β/goat polyclonal/AF2400; Brachyury/goat polyclonal/AF2085), DAKO (SMA/mouse monoclonal/M0851), Zymed (Nkx2.1/ mouse monoclonal /18-0221/1:100), Developmental Studies Hybridoma Bank (SSEA-4/mouse monoclonal/MC-813-70/1:200) and TAKARA (E-cadherin/rat monoclonal/M108/1:50). The staining for Bf1 was performed using rabbit-raised antibodies as described previously[3]. Chromosomal G-band analysis was done as described previously[30] by pretreating hES cells with 0.06 μg/ml colecemid for 4 h and incubating them in 0.075 M KCl for 10 min.

Teratoma formation, *in vitro* mesoendodermal induction and RT-PCR. Teratoma formation experiments were done by injecting ~5×10^5 hES cells (maintained in the presence of Y-27632 and detached from the feeder layer as cell clumps in the presence of Y-27632) subcapsularly into the testes of 7-week-old SCID mice using a Hamilton syringe. For *in vitro* differentiation into mesodermal progenitors (**Fig. 1g,i**), hES cells were plated on a plastic dish coated with collagen IV, and cultured in DMEM/F12 supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids and 0.1 mM 2-ME under 2% CO_2 for 6 d. On day 6, 15–25% of the cells were positive for Brachyury staining. For endodermal progenitors (**Fig. 1h,k**), hES cells were cultured with 1% FCS and 100 ng/ml activin (the rest of the ingredients were the same as in **Fig. 1g**) on a collagen IV-coated dish for 6 d. Hnf3β expression was found in ~10% of cells cultured with 10% FCS on day 6. RT-PCR was performed using the following primers: glyceldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward, 5′-GAGTCAACGGATTTGGTCGT-3′; reverse, 5-TGTGGTCATGAGTCCTTCCA-3′; 513-bp product), *T* (brachyury) (forward, 5′-GCAAAAGCTTTCCTTGATGC-3′; reverse, 5′-ATGAGGATTTGCAGGTG GAC-3′; 144-bp product), *Sox17* (forward, 5′-CGCACGGAATTTGAACAG TA-3′; reverse, 5′-CAGTAATATACCGCGGAGCTG-3′; 149-bp product), and *Meox1* (forward, 5′-TGAAGTGGAAGCGTGTGAAG-3′; reverse, 5′-GGTAG GGGGCTCAGTCCTTA-3′; 139-bp product).

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

K.W. and Y.S. designed the project; Y.S. wrote the report; all authors performed experiments.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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