Inhibition of Caspase-mediated Anoikis Is Critical for Basic Fibroblast Growth Factor-sustained Culture of Human Pluripotent Stem Cells

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Apoptosis and proliferation are two dynamically and tightly regulated processes that together maintain the homeostasis of renewable tissues. Anoikis is a subtype of apoptosis induced by detachment of adherent cells from the extracellular matrix. By using the defined mTeSR1 medium and collecting freshly detached cells, we found here that human pluripotent stem (PS) cells including embryonic stem (ES) cells and induced pluripotent stem cells are subject to constant anoikis in culture, which is escalated in the absence of basic fibroblast growth factor (bFGF). Withdrawal of bFGF also promotes apoptosis and differentiation of the remaining adherent cells without affecting their cell cycle progression. Insulin-like growth factor 2 (IGF2) has previously been reported to act downstream of FGF signaling to support self-renewal of human ES cells. However, we found that IGF2 cannot substitute bFGF in the TeSR1-supported culture, although endogenous IGF signaling is required to sustain self-renewal of human ES cells. On the other hand, all of the bFGF withdrawal effects observed here can be markedly prevented by the caspase inhibitor z-VAD-FMK. We further demonstrated that the bFGF-repressed anoikis is dependent on activation of ERK and AKT and associated with inhibition of Bcl-2-interacting mediator of cell death and the caspase-ROCK1-myosin signaling. Anoikis is independent of pre-detachment apoptosis and differentiation of the cells. Because previous studies of human PS cells have been focused on attached cells, our findings revealed a neglected role of bFGF in sustaining self-renewal of human PS cells: preventing them from anoikis via inhibition of caspase activation.

Fibroblast growth factor (FGF)2 signaling plays important roles in the regulation of early embryogenesis as well as in embryonic stem (ES) cell self-renewal and differentiation. It supports the self-renewal of human ES cells but is required for differentiation of mouse ES cells into a number of lineages (1, 2). Basic FGF (bFGF or FGF2), at 4 ng/ml, was first used to supplement the medium used to culture human ES cells on mouse embryonic fibroblast feeder cells (3) and then was used to supplement medium conditioned on mouse embryonic fibroblasts for the feeder-free culture of human ES cells on Matrigel (BD Biosciences, San Jose, CA) (4). We have previously found that high dose (40 ng/ml) bFGF can synergize with Noggin, an antagonist of bone morphogenetic proteins, to maintain human ES cell culture without the need for feeders or feeder-conditioned medium (5). Bone morphogenetic proteins belong to the transforming growth factor β (TGFβ) superfamily and can induce human ES cell differentiation to trophoblast (6) or primitive endoderm (7), depending on the culture contexts. Noggin is no longer necessary when the bFGF concentration is increased to 100 ng/ml to compensate for the degradation of bFGF in medium (8). FGF signaling also works concertedly with TGFβ signaling to inhibit bone morphogenetic protein signaling (9) and synergizes with TGFβ and WNT signaling to support human ES cell culture (1, 2). The defined medium TeSR1 was formulated as serum-free, animal-free medium that supports feeder-free culture of human ES cells, which contains bFGF (100 ng/ml), TGFβ1, and lithium chloride (an activator of WNT signaling) (10). It was later commercialized as mTeSR1 with bovine serum albumin to replace human serum albumin (11) (Stem Cell Technologies, Inc., Vancouver, Canada). Other defined media have included bFGF, as well, to support human ES cell culture (12–14). bFGF-supplemented media have also been used to derive and culture human induced pluripotent stem (iPS) cells (15, 16).

Extensive studies have been carried out to explore the mechanism whereby FGF signaling acts on human ES cells. Many FGF receptors and ligands are expressed in human ES cells (17, 18) with FGFFR1 (19) and FGF4 (20) being the most abundant species. Expression of endogenous bFGF decreases during human ES cell differentiation (21). Inhibition of FGF receptors with SU5402 decreases phosphorylation/activation of ERK in human ES cells and induces differentiation (22), whereas exogenous bFGF increases phosphorylation/activation of ERK in the cells (18, 19). MEK/ERK cascade cooperates with phosphatidylinositol 3-kinase/AKT cascade (also downstream of FGF receptor signaling) to maintain

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self-renewal of the cells, and inhibition of MEK/ERK activity causes a loss of the self-renewal capacity of human ES cells (23). Moreover, it has been shown that bFGF may also work by stimulating differentiated human ES cells to produce IGF2, which then activates IGF receptors on adjacent undifferentiated ES cells to sustain their self-renewal, the so-called paracrine mechanism (24).

