Human embryonic stem cell-specific role of YAP in maintenance of self-renewal and survival

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Research Article

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Abstract

Human embryonic stem cells (hESCs) have unique characteristics, i.e., self-renewal and pluripotency, that are clearly distinct from those of other types of cells. These characteristics of hESCs are tightly regulated by complex signaling mechanisms. In this study, we show that the yes-associated protein (YAP) functions in an hESC-specific manner to maintain self-renewal and survival in hESCs. hESCs reacted highly sensitively to YAP downregulation for cell survival. Interestingly, hESC displayed dynamic changes of YAP expression in response to YAP downregulation. Our results also demonstrated that YAP is critical for the maintenance of self-renewal. Meanwhile, we found that doxycycline upregulated YAP in hESCs and attenuated the decrease in cell survival induced by YAP downregulation. However, neither the decrease in the expression of self-renewal markers triggered by YAP downregulation nor neural/cardiac differentiation was affected by doxycycline treatment. Collectively, the results of this study revealed the mechanism underlying the role of YAP as well as a novel function of doxycycline in hESCs.

Introduction

The characteristics of human pluripotent stem cells (hPSCs), including self-renewal, which consists in sustained proliferation and immortality for an extended period in culture, and pluripotency, which is the potential to give rise to cells of all three germ layers, provide an inexhaustible source to study the early mammalian developmental processes, the development of cell therapies, their applications in drug discovery, etc. [1–3]. To understand the molecular mechanisms underlying the self-renewal capacity of hPSCs, it would be useful to identify the cellular components that permit its precise regulation. To date, several pathways that contribute to the regulation of hPSC self-renewal have been identified, and the roles of various factors, such as extrinsic stimuli, intrinsic signal molecules, transcription factors, and epigenetic modifiers, in hPSCs have been elucidated [1, 4]. In the present study, we focused on the role of the yes-associated protein (YAP) in the maintenance of survival and self-renewal in hPSCs.

YAP is as a transcriptional regulator in the Hippo signaling pathway that regulates genes involved in many cellular functions, including proliferation, organ-size control, and tumorigenesis [5]. In particular, studies of the function of YAP have been actively conducted in PSCs. Moreover, YAP is highly expressed in embryonic stem cells (ESCs) [6]. Previous studies demonstrated that YAP promotes stem cell self-renewal and pluripotency, and that loss of YAP leads to the loss of pluripotency in human and mouse ESCs (hESCs and mESCs, respectively) [7–9]. In addition, it was reported that YAP prevents hESC differentiation and that YAP overexpression suppresses mESC differentiation [9]. Conversely, another study reported that YAP is dispensable for self-renewal, depletion of YAP inhibits differentiation, and overexpression of YAP stimulates differentiation in mESCs [10]. It has also been shown that YAP depletion does not affect any of the normal stem cell characteristics in human induced pluripotent stem cells (hiPSCs) [11]. These results suggest that the function of YAP is controversial.

In the present study, we investigated how self-renewal, survival and differentiation are affected by the up- or downregulation of YAP in hESCs. Importantly, we demonstrated that the role of YAP is hESC specific.

This study will advance the understating of the self-renewal and pluripotency of hESCs.

Materials And Methods

Human embryonic stem cell culture

The SNUhES31 hESC line was obtained from the Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University Hospital, South Korea. hESCs were cultured on mouse embryonic fibroblasts treated with 10 μg/mL mitomycin-C (Roche, Mannheim, Germany) and were maintained in hESC medium comprising 20% knockout serum replacement (Life Technologies, Carlsbad, CA, USA), 1% Minimum Essential Medium-Nonessential Amino Acids (MEM-NEAA; Life Technologies), 1% Glutamax (Life Technologies), and 7 μL/L β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) in -12 (Life Technologies) containing 20 ng/mL of bFGF (R&D Systems, Minneapolis, MN, USA). For feeder-free hESC culture, cells were detached from the feeder cells using 1 mg/mL dispase (Life Technologies) and cultured in Essential-8 Medium (Life Technologies) on Geltrex (Life Technologies)-coated culture plates. Cells were subcultured as small clusters every 4 days using 0.5 mM EDTA solution.

Genetic regulation of YAP expression by lentiviral vectors

YAP shRNA expression vectors were used to specifically downregulate YAP. YAP target sequences (5¢–TGACTCAGGATGGAGAAATTT–3¢ for shYAP and 5¢–GACTCAGGATGGAGAAATTTTA–3¢ for shYAP #2) were acquired from the GPP web portal (http://portals.broadinstitute.org/gpp/public/) and cloned into the pLKO.1-TRC cloning vector. The pLKO.1-TRC shRNA vector was used as a control. Both vectors were gifts from Dr. David Root, University of Coloado in Boulder (Addgene plasmids #10878 and #10879) [12]. For overexpression of YAP, we used YAP5SA that was mutated five serine amino acid to alanine to prevent protein degradation by phosphorylation. To construct a lentiviral vector overexpressing YAP5SA, we subcloned the YAP5SA gene into the lentiviral vector with regulation under the cytomegalovirus early enhancer/chicken β actin (CAG) promoter. The YAP5SA vector was a gift from Dr. Kunliang Guan, University of California, San Diego (Addgene plasmid #33093) [13]. The lentiviral vectors were kindly provided by Dr. Yibing Qyang (Yale Cardiovascular Research Center, Yale School of Medicine). For the production of lentiviral particles, subcloned lentiviral plasmids were co-transfected with the lentivirus-packaging plasmids (VSV-G-expressing envelope plasmid and another plasmid containing the *gag*, *pol*, and *rev* genes (kindly provided Dr. Yibing Qyang) into HEK293T cells using the X-tremeGene HP DNA transfection reagent (Roche Applied Science, Penzburg, Germany) at 37°C in 5% CO_2 for 24 h. The virus-containing medium was collected daily for 3 days after transfection and concentrated by ultracentrifugation at 55,200 × *g* at 4°C for 2 h (Hitachi, Ltd., Tokyo, Japan). To transfect hESCs with lentiviral particles, 2.5×10^5 hESCs dissociated into single cells were plated in a 24-well plate with concentrated virus-containing medium at a low titer (4×10^6 IU/mL) or a high titer (2×10^7 IU/mL) for 24 h at 37°C, followed by 2 days of culture. Cells were selected by treatment with 2 μg/mL of puromycin (Life Technologies).

