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RESEARCH ARTICLE

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# Yes-associated protein activation potentiates glycogen synthase kinase-3 inhibitor-induced proliferation of neonatal cardiomyocytes and iPS cell-derived cardiomyocytes

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## Abstract

Because mammalian cardiomyocytes largely cease to proliferate immediately after birth, the regenerative activity of the heart is limited. To date, much effort has been made to clarify the regulatory mechanism of cardiomyocyte proliferation because the amplification of cardiomyocytes could be a promising strategy for heart regenerative therapy. Recently, it was reported that the inhibition of glycogen synthase kinase (GSK)-3 promotes the proliferation of neonatal rat cardiomyocytes (NRCMs) and human iPS cell-derived cardiomyocytes (hiPSC-CMs). Additionally, Yes-associated protein (YAP) induces cardiomyocyte proliferation. The purpose of this study was to address the importance of YAP activity in cardiomyocyte proliferation induced by GSK-3 inhibitors (GSK-3Is) to develop a novel strategy for cardiomyocyte amplification. Immunofluorescent microscopic analysis using an anti-Ki-67 antibody demonstrated that the treatment of NRCMs with GSK-3Is, such as BIO and CHIR99021, increased the ratio of proliferative cardiomyocytes. YAP was localized in the nuclei of more than 95% of cardiomyocytes, either in the presence or absence of GSK-3Is, indicating that YAP was endogenously activated. GSK-3Is increased the expression of β-catenin and promoted its translocation into the nucleus without influencing YAP activity. The knockdown of YAP using siRNA or pharmacological inhibition of YAP using verteporfin or CIL56 dramatically reduced GSK-3I-induced cardiomyocyte proliferation without suppressing β-catenin activation. Interestingly, the inhibition of GSK-3 also induced the proliferation of hiPSC-CMs under sparse culture conditions, where YAP was constitutively activated. In contrast, under dense culture conditions, in which YAP activity was suppressed, the proliferative effects of GSK-3Is on hiPSC-CMs were not detected. Importantly, the activation of YAP by the knockdown of α-catenin restored the proproliferative activity of GSK-3Is. Collectively, YAP activation potentiates the GSK-3I-induced

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proliferation of cardiomyocytes. The blockade of GSK-3 in combination with YAP activation resulted in remarkable amplification of cardiomyocytes.

KEYWORDS
cardiomyocyte, catenins, cell proliferation, regeneration, signal transduction

## 1 | INTRODUCTION

As mammalian cardiomyocytes largely lose cell proliferative activity immediately after birth, cardiomyocyte loss results in cardiac dysfunction, leading to heart failure. Therefore, the induction of cardiomyocyte proliferation could be a promising therapeutic strategy against heart failure (Bergmann et al., 2015). To date, several studies have been conducted to identify signaling pathways that promote cardiomyocyte proliferation. Several pathways, including the Yes-associated protein (YAP) pathway and the inhibitory pathway of glycogen synthase kinase (GSK)-3 signaling, have been identified as candidates for cardiomyocyte proliferation therapy (Singh et al., 2019; Zheng et al., 2021).

Recently, much attention has been paid to GSK-3 inhibitors (GSK-3Is) because the inhibition of GSK-3 by 6-bromoindirubin-3′-oxine (BIO) activates the β-catenin-signaling pathway and promotes cell cycle progression in neonatal rat cardiomyocytes (NRCMs) and human iPS cell-derived cardiomyocytes (hiPSC-CMs) (Tseng et al., 2006; Uosaki et al., 2013). Similarly, CHIR-99021, another GSK-3I, promotes cardiomyocyte proliferation via the β-catenin pathway (Sharma et al., 2018; Titmarsh et al., 2016). Although GSK-3Is strongly promote the proliferation of NRCMs and hiPSC-CMs in vitro, the administration of these drugs to adult mammals results in a limited induction of proliferating cardiomyocytes in vivo (Buikema et al., 2020), suggesting that other signaling pathways potentiate the GSK-3I-mediated proliferation of NRCMs and hiPSC-CMs.

