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

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Abstract

Human embryonic stem cells (hESCs) have unique characteristics, such as self-renewal and pluripotency, which are distinct from those of other cell types. These characteristics of hESCs are tightly regulated by complex signaling mechanisms. In this study, we demonstrate that yes-associated protein (YAP) functions in an hESC-specific manner to maintain self-renewal and survival in hESCs. hESCs were highly sensitive to YAP downregulation to promote cell survival. Interestingly, hESCs displayed dynamic changes in YAP expression in response to YAP downregulation. YAP was critical for the maintenance of self-renewal. Additionally, the function of YAP in maintenance of self-renewal and cell survival was hESC-specific. Doxycycline upregulated YAP in hESCs and attenuated the decreased cell survival induced by YAP downregulation. However, decreased expression of self-renewal markers triggered by YAP downregulation and neural/cardiac differentiation were affected by doxycycline treatment. Collectively, the results reveal the mechanism underlying the role of YAP and the novel function of doxycycline in hESCs.

Keywords
Human embryonic stem cells · Yes-associated protein · Self-renewal · Survival · Doxycycline

Abbreviations
bFGF Basic fibroblast growth factor
CAG Cytomegalovirus early enhancer/chicken β actin
cDNA Complementary DNA
cTNT Cardiac troponin T
DMEM/F-12 Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12
EB Embryoid body
EDTA Ethylenediaminetetraacetic acid
FOX2 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
MESPI Mesoderm Posterior BHLH Transcription Factor 1
Nkx2.5 NK2 Homeobox 5
NEAA Nonessential amino acids
OCT4 Octamer-binding transcription factor 4
PAX6 Paired Box 6

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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

Introduction

Human pluripotent stem cells (hPSCs) exhibit self-renewal, in which proliferation is sustained and immortality for an extended period in culture. The cells are also pluripotent and can differentiate to form cells of all three germ layers. hPSCs provide an inexhaustible source to study early mammalian developmental processes, develop cell therapies, and enable drug discovery [1–3]. To understand the molecular mechanisms underlying the self-renewal capacity of hPSCs, it would be useful to identify cellular components that permit precise regulation. Several pathways that contribute to the regulation of hPSC self-renewal have been identified. In addition, the roles of various factors, including extrinsic stimuli, intrinsic signal molecules, transcription factors, and epigenetic modifiers, have been elucidated in hPSCs [1, 4]. In the present study, we focused on the role of the yes-associated protein (YAP) in the maintenance of hPSC survival and self-renewal.

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

In the present study, we investigated the effect of YAP up- or downregulation in hESCs. Importantly, we demonstrate that YAP is specific to hESCs. The findings advance the understanding of the self-renewal and pluripotency of hESCs.

Materials and methods

hESC 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 DMEM/F-12 (Life Technologies) containing 20 ng/mL basic fibroblast growth factor (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 plates coated with Geltrex (Life Technologies). Cells were subcultured as small clusters every 4 days using 0.5 mM EDTA solution.

Genetic regulation of YAP expression by lentiviral vectors

YAP short hairpin RNA (shRNA) expression vectors were used to specifically downregulate YAP. YAP target sequences (5′–TGACTCAGGATGGAGAATTT–3′ for shYAP and 5′–GACTCAGGATGGAGAATTTA–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 Colorado in Boulder (Addgene plasmids #10878 and #10879) [12]. For overexpression of YAP, we used YAP5SA that was mutated from five serine amino acids to alanine to prevent protein degradation by phosphorylation. To construct a lentiviral vector overexpressing YAP5SA, we subcloned the YAP5SA gene into the lentiviral vector under the regulation of the cytomegalovirus early enhancer/chicken β-actin (CAG) promoter. The YAP5SA vector was a gift from Dr. Kun-Liang Guan, David Root, University of Colorado in Boulder (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, YAP shRNA or control shRNA plasmids were co-transfected with the lentivirus-packaging plasmids (vesicular stomatitis virus G-expressing envelope plasmid and another plasmid containing the gag, pol, and rev genes
(kindly provided by Dr. Yibing Qyang) into HEK293T cells using the X-tremeGene HP DNA transfection reagent (Roche Applied Science, Penzburg, Germany) at 37 °C in a 5% CO₂ atmosphere 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 × 10⁵ hESCs dissociated into single cells were plated in a 24-well plate with concentrated virus-containing medium at a low titer (4 × 10⁶ IU/mL) or high titer (2 × 10⁷ IU/mL) for 24 h at 37 °C, followed by 2 days of culture. The cells were selected by treatment with 2 μg/mL puromycin (Life Technologies).