Western blot analysis

Cells were lysed in lysis buffer (iNtRON Biotechnology, Seongnam, Korea) by sonication (Vibra-Cell, Sonics, Newtown, CT, USA) on ice. Cell lysates were separated by 10%–12% SDS-PAGE and transferred PVDF membranes (Millipore, Burlington, MA, USA). Blots were washed with TBST (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20; Affymetrix, Santa Clara, CA, USA), blocked with 5% skim milk (Millipore) for 1 h, and incubated with the primary antibodies. The following primary antibodies were used: rabbit anti-YAP (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-p-YAP (cell signaling technology, Danvers, MA, USA), rabbit anti-Oct4 (Santa Cruz Biotechnology), rabbit anti-Sox2 (cell signaling technology), rabbit anti-Nanog (cell signaling technology), and mouse anti-β-actin (Santa Cruz Biotechnology). Primary antibodies were detected using goat anti-rabbit (Santa Cruz Biotechnology) or goat anti-mouse (Santa Cruz Biotechnology) IgG conjugated with horseradish peroxidase (HRP). The bands were visualized using an enhanced chemiluminescence solution (Thermo Scientific, Waltham, MA, USA). Images were acquired using an ImageQuant LAS 4000 Mini system (GE Healthcare, Chicago, IL, USA).

Cell counting kit-8 (CCK-8) assay

hESCs were plated at a density of 1×10^4 cells/well in 96-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and cultured under each condition. CCK-8 solution was added to each well at a 1:10 dilution, followed by further incubation at 37°C for 3 h. Absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the RNeasy Plus RNA Extraction Kit (Qiagen, Germantown, MD, USA). Reverse transcription was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Reverse transcription products (2.5 ng cDNA) were amplified using the FastStart Essential DNA Green Master PCR Kit (Roche Applied Science, Penzburg, Germany) and primers. The primers used here were as follows: 5¢–GTTGGAGAAGGTGGAACCAA–3¢ (forward) and 5¢–CTCCTTCTGCAGGGCTTTC–3¢ (reverse) for *Oct-4*; 5¢–CAGAAGGCCTCAGCACCTAC–3¢ (forward) and 5¢–ATTGTTCCAGGTCTGGTTGC–3¢ (reverse) for *Nanog*; 5¢–CCTAAGGAACCACCGGTCA–3¢ (forward) and 5¢–AGCATGGACAGACAAGCAGA–3¢ (reverse) for *Brachyury T*; 5¢–GATGGAGCCAAGCCCAC–3¢ (forward) and 5¢–CACAGAGACGGCGTCAGT–3¢ (reverse) for *MESP1*; 5¢–GTTGTCCGCCTCTGTCTTCT–3¢ (forward) and 5¢–TCTATCCACGTGCCTACAGC–3¢ (reverse) for *Nkx2.5*; and 5¢–TTCACCAAAGATCTGCTCCTCGCT–3¢ (forward) and 5¢–TTATTACTGGTGTGGAGTGGGTGT–3¢ (reverse) for *TNNT2*. Samples were cycled 45 times using a LightCycler® 96 Real-Time System (Roche Applied Science). RT-qPCR was performed using the following parameters: 5 min at 95°C, 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. All RT-qPCR experiments were performed in triplicates. The cycle threshold was calculated using default settings with real-time sequence-detection software (Roche Applied Science).

Neural differentiation

Neural differentiation was induced by transferring approximately 9×10^3 cells in 20 μL of hESC medium without bFGF and supplemented with 50 μM Y-27632**,** 5 μM dorsomorphin (DM) (Sigma-Aldrich), and 5 μM SB431542 (Sigma-Aldrich) onto the lid of a 100 mm petri dish followed by culture for 2 days as a hanging drop, to form embryoid bodies (EBs). The day of hanging-drop preparation was defined as EB day 0. On EB day 2, the EBs were transferred to a 100-mm petri dish and cultured for an additional 4 days in suspension in medium with same composition. On EB day 6, the EBs were attached to a 60-mm tissue-culture dish coated with Matrigel (BD Biosciencesplate, San Jose, CA, USA) and cultured in neural induction medium [1× N2 supplement (Life Technologies) and 1× nonessential amino acids (Life Technologies) in DMEM/F12 medium] for 4 days. The cells were analyzed on EB day 10.