YAP plays an important role in controlling organ size by regulating cell proliferation (Dong et al., 2007; Zhao et al., 2007). Dephosphorylated YAP translocates to the nucleus and enhances cell cycle activity (Lin et al., 2014). YAP is essential for normal heart growth (von Gise et al., 2012). In cardiomyocytes, YAP activity is regulated by a wide range of signaling pathways, including the Hippo and α-catenin pathways (Heallen et al., 2011; Li et al., 2015). Interestingly, the activation of YAP promotes the proliferation of neonatal cardiomyocytes and hiPSC-CMs (Mills et al., 2017; Xin et al., 2011); however, the biological significance of YAP in GSK-3I-induced cardiomyocyte proliferation remains to be fully addressed.

In this study, we examined the effects of YAP activity on the GSK-3I-induced proliferation of NRCMs. YAP was endogenously activated in NRCMs, in the presence or absence of GSK-3Is. GSK-3Is robustly increased the number of proliferating cardiomyocytes, whereas the inhibition of endogenous YAP activity remarkably suppressed the cardiomyocyte proliferation induced by GSK-3Is in NRCMs. The proproliferative effects of GSK-3Is were also observed in hiPSC-CMs under sparse culture conditions. In contrast, under dense culture conditions, YAP activity was suppressed, and GSK-3Is could not significantly induce proliferation. However, the activation of YAP by α-catenin knockdown restored the proliferative effects of GSK-3Is. These data suggest that YAP activation potentiates cardiomyocyte proliferation via GSK-3Is.

## 2 | MATERIALS AND METHODS

### 2.1 | Inhibitors

BIO (Selleck Chemicals) and CHIR99021 (Cayman Chemicals) were used as GSK-3Is. YAP inhibitors, verteporfin and CIL56, were purchased from Cayman Chemicals.

### 2.2 | NRCM culture

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals with the approval of the Animal Experimentation Committee of Osaka University and Institutional Animal Care (the approval number: Douyaku R03-16). Primary cultures of cardiac myocytes were prepared from the ventricles of 1–2-day-old Wistar rats, as described previously (Wahyuni et al., 2021). Briefly, hearts were digested with a solution containing 0.1% collagenase type IV and 0.1% trypsin. To remove non-cardiomyocytes, total cells were precultured in culture dishes for 90 min, and floating cells were used as NRCMs. NRCMs were cultured in DMEM supplemented with 10% FBS. All experiments were performed 24 h after serum depletion. For immunoblot and immunofluorescence microscopic analyses, cells were seeded on 24-well plates at $2.0–2.5 \times 10^5$ cells/1.9 $cm^2$ well.

### 2.3 | Cardiac differentiation of human-induced pluripotent stem cells (hiPSCs)

hiPSCs (253G1; Riken) were used in this study (Nakagawa et al., 2008). Cardiomyogenic differentiation was induced as previously described with some modifications (Matsuura et al., 2012). Briefly, the cells were cultured in StemPro 34 medium (Thermo Fisher Scientific) containing 2 mM L-glutamine, 50 μg/ml ascorbic acid, and 400 μM 1-thioglycerol. hiPSCs were dissociated using Accumax (Nacalai Tesque), transferred to a bioreactor, and supplemented with several human recombinant proteins, including BMP4, activin A, bFGF, and VEGF, and small molecules such as IWR-1 and IWP-2

(Sigma-Aldrich/Merck) on Days: 0–1, BMP4; 1–4, activin A, BMP4, and bFGF; 4–6, IWR-1 and IWP-2; and after Day 6, VEGF and bFGF. hiPSC-CMs were cultured in DMEM with 10% FBS and then in serum-free medium for 24 h before use.

### 2.4 | Transfection of siRNA

Twenty-four hours after seeding, the cells were transfected with siRNA for 48 h using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's protocol. The medium was replaced with serum-free medium. The siRNA sequences used in this study are listed in Table S1.