We have previously found that withdrawal of bFGF from TeSR1 medium causes a rapid decline of human ES cell proliferation and a slow reduction in the expression of pluripotency genes (9). However, the detailed mechanism by which bFGF maintains self-renewal of human ES cells and promotes their proliferation remains elusive. We hypothesized that bFGF may also act on human ES cells through another mechanism irrelevant to regulation of the pluripotency genes. With this in mind, we studied cell cycle progression and apoptosis including cell death that is caused by the detachment (anoikis) of human ES and iPS cells. Our results suggest that these human pluripotent stem (PS) cells are subject to constant anoikis in culture, which is inhibited by bFGF via repression of caspase activities, and bFGF withdrawal-induced differentiation of the remaining adherent cells is also mediated by caspase.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Human PS cells including H14 (25) and CT2 ES cell lines and HDFa-YK26 (named YK26 in this study) and IMR90-TZ1 (named TZ1 in this study) iPS cell lines were used in this study. Human dermal fibroblasts, adult (HDFa), were purchased from Invitrogen for YK26 derivation, and IMR90 fibroblasts were purchased from American Type Culture Collection (Manassas, VA) for TZ1 derivation. CT2 (26), YK26, and TZ1 were derived in this laboratory as previously described (15, 16, 25). The cells were routinely cultured on plates coated with Matrigel (BD Biosciences, San Jose, CA) in human PS medium, i.e. Dulbecco’s modified Eagle’s medium/F-12 containing 20% KnockOut serum replacer, 0.1 mM nonessential amino acids, 1 mM L-glutamine (all from Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich) that was conditioned on mouse embryonic fibroblasts and then supplemented with 4 ng/ml bFGF (Millipore, Billerica, MA) (4). The cells were adapted to mTeSR1 (10) (Stem Cell Technologies, Vancouver, Canada) for at least one passage prior to the experiments. For treatment with bFGF or IGF2, the cells were cultured in customized mTeSR1 (Stem Cell Technologies) containing 0, 10, or 100 ng/ml bFGF (T1/F0, T1/F10, or T1) or 30 ng/ml of IGF2 (Upstate Biotechnology, Lake Placid, NY) for various days with a daily medium change. In some experiments, chemical inhibitors were used, including z-VAD-FMK (Biomol International, Plymouth Meeting, PA) to inhibit pan-caspase activities, Y27632 (Wako Pure Chemical Industries, Tokyo, Japan) to inhibit RhoA-associated kinase 1 (ROCK1), PD173074 (Stemgent, San Diego, CA), and SU5402 (Pharmacia & Upjohn Co., Kalamazoo, MI) to inhibit FGF receptors, U0126 (LC Laboratories, Woburn, MA) to inhibit ERK, and LY294002 (also LC Laboratories) to inhibit AKT. The anti-IGF1R antibody clone 1H7 (BD Bioscience, San Jose, CA) was used to inhibit binding of IGF1 and IGF2 to IGF1 receptor (24).

Cell Proliferation and Viability Assays—After removal of consumed medium, human PS cells were washed with Dulbecco’s modified Eagle’s medium/F-12 twice before the addition of fresh medium. Freshly detached and/or remaining attached cells were collected at various times post-medium change for subsequent analyses. The attached cells were harvested by using Accutase (Innovative Cell Technologies, San Diego, CA). The cells were stained with ViaCount (Millipore) followed by proliferation and viability assays on the Guava EasyCyte flow cytometry system. The samples were prepared in triplicate, the cells were counted twice, and the data were analyzed with the Guava ViaCount software. Floating cell ratio (%) = floating cell number/ (floating cell number + attached cell number) × 100. The percentage data from replicates were analyzed by using Student t test. At least three independent experiments were analyzed. The data were expressed as the means ± standard deviation.

Flow Cytometry—Human PS cells were processed for flow cytometry (FACS) analysis to detect cells that were positive for OCT4 and active caspase-3 (aCasp3). The cells were dissociated with Accutase, fixed, and permeabilized with Fix/Perm kits before staining with anti-OCT4 antibody conjugated with allogloccyanin and anti-aCasp3 antibody conjugated with phycoerythrin (all from BD Biosciences). Apoptotic cells and dead cells were determined by live staining of the cells with annexin V-phycocerythrin and 7-aminoactinomycin D (7-AAD) (BD Bioscience), respectively. Analysis of cell cycle progression (displayed as supplemental data) was carried out via 5-bromo2'-deoxy-uridine (BrdUrd) and 7-AAD staining as follows. The live cells were incubated with 10 μM BrdUrd for 2 h. The cells were then fixed, permeabilized, and stained with anti-BrdUrd antibody conjugated with fluorescein isothiocyanate and 7-AAD following the manufacturer’s instructions (BD Bioscience). The data were collected on FACS LSR II flow cytometer using FACS Diva software (BD Biosciences). Post-acquisition analysis was performed with FlowJo software (Treestar, Ashland, OR). Data analysis was performed as above. For some experiments, representative scatter plots were displayed.