Cardiac differentiation

Cardiac differentiation was induced as previously described [14]. Feeder-free hESCs were dissociated into single cells by incubation with Accutase® for 5–8 min, then plated onto Matrigel-coated plates at a density of 1.5×10^5 cells/cm^2 in mTeSR™1 supplemented with 5 μM Y27632, which is a Rho-associated protein kinase inhibitor. We set this day as day −4. The following day, we changed the medium to mTeSR™1 without Y27632, and refreshed the medium every day for an additional 2 days. At day −1, we changed the medium to mTeSR™1 containing Matrigel (1:60 dilution). To induce cardiac differentiation, we replaced the mTeSR™1 medium with RPMI/B-27 minus insulin medium (RPMI1640 and B-27 minus insulin supplement) supplemented with 10 μM CHIR99021, which is a GSK-3 inhibitor (Day 0), followed by culture for 24 h. The culture medium was then replaced with RPMI/B-27 minus insulin medium without supplement (day 1) and cultured for 2 days. At day 3, we replaced the medium with RPMI/B-27 minus insulin medium supplemented with 7 μM XAV939, which is a tankyrase inhibitor, and 5 μM IWP2, which is a porcupine inhibitor, followed by culture for 48 h. The culture medium was replaced with RPMI/B-27 minus insulin medium and cultured starting at day 5. At day 7, the medium was replaced with RPMI/ B-27 (RPMI1640 and 50X B-27 supplement), and was changed every other day thereafter. Analyses were performed at day 10.

Flow cytometry

Cells were dissociated with Accutase® for 10 min and fixed with 3.2% paraformaldehyde in PBS. Subsequently, cells were blocked and permeabilized with 10% normal goat serum in PBST for 1 h at room temperature. Cells were then incubated with the primary antibody overnight at 4°C, followed by the Alexa 488-conjugated secondary antibody for 3 h at room temperature. Samples were analyzed using a FACS Aria III Flow cytometer (BD Biosciences, NJ, USA).

Statistical analysis

The results were reported as means ± S.E.M. Differences between mean values were analyzed using Student's *t*-test. Significance was set at $P < 0.05$.

Results

Effect of shYAP on hESC viability

To examine the effect of YAP downregulation in hESCs, we transfected cells with low- or high-titer shYAP. As shown in Fig. 1A, YAP expression was significantly downregulated by shRNA transfection at a low titer (shYAP-low, 4×10^6 IU/ml) and high (shYAP-high, 2×10^7 IU/mL) titers in a dose-dependent manner. Although a significant level of cell death was induced by shYAP-high, shYAP-low did not cause significant changes in cell viability (Fig. 1B). These results were confirmed by another experiment using shRNA with different targeting sequence (shYAP#2). YAP expression was significantly downregulated by shYAP#2 at a low (4×10^6 IU/mL) and high (2×10^7 IU/mL) titers in a dose-dependent manner. A significant level of cell death was induced by shYAP#2-high, whereas shYAP#2-low did not cause significant changes in cell viability (Supplementary Fig. 1A and 1B). These results were were consistent with those of the experiments using shYAP. To examine whether shYAP-high affects cell viability in other types of cells, we transfected HEK293T and A549 cells with a high titer of shYAP. As shown in Supplementary Fig. 2A, YAP expression was significantly downregulated by shYAP-high. However, unlike in hESCs, no significant cell death was observed in these cell types (Supplementary Fig. 2B). These results suggest that hESCs viability is highly sensitive to downregulation of YAP expression.

Effect of shYAP on hESC self-renewal

The influence of YAP downregulation on self-renewal of hESCs was assessed. We observed that shYAP-low significantly downregulated the expression levels of the hESC self-renewal marker proteins Oct4, Nanog, and Sox2 (Fig. 2A). We selected hESCs transfected with shYAP-low using puromycin treatment (10 μg/mL), as described in the Materials and Methods, and maintained these cells for an additional 4 days (post-transfection day 7). As shown in Fig. 2B, differentiated cell morphological changes were observed in hESCs transfected with shYAP-low. In experiments aimed at examining whether these cells maintain shYAP activity, YAP expression levels were paradoxically increased in the shYAP-low group (Fig. 2C). When these cells were cultured for a longer duration (post-transfection day 30), differentiated-cell morphological changes and decreased expression of Oct4, Nanog, and Sox2 were observed in the shYAP-low group (Fig. 2D and Fig. 2E). There was no change in the increased YAP expression levels in the shYAP-low group after culturing for a longer duration (Fig. 2F). However, unlike in hESCs, a paradoxical increase in YAP expression was not observed in shYAP-transfected A549 and HEK293T cells (Supplementary Fig. 2C). Taken together, these results suggest that the YAP protein is important for the maintenance of self-renewal in hESCs, and that YAP downregulation induces a paradoxical increase in YAP expression specifically in hESCs. Because our results indicated that the morphology of hESC was changed to a differentiated form and that the expression of self-renewal marker proteins was decreased by shYAP-low, we examined differentiation markers in different lineages. The expression levels of genes specific to the endoderm (*SOX17* and *FOXA2*), ectoderm (*PAX6* and *Nestin)*, and mesoderm (*Brachyury T*, *MEF2C*, and *NKX 2.5*) were commonly increased in the shYAP-low group (Fig. 2G – 2I). These results suggest that YAP downregulation induces non-specific hESC differentiation without directionality.