Western blot analysis

Cells were lysed in lysis buffer (iNtRON Biotechnology, Seongnam, Korea) by sonication (Vibra-Cell; Sonics, Winooski, VT, USA). Cell lysates were separated by 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (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 (sc-2004, 1:10 000; Santa Cruz Biotechnology) or goat antimouse (sc-2005, 1:10 000; 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 × 10⁴ 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 an iScript™ complementary DNA (cDNA) Synthesis Kit (Bio-Rad, Hercules, CA, USA). Reverse transcription products (2.5 ng cDNA) were amplified using a FastStart Essential DNA Green Master PCR Kit (Roche Applied Science, Penzburg, Germany) and primers. The primers used were 5′–GCATTCCTCAATTTAGAACGGCGTGA–3′ (forward) and 5′–GCCCTTGCAGCCAGAATACACATT–3′ (reverse) for FOXA2, 5′–TATGGTGTGGGCCAAAGACGAA–3′ (forward) and 5′–CCGCTTCCTCCTGAAAGTCAA–3′ (reverse) for SOX17, 5′–GAGAGGAGGACAAGTCC–3′ (forward) and 5′–CACTTGGGAACTGAGAC–3′ (reverse) for Nestin, 5′–GCCTATGCCACCCCCAGT–3′ (forward) and 5′–TCACTTCCGGGAACTGAC–3′ (reverse) for PAX6; 5′–GTGGTTCGCCGCTCTGCTTCT–3′ (forward) and 5′–TCTATCCAGTGCTCTACAGC–3′ (reverse) for NKKX2.5, 5′–CCAAGGACTAATCTGATC–3′ (forward) and 5′–TTTCCTGTTCTCCCAAA–3′ (reverse) for MEF2C; and 5′–CTTCCCTGAGACCCAGTT–3′ (forward) and 5′–CAGGGTTGGGTACCTGTC–3′ (reverse) for Brachyury T. Samples were cycled 45 times using a LightCycler 96 Real-Time System (Roche Applied Science). RT-qPCR was performed for 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 triplicate. 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 × 10³ 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. Samples were cultured 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, EBs were attached to a 60-mm tissue culture dish coated with Matrigel (BD Biosciences, San Jose, CA, USA) and cultured for 4 days to induce neural induction in DMEM/F12 medium containing 1×N2 supplement and 1× nonessential amino acids (both from Life Technologies). The cells were analyzed on EB day 10. The neural differentiation protocol used in this study is depicted in Fig. 7a.
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 and 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 Rho-associated protein kinase inhibitor; this day was defined as day–4. The following day, the medium was changed to mTeSR™1 without Y27632. The medium was subsequently refreshed each day for the next 2 days. On day–1, the medium was changed 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, a glycogen synthase kinase-3 (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 supplementation (day 1), and the cells were cultured for 2 days. On day 3, we replaced the medium with RPMI/B-27 minus insulin medium supplemented with 7 μM XAV939, a tankyrase inhibitor, and 5 μM IWP2, a porcupine inhibitor, followed by culture for 48 h. The culture medium was replaced with RPMI/B-27 minus insulin medium, and the cells were cultured starting on day 5. On day 7, the medium was replaced with RPMI/B-27 (RPMI1640 and 50× B-27 supplement) and was changed every other day thereafter. Analyses were performed on day 10. The cardiac differentiation protocol used in this study is depicted in Fig. 7d.

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. The cells were incubated with the primary antibody overnight at 4 °C, followed by incubation with Alexa 488-conjugated secondary antibody for 3 h at room temperature. The samples were analyzed using a FACS Aria III flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