Fluorescence Imaging—To detect filamentous actin (F-Ac- tin), the cells were fixed with prewarmed 4% paraformaldehyde in phosphate-buffered saline for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. After washing, the cells were stained with phalloidin-fluorescein isothiocyanate (Sigma). aCasp3 was detected by the Image-iT-LIVE-RED-Caspase-3 detection kit (Invitrogen) following the manufacturer’s protocol. Diamidino-2-phenylindole was used to counterstain the nuclei. The phase and fluorescent images were taken under Zeiss Apotome microscope and analyzed with the Zeiss Axio- vision 4.7 software.

Western Blotting—The cells were washed with cold phosphate-buffered saline before harvest, lysed for 15 min on ice in a cell lysis buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and protease inhibitor mixture (Roche Applied Science). Total cell lysates
were resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked in 5% nonfat dry milk in TBS-T (0.1% Tween 20 in TBS containing 0.2 M Tris base and 1.5 M sodium chloride) and followed by blotting with antibodies against ROCK1, myosin light chain phosphorylated at cysteine 20 (p-MLC(S20)); NANOG and β-actin (Abcam, Cambridge, MA); ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA); AKT, phospho-AKT(S473), and phospho-ERK1/2 (Cell Signaling Tech. Danvers, MA); and BCL-2 and BIM (BD Bioscience). After washing with TBS-T three times, the membranes were blotted with corresponding secondary antibodies conjugated with horseradish peroxidase. The membranes were then developed in the ECL Buffer (Millipore), and the blots were visualized by using the Fujifilm LAS-3000 Imager (Stamford, CT).

RESULTS

Decline of Human PS Cell Proliferation upon bFGF Withdrawal Is Mainly Caused by Anoikis—To elucidate the mechanism by which bFGF sustains human PS cell culture, we used multiple human PS cell lines including H14 and CT2 ES cell lines and YK26 and TZ1 iPS cell lines in this study. First we confirmed that bFGF is required for proliferation of these cells by using the customized mTeSR1 media T1/F0, T1/F10, and T1. All four PS cell lines were seeded at an equivalent number (3.5 × 10^5) cells/well at day 0 in mTeSR1 (T1) medium, 12 h later, the consumed medium was replaced with customized mTeSR1 containing 0, 10, or 100 ng/ml bFGF (T1/F0, T1/F10, or T1). Attached cell number was counted at days 3 and 6 following a thorough wash and averaged from triplicates. B, bright field image of CT2 cells cultured T1/F0, T1/F10, or T1 for 3 days. C, cell cultures prepared as in A. Both floating and attached cells were collected at 24 h post-medium change on day 3 to calculate floating cell ratio. *, p < 0.05 versus T1/F0. D, live (7-AAD<sup>−</sup> and annexin V<sup>−</sup>) cell ratio was determined among floating cells collected, at various times post-medium change, from CT2 cells cultured in T1 or T1/F0. bFGF Inhibits Anoikis of Human ES and iPS Cells

FIGURE 1. Decline of human PS cell proliferation upon bFGF withdrawal is mainly caused by anoikis. A, human ES (CT2 and H14) or iPS (YK26 and TZ1) cells were plated at 3.5 × 10^5 cells/well at day 0 in mTeSR1 (T1) medium, 12 h later, the consumed medium was replaced with customized mTeSR1 containing 0, 10, or 100 ng/ml bFGF (T1/F0, T1/F10, or T1). Attached cell number was counted at days 3 and 6 following a thorough wash and averaged from triplicates. B, bright field image of CT2 cells cultured T1/F0, T1/F10, or T1 for 3 days. C, cell cultures prepared as in A. Both floating and attached cells were collected at 24 h post-medium change on day 3 to calculate floating cell ratio. *, p < 0.05 versus T1/F0. D, live (7-AAD<sup>−</sup> and annexin V<sup>−</sup>) cell ratio was determined among floating cells collected, at various times post-medium change, from CT2 cells cultured in T1 or T1/F0.
Through time lapse photography, we also observed rapid cell detachment in CT2 cell culture even in T1 (supplemental video clip).