Effect of chemical inhibition of YAP in hESCs

The response of hESCs to genetic regulation of YAP was also confirmed through chemical regulation. Verteporfin is an FDA-approved drug used in photodynamic therapy for macular degeneration. It was recently identified as an inhibitor of YAP signaling [15]. Verteporfin treatment at concentrations >50 nM for 24 h significantly downregulated YAP expression in a dose-dependent manner (Fig. 3A). Since phosphorylated YAP, which is a major downstream effector of the Hippo pathway, is sequestered in the cytoplasm and subsequently degraded [16,17], we investigated whether the decrease in YAP expression by verteporfin treatment is mediated by the phosphorylation of YAP. However, YAP phosphorylation was abruptly decreased by verteporfin treatment in a dose- and time-dependent manner (Fig. 3B and 3C). Although further studies are required to identify the exact mechanism underlying this observation, our results suggest that the downregulation of YAP by verteporfin is not mediated by YAP phosphorylation. Next, we examined the effects of verteporfin on hESC survival. As shown in Fig. 3D, significant cell death was observed at a verteporfin concentration of 300 nM for 24 h, and at concentrations above 100 nM for 72 h. In contrast, verteporfin concentration < 50 nM did not induce significant cell death. In HEK293T and A549 cells, treatment with 300 nM verteporfin for 24 h significantly decreased YAP expression, whereas cell viability was not affected by 300 nM verteporfin, even after treatment for 72 h in both cell types (Supplementary Fig. 3A and 3B). Treatment of hESCs with 300 nM verteporfin for 24 h significantly downregulated Oct4, Nanog, and Sox2 expression (Fig. 3E). As in shYAP-low, a paradoxical increase in YAP expression was induced by treatment with verteporfin concentration < 50 nM for 72 h, but the decreased expression of Oct4, Nanog, and Sox2 was maintained under these conditions (Fig. 3F and 3G). In agreement with the results of the genetic regulation of YAP, these findings indicate that strong downregulation of YAP by a chemical inhibitor also inhibits cell survival, and that weak downregulation of YAP at a level that does not affect cell survival inhibits self-renewal with induction of a paradoxical increase of YAP expression in hESCs.

Effect of YAP overexpression on self-renewal and proliferation

Next, we examined the effect of YAP overexpression in hESCs. Cells were transfected with a low- or high-titer YAP5SA. As shown in Fig. 7A, YAP expression was significantly upregulated by YAP5SA transfection at low- (YAP5SA-low, 4×10^6 IU/mL) and high- (2×10^7 IU/mL) titers in a dose-dependent manner. CCK-8 assay results showed that YAP5SA significantly induced hESC proliferation (Fig. 7B). YAP5SA also significantly upregulated the expression of the hESC self-renewal marker proteins (Fig. 7C). These collective findings confirmed that YAP plays a role in the maintenance of self-renewal and proliferation of hESCs.

Effect of doxycycline on YAP expression, self-renewal, and survival in hESCs

Doxycycline had a novel effect of YAP upregulation in hESCs. The significantly increased YAP expression occurred in dose- and time-dependent manners (Fig. 4A and 4B). YAP phosphorylation was significantly downregulated by doxycycline treatment (Fig. 4C). Moreover, doxycycline-induced YAP upregulation was

not observed in HEK293T or A549 cells (Supplementary Fig. 4A and 4B). These results suggest that doxycycline-induced YAP upregulation is directly affected by changes in YAP phosphorylation and is specific to hESCs. Next, we examined the effect of doxycycline on the survival and self-renewal of hESCs. As 300 nM verteporfin induced significant cell death, we examined the effect of doxycycline under these conditions. The decrease in YAP expression induced by 300 nM verteporfin was significantly reversed by doxycycline (Fig. 5A). Moreover, the 300 nM verteporfin-induced decrease in cell viability was recovered by doxycycline treatment (Fig. 5B). These results suggest that doxycycline can prevent verteporfin-induced cell death by upregulating YAP expression. In addition, Oct4, Nanog, and Sox2 expression levels were not affected by the 24-h doxycycline treatment (Fig. 5C). However, doxycycline treatment for 72 h significantly upregulated Oct4, Sox2, and YAP expression (Fig. 5D). These results indicate that doxycycline-induced YAP upregulation may promote self-renewal of hESCs. Previous studies have reported that ectopic YAP expression stimulates the expression of genes, including *Oct4*, *Nanog*, and *Sox2*, that are important for self-renewal of mESCs [9]. However, verteporfin-induced downregulations of Oct4, Sox2, and Nanog were not affected by doxycycline treatment for 24 or 72 h (Fig. 5F and 5E). The collective results indicate that doxycycline inhibits cell death induced by YAP downregulation in hESCs. However, the downregulation of self-renewal markers triggered by YAP downregulation is not reversed, even with a doxycycline-mediated increase in the expression of YAP. These results agree with the finding that the decreased expression levels of self-renewal markers induced by YAP downregulation were not affected by the paradoxical increase in YAP expression. Therefore, we examined the effect of doxycycline on hESC differentiation into specific lineages.