The results are reported as mean ± S.E.M. Differences between mean values were analyzed using Student’s t-test. Statistical 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 titer (shYAP-high, 2 × 10^7 IU/mL) in a dose-dependent manner. Although significant 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 sequences (shYAP#2). YAP expression was significantly downregulated by shYAP#2 at 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. S1a and S1b). These results were consistent with those
of the experiments using shYAP. To examine whether shYAP-high affects cell viability in other cell types, we transfected HEK293T and A549 cells with a high titer of shYAP. As shown in Supplementary Fig. S2a, YAP expression was significantly downregulated by shYAP-high. However, unlike in hESCs, no significant cell death was observed in these cell types (Supplementary Fig. S2b). 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, morphological changes in differentiated cells 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), morphological changes in differentiated cells and decreased expression of OCT4, NANOG, and SOX2 were observed in the shYAP-low group (Fig. 2c and e). 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. S2c). 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 NKK 2.5) were increased in the shYAP-low group (Fig. 2g–i). 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 the genetic regulation of YAP was confirmed through chemical regulation. Verteporfin is a Food and Drug Administration (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- and time-dependent manner (Fig. 3A and B). 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. 3C and D). 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. 3E, 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 concentrations < 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. S3a and S3b). Treatment of hESCs with 300 nM verteporfin for 24 h significantly downregulated OCT4, NANOG, and SOX2 expression (Fig. 3F). As in shYAP-low, a paradoxical increase in YAP expression was induced by treatment with verteporfin at a concentration below 50 nM for 72 h, but the decreased expression of OCT4, NANOG, and SOX2 was maintained under these conditions (Fig. 3G and H). 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 by inducing a paradoxical increase in 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 low or high titer YAP5SA. As shown in Fig. 4a, YAP expression was significantly upregulated by YAP5SA transfection at low titer (YAP5SA-low, 4 × 10^6 IU/mL) and high titers (2 × 10^7 IU/mL) in a dose-dependent manner. CCK-8 assay results showed that YAP5SA significantly induced hESC proliferation (Fig. 4b). YAP5SA also significantly upregulated the expression of hESC self-renewal marker proteins (Fig. 4c). These collective findings confirmed that YAP plays a role in the maintenance of self-renewal and proliferation of hESCs. Interestingly and paradoxically, YAP expression levels were decreased in the YAP5SA group when these cells were cultured for a longer duration (post-transfection day 14). The expression levels of OCT4, NANOG, and SOX2 were also decreased in the YAP5SA group (Fig. 4d). These results suggest that YAP upregulation induces a paradoxical decrease in YAP expression and the expression of self-renewal marker proteins was decreased because of decreased YAP expression in 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. 5a and b). YAP phosphorylation was significantly downregulated by doxycycline treatment (Fig. 5c). The effect of doxycycline on YAP phosphorylation was transient, as YAP phosphorylation was recovered 4 h after doxycycline
Fig. 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, D Cells were treated with 300 nM verteporfin for the indicated time (0–240 min). YAP and p-YAP expression was detected using Western blot analysis. *P<0.05; **P<0.001 vs. 0 min. E 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 denotes 200 μm. *P<0.05; ***P<0.005; ****P<0.001 vs. 0 nM. F Cells were incubated with or without 300 nM verteporfin for 24 h and OCT4, NANOG, and SOX2 expression was detected using Western blot analysis. G Cells were treated with verteporfin at the indicated concentration (0–50 nM) for 72 h, and YAP expression was detected using Western blot analysis. H 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
treatment. Moreover, doxycycline-induced YAP upregulation was not observed in HEK293T or A549 cells (Supplementary Fig. S4a and S4b). 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. 6a). Moreover, the 300 nM verteporfin-induced decrease in cell viability was recovered by doxycycline treatment (Fig. 6b). 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. 6c). However, doxycycline treatment for 72 h significantly upregulated OCT4, SOX2, and YAP expression (Fig. 6d). 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, NANOG, and SOX2 were not affected by doxycycline treatment for 24 or 72 h (Fig. 6f and e). 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 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 d, no significant differences in differentiation into cTNT-positive cells were observed between control and YAP5SA transfected hESCs by immunocytochemistry and flow cytometry. We next examined the expression levels of YAP in neural or cardiac cells differentiated from control and YAP5SA-transfected hESCs. As shown in Fig. 8e and f, there were no significant differences in YAP expression levels between control and YAP5SA-transfected cells in both cell types. 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

Fig. 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+. b Cells were pretreated with 300 nM verteporfin for 30 min and then incubated with or without 1 μg/mL doxycycline for 24 h. Brightfield images were acquired, and cell viability was measured using a CCK-8 assay. Scale bar denotes 200 μm. \(\#P < 0.05\); \(\###P < 0.005\) vs. doxycycline+/verteporfin–; \(\#P < 0.05\) vs. doxycycline+/verteporfin+. 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. 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.

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Fig. 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 Schematic of the protocol used to differentiate hESCs into neural lineages. b PAX6 expression was detected using immunocytochemistry at day 10 after cell differentiation. Scale bar denotes 200 μm. c 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 mean ± SEM. Cardiac differentiation was induced in the absence or presence of 1 μg/mL doxycycline during the undifferentiated and/or differentiated stage. d Schematic of the protocol used to differentiate hESCs into cardiac lineages. e cTNT expression was detected using immunocytochemistry at day 10 after cell differentiation. f 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 mean ± SEM. hESC-derived neural (g) or cardiac (h) cells were treated with 1 μg/mL doxycycline for 24 h, and YAP expression was detected using Western blot analysis.
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. 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 discovered during the establishment of the conditional YAP knockdown system. Before establishing the doxycycline-induced YAP knockdown system, we tested whether doxycycline treatment affected YAP expression. Doxycycline significantly increased YAP expression. This finding is both interesting and controversial, because many researchers have used a conditional gene regulation system that generally uses doxycycline even in differentiated neural or cardiac cells from hESCs. 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 under any 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.

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

Declarations

Conflict of interest No competing interests were disclosed by authors.

Ethical approval and consent to participate All experiments involving human embryonic stem cells (hESCs) were approved by the Public Institutional Bioethics Committee (approval no. PI-201910-41-001) designated by the Ministry of Health and Welfare and the Korea Centers for Disease Control and Prevention.

Consent for publication We have obtained consent to publish this paper from all the participants of this research.

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