bFGF has been shown to prevent apoptosis and regulate cell adhesion in many other cell types (27, 28). For adherent cells, apoptosis can happen as anoikis, which is induced by cell detachment from the extracellular matrix (29, 30). Thus, it is possible that a high level of anoikis may account for the decline in cell proliferation when the PS cells are cultured in the absence of bFGF. To test this, both attached and floating cells in the PS cell cultures were collected and counted 24 h post-medium change on day 3 in the test media. The floating cell ratio reached 40–60% in T1/F0 but was only approximately half of that in T1/F10 or T1 (Fig. 1C). We then performed live cell staining on the detached cells with annexin V (for apoptotic cells) and 7-AAD (for dead cells) followed by FACS analysis. Surprisingly we found that more than 75% of the cells remained alive (7-AAD− and annexin V−) at 3 h post-medium change regardless of whether they were cultured in T1 or T1/F0, but the live cell ratio dropped rapidly in both groups when the cell collection was delayed (Fig. 1D). These data strongly suggest that human PS cells can detach from the extracellular matrix before apoptosis, and the cell loss upon bFGF withdrawal is mainly caused by anoikis, whereas the subsequent apoptosis induced by detachment is bFGF-independent.

It has been known that anoikis-committed cells often show disruption of cytoskeletal F-Actin and activation of cortical F-Actin (31). Indeed, after 3 days in the test media, we found that ~88% of T1-cultured cells had F-Actin stress fiber across the cells (a proattachment sign), and this ratio dropped to 15% for the T1/F0-cultured cells; in contrast, cortical ring-like F-Actin structures (a proanoikis sign) appeared more in the cells cultured in T1/F0 than T1 (Fig. 2, A–C). This morphological evidence indicates that human PS cells cultured in T1/F0 are more poised to detach than in T1. Because the initiation and execution of anoikis depends on the activation of caspases including caspase-3, which is also an early apoptosis marker (32), we analyzed aCasp3 in freshly detached OCT4− cells at various times post-medium change on day 3 in the test media. The aCasp3 ratio was ~37 and ~53% for culture in T1 and T1/F0, respectively, at 1.5 h post-medium change, and increased to ~50 and ~80% for T1 and T1/F0, respectively, at 3 h (Fig. 2D). In addition, we also noticed that, regardless of whether they were cultured in T1 or T1/F0, ~40% of the detached cells remained

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bFGF Inhibits Anoikis of Human ES and iPS Cells

A

FIGURE 3. bFGF inhibits apoptosis and caspase-3 activation of attached human PS cells. A, CT2 cells were prepared as for Fig. 1A. Both attached and floating cells were harvested separately or together on day 3 at 24 h post-medium change for determination of live (7-AAD- and annexin V-) cell ratio via FACS. B, a bar chart is shown for live cell ratio of both the attached and floating cells collected together from CT2 cultures as in A. *, p < 0.01 versus T1/F0. C, H14 cells were cultured in T1 or T1/F0 and fixed on day 3, followed by staining for aCasp3 (red) and counterstaining with diamidino-2-phenylindole (DAPI, blue). D, CT2 cells were prepared as for A. On day 3, the attached cells were collected and fixed for FACS detection of aCasp3- cell ratio, and the data were gated on OCT4+ cells. E, experiments as described for D were performed on all 4 lines of PS cells and shown in a bar chart. *, p < 0.05, versus T1/F0.

OCT4 positive at 1.5 h post-medium change, which quickly dropped to ∼10% at 3 h. Altogether, these data indicate that anoikis actively occurs in human PS cells culture, and bFGF can inhibit anoikis, thus reducing cell loss and promoting cell proliferation.

bFGF Sustains OCT4+ Cell Ratio of Human PS Cells without Affecting Cell Cycle Progression—It has been shown that undifferentiated ES cells have faster cell cycle progression than differentiated ones (33, 34), which was confirmed by us (supplemental Fig. S1A). As described above, bFGF can maintain the pluripotency of human ES cells (1, 2). Therefore, it is possible that the reduced proliferation of human PS cells may also result from differentiation of the cells upon bFGF withdrawal. This proved true because bFGF prevented the slow decline of OCT+ cell population in the four PS cell lines tested in this study (supplemental Fig. S2) as reported previously (9). It is noteworthy that the decrease in bFGF concentration from 100 to 10 ng/ml caused no remarkable changes of either the OCT4+ cell ratios of all the four cell lines (supplemental Fig. S2) or the total cell numbers of H14 and TZ1 (Fig. 1A). This suggests that bFGF at 10 ng/ml is sufficient to maintain the total and pluripotent cell numbers of human PS cells within the test time of 6 days. It has been known through current (supplemental Fig. S2) and previous (8–10) experiments that different degrees of OCT4- cell ratio reduction can be observed among various human PS cell lines cultured in the absence of bFGF. For example, the ratio remained above 90% for YK26 cells cultured in T1/F0 for 6 days (supplemental Fig. S2). However, like the other cell lines, YK26 still encountered severe cell loss upon bFGF withdrawal (Fig. 1A). Thus, differentiation of PS cells may contribute little to the decline of cell proliferation at least at the early time of bFGF withdrawal.