Effect of doxycycline on hESC differentiation

The efficiencies of hESC differentiation into neural or cardiac lineages in doxycycline-untreated control cells, doxycycline-treated cells at the undifferentiated stage, and doxycycline-treated cells in the undifferentiated and differentiated periods were assessed. When the effect of doxycycline on neural differentiation was examined, no obvious differences in the differentiation of hESCs into cells positive for the paired box 6 (PAX6) neural-progenitor-specific marker [18] were observed among the three groups by immunocytochemistry (Fig. 6A). Flow cytometry revealed no difference in the percentage of PAX6-positive cells (Fig. 6B). Next, we examined the effects of doxycycline on cardiac differentiation. As shown in Fig. 6C and 6D, no significant differences in differentiation into cells positive for cardiac troponin T (cTnT), a cardiac-specific marker, were observed among the three groups in immunocytochemistry and flow cytometry. The collective results indicate that doxycycline does not affect the differentiation of hESCs into neural or cardiac cell lineages.

Effect of YAP overexpression on hESC differentiation

The efficiencies of differentiation into neural or cardiac lineages in control and YAP5SA transfected hESCs were assessed. Examination of the effects of YAP5SA on neural differentiation revealed no obvious differences in the differentiation of hESCs into cells positive for PAX6 in control and YAP5SA transfected hESCs by immunocytochemistry (Fig. 8A). Flow cytometry revealed no difference in the

percentage of PAX6-positive cells (Fig. 8B). Next, we examined the effects of YAP5SA on cardiac differentiation. As shown in Fig. 8C and 8D, no significant differences in differentiation into cTnT-positive cells were observed between control and YAP5SA transfected hESCs by immunocytochemistry and flow cytometry. The collective findings indicate that YAP overexpression does not affect the differentiation of hESCs into neural or cardiac cell lineages.

Discussion

The observed genetic and chemical regulation of YAP demonstrate that this protein is essential for the self-renewal and survival of hESCs. These experiments yielded several interesting findings specific to hESCs. YAP downregulation via shRNA (shYAP-high) induced significant cell death at a level that did not affect other cell types (A549 and HEK293T cells). It has been reported that responses to YAP downregulation depend on cell type. For example, the survival of uveal melanoma and mesothelioma cells is not associated with YAP expression levels [19], whereas YAP downregulation affects cell survival in other cell types [20, 21]. It has also been reported that hESC expansion and survival are impaired by YAP attenuation [3]. In this study, hESC survival was affected by shRNA and also by chemical inhibition of YAP at a much lower degree of YAP downregulation than other cell types. For example, the degree of cytotoxicity detected in hESCs in the presence of 300 nM verteporfin was comparable to that observed at a concentration of 8 μM in human umbilical vein endothelial cells, endothelial cells, and pancreatic cancer cells [20]. Furthermore, the survival of A549 and HEK293T cells was not affected by treatment with 300 nM verteporfin. Based on these results, we conclude that hESCs are much more sensitive to YAP downregulation and that YAP is more important for cell survival in hESCs than in other cell types.

At the level of YAP downregulation, which did not significantly affect hESC survival by shRNA (shYAP-low) and verteporfin (50 nM), morphological changes associated with differentiation and decreased expression of self-renewal markers, including Oct4, Nanog, and Sox2, were observed in hESCs. Previous studies have demonstrated the role of YAP in the self-renewal of mouse and human ESCs [7, 9]. However, to the best of our knowledge, this is the first direct evidence of a decrease in the expression of self-renewal marker proteins after YAP downregulation in hESCs. When hESCs transfected with shYAP-low were cultured for a prolonged period, the apparent differentiation aspects were maintained. However, YAP expression was significantly increased in hESCs under these conditions. The YAP expression level in these cells was higher than that in the control hESCs. Because this paradoxical upregulation of YAP was not observed in HEK293T and A549 cells, we concluded that this is an hESC-specific phenomenon that presumably stems from the sensitivity of hESCs to and the resistance to cell death caused by the downregulation of YAP. Moreover, the downregulation of self-renewal marker proteins was not reversed despite the re-increase in YAP expression. These results suggest that once hESC differentiation is in progress, it cannot be prevented even if the YAP expression level increases. Thus, hESCs are highly sensitive to the reduction in YAP expression, and YAP is an essential protein for the maintenance of self-renewal and survival of hESCs. Additionally, forced YAP expression significantly increased cell proliferation and expression of self-renewal markers in hESCs. These results strongly support the above findings, verifying the role of YAP in hESCs.

In this study, we observed that doxycycline enhanced self-renewal and survival. These effects of doxycycline on hESCs have been previously reported [22]. However, this study is the first to reveal that the effect of doxycycline is mediated by the promotion of YAP expression. The effect of doxycycline on YAP expression was accidentally discovered during the establishment of the conditional YAP knockdown system. Before establishing the doxycycline-induced YAP knockdown system, we simply tested whether doxycycline treatment affected YAP expression. Doxycycline significantly increased YAP expression. Although this was an interesting finding, at the same time it was sufficient to cause controversy because many researchers use a conditional gene regulation system that generally uses doxycycline. Doxycycline-induced YAP upregulation was observed in hESCs but not in HEK293T and A549 cells. Therefore, we conclude that the effect of doxycycline on YAP expression is probably specific to hESCs, which should be considered before performing experiments to control YAP expression in hESCs using a doxycycline-induced system. Doxycycline inhibited the verteporfin-induced decrease in YAP expression, and verteporfin-induced cell death was significantly attenuated by doxycycline treatment. Previously, the diverse effects of doxycycline were reported to depend on cell type. Doxycycline generally shows anticancer activity in various cancer cells, including cervical cancer and hepatocellular carcinoma cells [23, 24], whereas protective effects have also been reported in other cell types, including ESCs and thymic epithelial cells [25, 26]. Although further studies are required to determine the mechanisms underlying the cell type-dependent effect of doxycycline, we conclude that doxycycline-induced YAP upregulation can enhance cell survival, at least in hESCs, because we also observed that YAP expression depended on the cell type and was important for hESC survival. Doxycycline also upregulated Oct4 and Sox2 expression. As YAP is essential for the self-renewal and survival of hESCs, these results were expected. Previous reports have suggested that YAP–TEAD transcriptional complexes directly activate Oct4 and Sox2 expression, and the subsequent expansion of pluripotent cells [9]. These authors suggested that genetically forced expression of YAP inhibits differentiation and is sufficient to maintain stem cell characteristics in mESCs. However, doxycycline treatment did not prevent the verteporfin-induced decrease in the expression of self-renewal markers, despite its YAP induction activity. Doxycycline treatment also had no significant effect on neural or cardiac differentiation in each differentiation condition. Additionally, neural and cardiac differentiation was not affected by YAP overexpression. These results agree with the aforementioned conclusion that once hESC differentiation is in progress, it cannot be prevented despite the induction of YAP upregulation. Therefore, we conclude that although YAP is important for the maintenance of self-renewal and survival in undifferentiated hESCs, the influence of increased YAP expression on differentiation is not significant under differentiation conditions.

In summary, our findings demonstrate the importance of YAP for the maintenance of self-renewal and survival in hESCs. hESCs were specifically sensitive to YAP downregulation with respect to survival, and the downregulated YAP levels could be dynamically altered in hESCs. The self-renewal and survival of hESCs was promoted by doxycycline through the enhancement of YAP expression, whereas their differentiation was not affected by doxycycline. Doxycycline can supplement the maintenance of self-renewal and survival of hESC cultures. This study advances the understanding of the cellular physiology of hESCs.

Abbreviations

bFGF	Basic fibroblast growth factor
cDNA	Complementary DNA
CAG	Cytomegalovirus early enhancer/chicken β actin
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
EB	Embryoid body
EDTA	Ethylenediaminetetraacetic acid
FOXA2	Forkhead Box A2
HEK	Human embryonic kidney
hESCs	Human embryonic stem cells
hiPSCs	Human induced pluripotent stem cells
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
MEF2C	Myocyte Enhancer Factor 2C
MEM	Minimum essential medium
MESP1	Mesoderm Posterior BHLH Transcription Factor 1
Nkx2.5	NK2 Homeobox 5
NEAA	Nonessential amino acids
OCT	Optimal cutting temperature
PAX6	Paired Box 6
PBST	0.1% Triton X-100 in phosphate-buffered saline
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	Standard error of the mean

shRNA	Short hairpin RNA
SOX2	Sex-determining region Y-box 2
TNNT2	Troponin T2, Cardiac Type
YAP	Yes-associated protein

Declarations

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mu Seog Choe, Chang Min Bae, So Jin Kim, Yu Jin Kown, and Won-young Choi*. The first draft of the manuscript was written by Min Young Lee and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript*

Conflicts of interest: No competing interests were disclosed by authors.