Because bFGF is a well known, potent mitogen on many other cell types (28), we then asked whether bFGF promotes human PS cell proliferation by accelerating cell cycle progression. CT2 cells were plated at equal number (3.5 × 10^5/well) in T1 medium. After overnight culture, the medium was replaced with T1/F0, T1/F10, or T1 and refreshed daily for up to 4 days. The cells were labeled with BrdUrd for 2 h on various days and fixed for intracellular staining with anti-OCT4 antibody for pluripotency assay, anti-BrdUrd antibody for DNA synthesis determination, and 7-AAD for DNA content measurement. OCT4+ cells were gated via FACS for analysis of cell cycle progression by analyzing the BrdUrd and 7-AAD staining profiles. The BrdUrd+ , BrdUrd+/7-AADlow+ , and BrdUrd+/7-AADhigh+ populations represented cells in the S, G2/M, and G1/M phases, respectively (supplemental Fig. S1B). No statistically significant differences in distribution of cell cycle phases were observed among the cells cultured in T1, T1/F10, or T1/F0 on each of the 4 days (supplemental Fig. S1C). Similar results were observed with other cell lines (data not shown) and in feeder-conditioned medium (34). This suggests that bFGF does not affect cell cycle progression of human PS cells.

bFGF Inhibits Apoptosis and Caspase-3 Activation of Attached Human PS Cells—To discriminate anoikis from regular apoptosis, we analyzed whether bFGF also inhibits apoptosis of attached human PS cells. CT2 cells were cultured in T1/F0, T1/F10, or T1 medium for 3 days before examining the live cell ratio of the attached cells or the mixture of both the attached and floating cells.
at 24 h post-medium change. The live cell ratio was increased by bFGF dose-dependently, which is more obvious with the mixture than with the attached cell fraction alone (Fig. 3, A and B). For example, compared with T1/F0, T1 increased the live cell ratio \( \sim 1.3\text{-fold} \) among the attached cells but \( \sim 2.2\text{-fold} \) among the mixture. This further suggests that anoikis is escalated when bFGF concentration declines, because the increased number of detached cells in the absence of bFGF severely reduced the live cell ratio of the mixture.

Immunostaining shows that the early apoptosis marker, aCasp3, was detected less in CT2 cells cultured in T1 than T1/F0 (Fig. 3C). This was confirmed by FACS analysis for aCasp3+ cell ratio of OCT4+ cells gated from attached CT2 (Fig. 3D) as well as H14, TZ1, and YK26 cells cultured in T1, T1/F10, or T1/F0 (Fig. 3E). These data suggest that bFGF not only prevents detachment of human PS cells but also protects the remaining attached cells from apoptosis. However, because the majority of the detached PS cells soon became aCasp3+ (Fig. 2D) and annexin V+ (Fig. 1D), whereas only 1–8% of the attached PS cells are aCasp3+ in the absence of bFGF (Fig. 3, C–E), this again implies that cell detachment occurs before apoptosis, and anoikis is the major cause of the cell loss upon bFGF withdrawal.

Caspase Inhibitor Markedly Rescues Human PS Cells from bFGF Withdrawal Effects—It has been known that activated caspases such as aCasp3 are required for both anoikis and apoptosis in other cell types (35, 36), and recently caspases have been shown to promote mouse ES cell differentiation by degrading Nanog (37). As described above, we observed elevated aCasp3+ cell ratio among human PS cells upon bFGF withdrawal (Fig. 3, C–E), which was accompanied by increased anoikis, predetachment apoptosis, and differentiation. To examine whether caspases are required for these bFGF withdrawal effects on human PS cells, we used the pan-caspase inhibitor z-VAD-FMK to antagonize these effects on human PS cells. As predicted, z-VAD-FMK treatment for 3 days remarkably lowered the aCasp3+ cell ratio of CT2 cells cultured in either T1 or T1/F0 (Fig. 4A). It also partially reversed the attached cell number reduced by bFGF withdrawal (Fig. 4B) and decreased the floating cell ratio (Fig. 4C).

Interestingly, z-VAD-FMK also markedly increased the attached cell number and reduced the floating cell ratio in the T1 cultures. The floating cell ratio dropped from \( \sim 26\% \) to \( \sim 7\% \) (Fig. 4C), and the attached cell number increased by \( \sim 50\% \) (Fig. 4B) for T1-cultured cells treated with 100 \( \mu \text{M} \) z-VAD-FMK. However, the treatment of the caspase inhibitor did not change the live cell ratio of the attached cells cultured in either T1/F0 or T1 (Fig. 4E). These data suggest that caspases initiate PS cell detachment (anoikis) before apoptosis takes place and also provide an additional line of evidence that anoikis rather than predetachment apoptosis is the major cause of the reduced cell proliferation upon bFGF withdrawal. Finally, consistent with the report on mouse ES cells (37), z-VAD-FMK also apparently prevented CT2 cell differentiation caused by bFGF withdrawal as shown by the increased OCT4+ cell ratio (which also contained reduced aCasp3+ cell ratio) (Fig. 4, D and F). This implicates that activation of caspases promotes differentiation of human PS cells as well as mouse ES cells.

IGF2 Cannot Substitute bFGF to Prevent Human PS Cell Anoikis—Bendall et al. (24) have reported that, in feeder-conditioned medium (containing serum replacement), bFGF can stimulate differentiated human ES cells to produce IGF2, which then support self-renewal of adjacent undifferentiated human ES cells; IGF2 can substitute bFGF to sustain prolonged culture of human ES cells in unconditioned medium. To test whether this is also true for the activities of bFGF in the defined T1 medium, we cultured CT2 cells for 3 days in T1/F0 supplemented with human recombinant IGF2 at 30 \( \mu \text{g/ml} \) as used by Bendall et al. To our surprise, IGF2 failed to prevent either the slow decline of OCT4+ cell ratio (Fig. 5A) or the drastic decrease of attached cell number (Fig. 5B), and neither did it prevent the increase of the floating cell ratio among the T1/F0-cultured CT2 cells (Fig. 5C). This suggests that IGF2 cannot substitute bFGF to support the culture of human ES cells in the defined medium.

To further analyze whether integral IGF signaling is required for sustaining human PS cell culture in T1, we blocked the signaling with IGF1 receptor blocking antibody 1H7 at 2 \( \mu \text{g/ml} \) as used by Bendall et al. (24). CT2 cells were cultured in T1 or T1/F0 with 1H7 or control IgG for 3 days. The blocking antibody affected neither the OCT4+ cell ratio (Fig. 5D) nor cell cycle progression (supplemental Fig. S3), but it slightly increased the aCasp3+ cell ratio (Fig. 5D). 1H7 also moderately reduced the attached cell number and increased the floating cell ratio, which was less severe than that caused by bFGF withdrawal; even in the presence of 1H7, bFGF still increased the attached cell number and reduced the floating cell ratio (Fig. 5, E and F). These data suggest that bFGF acts independently of IGF2; however endogenous IGF-sustained signaling is required for bFGF-supported human ES cell culture in the defined medium.

bFGF Effects Are Related to Activation of ERK and AKT, Inhibition of BIM and ROCK1–Myosin Signaling, and Degradation of NANOG—To analyze intracellular mechanisms for the bFGF activities observed in this study, we first tested whether bFGF acts specifically through FGF receptors. The addition of the FGF receptor inhibitor SU5402 to T1 medium increased aCasp3+ cell ratio (Fig. 6A), reduced attached cell number, and increased floating cell ratio among CT2 cells after 3 days of culture, although the anoikis was not as severe as that caused by bFGF withdrawal (Fig. 6B). Similar effects were observed with another FGF receptor inhibitor PD173074 (data not shown). The milder anoikis caused by the FGF receptor inhibitors may be due to incomplete blockage of FGF receptors by the chemical inhibitors.

bFGF has been reported to activate ERK and AKT in human ES cells to maintain self-renewal of the cells (18, 19, 23). Both ERK and AKT have been shown to inhibit apoptosis in other cell types (38, 39). We also found that both bFGF withdrawal and SU5402 treatment reduced phosphorylation/activation of ERK and AKT (Fig. 6C). We also found that SU5402 treatment or bFGF withdrawal could increase the level of BIM but not BCL-2. BIM is well known for its role to induce caspase activation and apoptosis (40). To further
examine the role of ERK and AKT in bFGF signaling, U0126 and LY294002 were used to inhibit ERK and AKT activity, respectively. As shown in Fig. 6D, U0126 and LY294002 significantly decreased the attached cell number and increased the floating cell ratio among CT2 cells treated for 3 days, although the effects were less severe compared with bFGF withdrawal. U0126 and LY294002 also slightly reduced OCT4+/cell ratio and increased aCasp3+ cell ratio among the treated cells (Fig. 6E). These suggest that bFGF may inhibit caspase and prevent anoikis through the activation of ERK and AKT and down-regulation of BIM.