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Figures

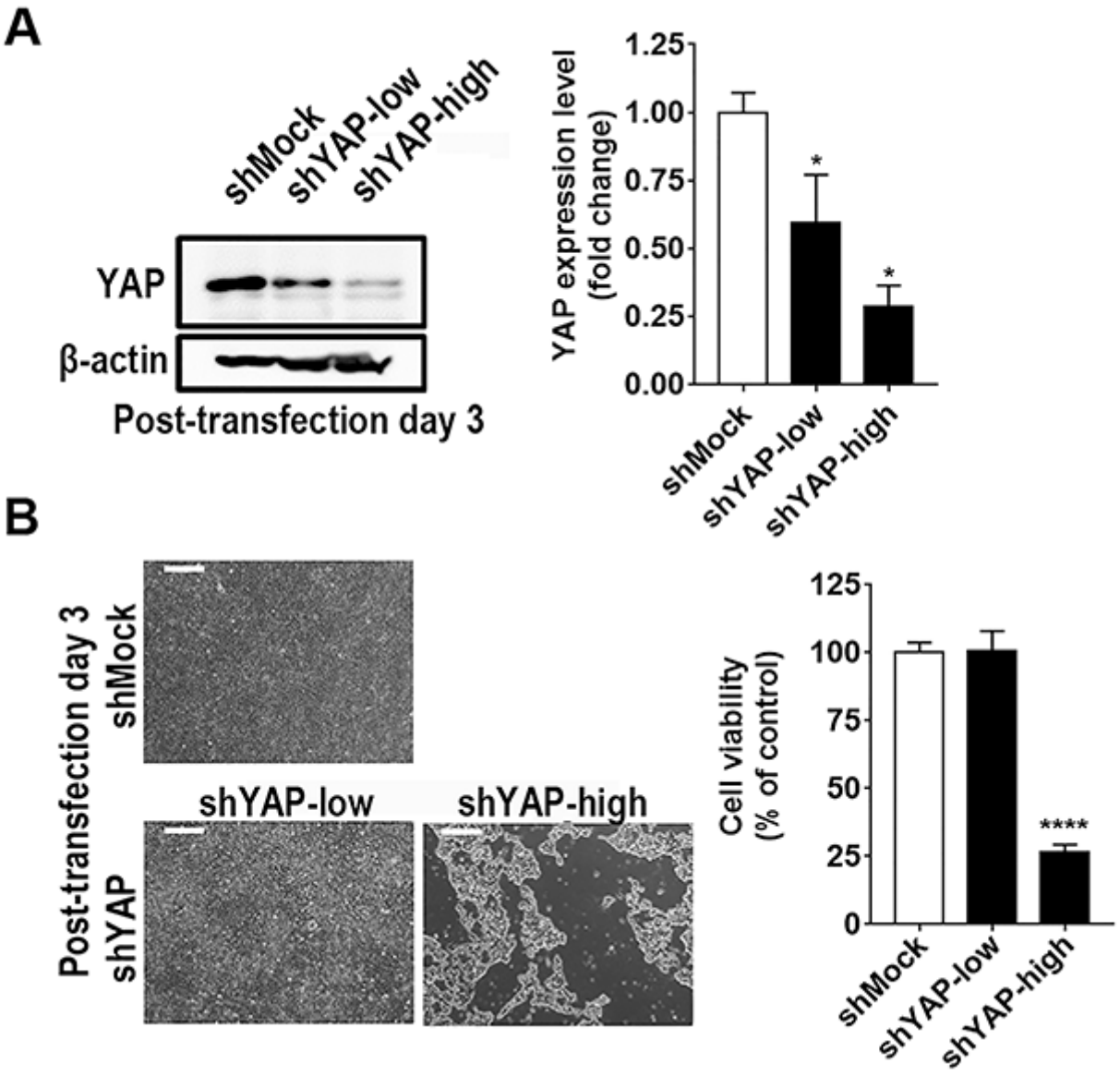

Figure 1

Effect of YAP knockdown on hESC viability. (A) hESCs were transfected with control (shMock) or YAP shRNA lentiviral particles at low titer (shYAP-low, 4×10^6 IU/mL) or high titer (shYAP-high, 2×10^7 IU/mL), and YAP expression at post-transfection day 3 was detected using western blot analysis. *$P < 0.05$ vs. shMock. (B) Cells were transfected with control (shMock) or YAP shRNA lentiviral particles at low titer (shYAP-low) or high titer (shYAP-high). Bright-field images were acquired and cell viability was measured using a CCK-8 assay at post-transfection day 3. Scale bar, 200 μm. ****$P < 0.001$. vs. shMock.

Figure 2

Effect of YAP knockdown on hESC self-renewal. (A) hESCs were transfected with control (shMock) or YAP shRNA at low titer (shYAP-low), and Oct4, Nanog, and Sox2 expression at post-transfection day 3 was

detected using western blot analysis. *P < 0.05; **P < 0.01 vs. shMock. Cells were transfected with control (shMock) or YAP shRNA at low titer (shYAP-low). (B) Bright-field images were acquired at post-transfection day 7. Scale bar, 200 μm. (C) YAP expression at post-transfection day 7 was detected using western blot analysis. *P < 0.05 vs. shMock. Cells were transfected with control (shMock) or YAP shRNA at low titer (shYAP-low). (D) Bright-field images were acquired at post-transfection day 30. Scale bar, 200 μm. (E) Oct4, Nanog, and Sox2 expression at post-transfection day 30 was detected using western blot analysis. (F) YAP expression at post-transfection day 30 was detected using western blot analysis. *P < 0.05 vs. shMock. The expression levels of genes specific to the endoderm (*SOX17* and *FOXA2*) (G), ectoderm (*PAX6* and *Nestin*) (H), and mesoderm (*Brachyury T*, *MEF2C*, and *NKX 2.5*) (I) at post-transfection day 30 were detected by quantitative real-time polymerase chain reaction. *P < 0.05; ****P < 0.001 vs. shMock.

Figure 3

Effect of verteporfin on hESCs. (A, B) hESCs were treated with verteporfin at the indicated concentrations (0–300 nM) for 24 h. YAP and p-YAP expression was detected using western blot analysis. *P < 0.05; **P < 0.001 vs. 0 nM. (C) Cells were treated with 300 nM verteporfin for the indicated time (0–240 min) and p-YAP expression was detected using Western blot analysis. *P < 0.05; **P < 0.001 vs. 0 min. (D) Cells were treated with verteporfin at the indicated concentrations (0–300 nM) for 24 or 72 h. Bright-field images were acquired and cell viability was measured using a CCK-8 assay. Scale bar, 200 μm. *P < 0.05; ***P < 0.005; ****P < 0.001 vs. 0 nM. (E) Cells were incubated with or without 300 nM verteporfin for 24 h and Oct4, Nanog, and Sox2 expression was detected using western blot analysis. (F) Cells were treated with verteporfin at the indicated concentration (0–50 nM) for 72 h and YAP expression was detected using western blot analysis. (G) Cells were incubated with or without 50 nM verteporfin for 24, 48, and 72 h and Oct4, Nanog, and Sox2 expression was detected using western blot analysis. *P < 0.05 vs. 0 nM.