It has been known that aCasp3 can activate ROCK1 via proteolytic cleavage, and activated ROCK1 can then phosphorylate myosin light chain (MLC), which finally promotes cell detachment by changing the configuration of the cytoskeleton formed together with F-Actin (41, 42). Agreeing...
with this mechanism, bFGF withdrawal caused cleavage of ROCK1 and phosphorylation of MLC, which was clearly rescued by z-VAD-FMK in a dose-dependent manner (Fig. 4G).

These data suggest that bFGF inhibits anoikis at least in part by repressing the caspase-ROCK1-myosin signaling.

As aforementioned, aCasp3 can induce mouse ES cell differentiation through cleavage and degradation of Nanog protein (37). We did not observe any cleaved form of NANOG protein in cell lysates from CT2 cultured in T1/F0 (or T1). This might result from failure of the antibody we used to detect the cleaved NANOG, instability of the cleaved NANOG, or rapid detachment (anoikis) of the cells with NANOG cleavage. However, we did see that the level of full-length NANOG protein decreased in the cells cultured in T1/F0 with 2 μg/ml IgG, or IGFR1 blocking antibody 1H7 for 3 days and assayed the same as in A–C.

![Figure 5](https://example.com/figure5.png)

FIGURE 5. bFGF acts independently of IGF2. A–C, CT2 cells were cultured in T1, T1/F0, or T1/F0 + 30 ng/ml IGF2 for 3 days. At 24 h post-medium change, both floating and attached cells were collected separately and tested for ratios of OCT4+ and aCasp3+ cells by FACS of the attached cells (A), attached cell number (B), and floating cell ratio (C). *, p < 0.01 versus T1 (D–F) CT2 cells were cultured in T1, T1/F0 with 2 μg/ml IgG, or IGFR1 blocking antibody 1H7 for 3 days and assayed the same as in A–C.

NANOG level declined at 8 h of the treatment (supplemental Fig. S4). This indicates that this is a post-translational event, and caspase activation caused by bFGF withdrawal may induce NANOG degradation, which then leads to the PS cell differentiation.

**ROCK Inhibitor Reduces Anoikis While Increasing Ratios of Apoptotic and Differentiated Human PS Cells**—It has been shown that treatment of human ES cells with the ROCK inhibitor Y27632 for overnight post-cell passaging dramatically enhances cell attachment and hence the cell colony number (43). We have also routinely used the ROCK inhibitor to increase cloning efficiency of human PS cells. Because ROCK1 induces phosphorylation of MLC, we confirmed that Y27632 indeed inhibited the MLC phosphorylation in CT2 cells (supplemental Fig. S5). Then we tested whether it would reduce anoikis of human PS cells by breaking the caspase-ROCK1-myosin signaling axis. We carried out these experiments on post-attachment CT2 cells. One day after passaging the cells in T1, the medium was replaced with T1 or T1/F0 with or without 10 μM Y27632 and refreshed daily for 3 days. More mixed cell morphologies for both differentiated and undifferentiated cells were observed in the cultures with Y27632 than without it (Fig. 7A). Y27632 slightly increased the number of attached cells in T1/F0 but not T1 (Fig. 7B) and moderately reduced the floating cell ratio in both T1 and T1/F0 (Fig. 7C). The mild rescue of the floating cell ratio by Y27632 may be due to its failure to inhibit anoikis caused by ROCK1-independent mechanism or apoptosis initiated before cell detachment. Consistent with the latter, more apoptotic cells were found among freshly detached cells (4 h post-medium change) from CT2 cell culture in T1/F0 plus Y27632 for 3 days (supplemental Fig. S6).

Unexpectedly, long term treatment of Y27632 reduced the OCT4+ cell ratio almost by half among the attached cells in T1 and remarkably increased aCasp3+ cell ratio of the gated OCT4+ cells from the attached cells by 8-fold (Fig. 7D). As shown in Fig. 7E, caspase activation has multiple consequences: cell anoikis (through ROCK1, myosin, etc.), differentiation (through NANOG degradation), or apoptosis. Because the ROCK inhibitor only represses anoikis but not the activated caspases, it can reduce anoikis (with increased cell attachment) but cannot inhibit cell differentiation and apoptosis (Fig. 7,
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A–D). Therefore, our data suggest that the ROCK inhibitor should only be used briefly to enhance the cloning efficiency of human PS cells. Prolonged use will increase the ratios of differentiated and apoptotic cells.

**DISCUSSION**

Apoptosis and proliferation are two dynamically and tightly regulated processes that together maintain the homeostasis of renewable tissues. Anoikis is a subtype of apoptosis induced by the loss of cell-matrix interaction, playing a critical role in many biological processes including development, tissue homeostasis, and cancer metastasis (32). Because of the adherent nature of human PS cells, previous studies have focused on attached cells with detached cells removed and ignored. By using the defined mTeSR1 medium and collecting both the attached and freshly detached cells at various times, we have demonstrated here that human PS cells undergo constant anoikis, a process probably used by the cells to maintain balanced cell densities by expelling extra (maybe also differentiated or abnormal) cells. The caspase-ROCK1-myosin signaling appears to be associated with the anoikis, which is under the tight control of bFGF via activation of ERK and AKT, decrease of BIM, and suppression of the caspase activities. Withdrawal of bFGF removes the control and causes extra loss of the cells and hence failure of human PS cell maintenance.

Inhibition of the caspase activity also enables bFGF to prevent the remaining attached human PS cells from apoptosis, which also contributes to maintenance of the homeostasis and increase of the cell number, although its impact on the cell proliferation is much less than the anti-anoikis effect of bFGF. It has been shown that bFGF prevents apoptosis in many other cell types (27, 28). Recently, Eisselleova et al. (31) reported that bFGF antagonizes irradiation- or hydrogen peroxide-induced apoptosis of human ES cells and increases the cloning efficiency of the cells by ~20% by promoting adhesion of newly passed cells (which also reduces the number of unattached cells) as shown previously (3). However, they did not address the inhibitory effect of bFGF on anoikis (cell detachment after adhesion) and apoptosis that spontaneously occurs among human ES cells, which is the key role of bFGF in sustaining human PS cell culture as revealed in this study. In the undefined medium used by Eisselleova et al., the proprietary serum replacement may contain components that favor cell attachment, thus masking the anti-anoikis effect of bFGF and resulting in a very mild increase of attached cell number in their experiments, as also observed in the report by Filipczyk et al. (34). The absence of the masking components in the defined mTeSR1 medium and analysis of the freshly detached cells have enabled us to identify the anti-anoikis effect of bFGF reflected by remarkable increase of attached cell number (10) (Fig. 1A) and decline of floating cells (Fig. 1C). We also confirmed that this effect is only obvious in mTeSR1 but not the undefined medium (data not shown).

Further, we provided evidence that the NANOG protein level decreases upon bFGF withdrawal even in the presence of a translation inhibitor and that the reduction of both the NANOG protein level and OCT4 + cell ratio in human PS cells cultured in the absence of bFGF can be evidently rescued by the caspase inhibitor z-VAD-FMK. Therefore, caspases may also promote NANOG protein degradation in human PS cells as in mouse ES cells (37), and bFGF may sustain the NANOG level via inhibition of its degradation rather than (or in addition to) stimulation of its transcription. This coincides with our previous observations that NANOG transcription does not decline.
obviously and rapidly in human ES cells cultured in the absence of bFGF except when TGFβ/H9252 signaling is inhibited at the same time (9).

Additionally, we tested whether the anti-anoikis activity of bFGF can be executed by IGF2 because the latter has been shown to act downstream of bFGF through differentiated human ES cells as a niche (24). We found that, although IGF signaling is required to sustain human PS cell self-renewal, IGF2 cannot substitute bFGF for its effects on human PS cells cultured in the defined medium. The discrepancies between our data and those reported by Bendall et al. (24) may result from the different media used in the two studies. The serum replacement in the medium used in their study contained proprietary components, some of which might synergize with IGF2 to support human ES cell culture. However, it is still hard to explain how as high as 98% undifferentiated (OCT4+/H11001) cells observed from T1-supported culture can be maintained by endogenous IGF2 produced, if any, by less than the 2% differentiated (OCT4−) cells. A more reasonable explanation is that both FGF and IGF signaling are directly required to sustain human PS cell self-renewal, although they may regulate each other in their target cells.

In summary, we have revealed in this study that 1) human pluripotent stem cells including ES and iPS cells are subject to constant anoikis, a critical mechanism that may help maintain their homeostasis; 2) activation of caspases is responsible for the anoikis as well as apoptosis and differentiation of the remaining attached cells; 3) the caspase-ROCK1-myosin signaling is associated to the anoikis; 4) bFGF protects human PS cells from all of these detrimental effects by inhibiting caspases through activation of ERK and AKT and inhibition of BIM (but independent of IGF2), with its anti-anoikis effect playing a dominant role in sustaining human PS cell proliferation; and 5) activated caspases causes NANOG degradation, which then leads to human PS cell differentiation. A model to accommodate all of these findings is schemed in Fig. 7E. It is important to point out, however, that not all apoptotic cells detach and not all detached cells are caused by anoikis (some cells die first and then detach). Because the pan-caspase inhibitor cannot completely replace bFGF to maintain long term propagation of human PS cells, bFGF must also act through other mechanisms in addition to inhibition of caspase activation. To this end, we have at least ascertained that bFGF does not accelerate the cell cycle progression of human PS cells to support their proliferation.

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