Figure 4

Effect of YAP overexpression on hESC self-renewal. (A) hESCs were transfected with control (CAG-mCherry) or YAP5SA (CAG-YAP5SA) lentiviral particles at low titer (YAP5SA-low, 4×10^6 IU/mL) or high titer (YAP5SA-high, 2×10^7 IU/mL). YAP expression at post-transfection day 3 was detected using western blot analysis. *P < 0.05 vs. control. (B) Cells were transfected with control or YAP5SA lentiviral particles at high titer (YAP5SA). Bright-field images were acquired and cell viability was measured using a CCK-8 assay at post-transfection day 3. Scale bar, 200 μm. ****P < 0.001. vs. control. (C) hESCs were transfected with control or YAP5SA lentiviral particles at high titer (YAP5SA). Oct4, Nanog, and Sox2 expression at post-transfection day 3 was detected using western blot analysis. *P < 0.05; **P < 0.01 vs. control.

Figure 5

Effect of doxycycline on YAP expression in hESCs. (A) hESCs were treated with doxycycline at the indicated concentrations (0–1 μg/mL) for 24 h. YAP expression was detected using western blot analysis. $^{**}P < 0.01$; $^{***}P < 0.005$ vs. 0 μg/mL. (B) Cells were treated with 1 μg/mL doxycycline for the indicated time (0–24h). YAP expression was detected using western blot analysis. $^{*}P < 0.05$; $^{***}P < 0.005$ vs. 0h. (C) Cells were treated with 1 μg/mL doxycycline for the indicated time (0–480 min) and p-YAP expression was detected using western blot analysis.

Figure 6

Effect of doxycycline on the survival and self-renewal of hESCs. (A) hESCs were pretreated with 300 nM verteporfin for 30 min and then incubated with or without 1 μg/mL doxycycline for 24 h. YAP expression was detected using western blot analysis. $^{**}P < 0.01$ vs. doxycycline–/verteporfin–; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.005$ vs. doxycycline+/verteporfin–; $^{++}P < 0.01$ vs. doxycycline+/verteporfin+. Cells were pretreated with or without 300 nM verteporfin for 30 min and then incubated with or without 1 μg/mL doxycycline for 24 h. Bright-field images were acquired and cell viability was measured using a CCK-8 assay. Scale bar, 200 μm. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.005$ vs. doxycycline+/verteporfin–; $^{+}P < 0.05$ vs. doxycycline+/verteporfin+. (C) Cells were treated with 1 μg/mL doxycycline for 24 h and Oct4, Nanog, and Sox2 expression was detected using western blot analysis. (D) Cells were treated with 1 μg/mL doxycycline for 72 h and YAP, Oct4, Nanog, and Sox2 expression was detected using western blot analysis. $^{*}P < 0.05$; $^{***}P < 0.005$ vs. 0 μg/mL. (E) Cells were pretreated with 300 nM verteporfin for 30 min and then incubated with or without 1 μg/mL doxycycline for 24 h. Oct4, Nanog, and Sox2 expression was detected using western blot analysis. (F) Cells were pretreated with 50 nM verteporfin for 30 min and then incubated with or without 1 μg/mL doxycycline for 72 h. Oct4, Nanog, and Sox2 expression was detected using western blot analysis. $^{*}P < 0.05$ vs. Control. ns, not significant.

Figure 7

Effect of doxycycline on the differentiation of hESCs into neural and cardiac lineages. Neural differentiation was induced in the absence or presence of 1 μg/mL doxycycline during the undifferentiated and/or differentiated stage. (A) PAX6 expression was detected using immunocytochemistry at day 10 after cell differentiation. Scale bar, 200 μm. (B) The numbers represent the percentage of PAX-6-positive cells within the indicated region. The percentage of PAX6-positive cells was analyzed in triplicate using flow cytometry. The values are reported as the mean ± SEM. Cardiac differentiation was induced in the absence or presence of 1 μg/mL doxycycline during the

undifferentiated and/or differentiated stage. (C) cTnT expression was detected using immunocytochemistry at day 10 after cell differentiation. (D) The numbers represent the percentage of cTnT-positive cells within the indicated region. The percentage of cTnT-positive cells was analyzed in triplicate using flow cytometry. The values are reported as the mean ± SEM.

Figure 8

Effect of YAP overexpression on the differentiation of hESCs into neural and cardiac lineages. Neural differentiation of hESCs transfected with control and YAP5SA lentiviral particles was induced. (A) PAX6 expression was detected using immunocytochemistry at day 10 after cell differentiation. Scale bar, 200 μm. (B) The numbers represent the percentage of PAX-6-positive cells within the indicated region. The percentage of PAX6-positive cells was analyzed in triplicate using flow cytometry. The values are reported as the mean ± SEM. Cardiac differentiation of hESCs transfected with control and YAP5SA lentiviral particles was induced. (C) cTnT expression was detected using immunocytochemistry at day 10 after cell differentiation. (D) The numbers represent the percentage of cTnT-positive cells within the indicated region. The percentage of cTnT-positive cells was analyzed in triplicate using flow cytometry. The values are reported as the mean ± SEM.

Supplementary Files

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