### 2.5 | Immunofluorescent microscopic analyses

Immunofluorescence microscopic analyses were performed as previously described (Wahyuni et al., 2021). Briefly, the cells were fixed with 4% paraformaldehyde in PBS. After membrane permeabilization, primary antibodies (Table S2) were applied overnight at 4°C, followed by reaction with Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies. The nuclei were stained with DAPI. Cell images were digitized using a fluorescence microscope (CV8000, Yokogawa). A researcher who was blinded to the assay conditions randomly selected more than 100 cardiomyocytes from each group. Fluorescence intensities were quantified using ImageJ (1.53e). Ki-67$^+$ cardiomyocytes were counted by a researcher who was blinded to the assay conditions. The ratio of Ki-67$^+$ cardiomyocytes to total cardiomyocytes was calculated.

### 2.6 | Immunoblot analyses

Immunoblot analyses were performed as previously described (Matsumoto et al., 2019). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Merck Millipore), which were incubated with primary antibodies (Table S3) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at 23–28°C. An ECL system (Promega) was used for detection. Signals were detected with ImageQuant LAS 4010 using the ImageQuant TL software (GE Healthcare). Protein expression levels were quantified by densitometry using ImageJ.

### 2.7 | Statistical analysis

All data are presented as mean ± SD. Multiple comparisons were performed by one-way ANOVA with Dunnett test or Tukey–Kramer test using Statcel Ver.4 (The Publisher *OMS*). $p < 0.05$ was considered to be statistically significant.

## 3 | RESULTS

### 3.1 | Blockade of endogenous YAP activity suppressed the proliferation of NRCMs induced by BIO, a GSK-3 inhibitor

As previously reported (Tseng et al., 2006), BIO treatment increased the ratio of Ki-67$^+$ cardiomyocytes in a dose-dependent manner (Figure 1a, b). As YAP plays an important role in cardiomyocyte proliferation (von Gise et al., 2012; Xin et al., 2013), we examined the effects of BIO on YAP activity. Immunofluorescence microscopic analysis revealed that YAP was localized in the nuclei of more than 95% of NRCMs in the presence or absence of BIO (Figure 1c, d), suggesting that endogenous YAP was not influenced by BIO. Consistently, BIO had no effect on the expression of YAP and phosphorylated YAP (p-YAP) in NRCMs (Figure 1e, f). We confirmed that CHIR99021, another GSK-3I, also induced cardiomyocyte proliferation (Figure 1g).

Next, NRCMs were transfected with a control siRNA or siRNAs targeting YAP. Immunoblot analysis revealed that YAP knockdown reduced YAP expression (Figure 2a, b). Consistently, the fluorescence intensity of nuclear YAP was lower in NRCMs transfected with siRNAs for YAP than in those transfected with control siRNA (Figure 2c, d). To address the importance of YAP in the BIO-induced proliferation of NRCMs, NRCMs were transfected with YAP siRNA, followed by treatment with BIO. YAP knockdown inhibited the basal proliferative activity of NRCMs in the absence of GSK-3Is, suggesting that YAP mediates NRCM proliferation independently of the effects of GSK-3Is. Importantly, the knockdown of endogenous YAP remarkably suppressed BIO-induced NRCM proliferation (Figure 2e). Consistently, the pharmacological inhibition of YAP by verteporfin or CIL56 reduced BIO-induced NRCM proliferation (Figure 2f). CHIR99021-induced cardiomyocyte proliferation was inhibited by transfection with YAP siRNA (Figure 2e). Thus, in addition to its independent effects, YAP exhibits proproliferative activity by enhancing the effects of GSK-3Is in NRCMs.

### 3.2 | Inactivation of YAP did not influence the β-catenin signaling pathway in NRCMs

Previous studies have reported that the GSK-3I-induced promotion of cardiomyocyte proliferation is mediated by the increased expression of β-catenin (Buikema et al., 2020; Quaife-Ryan et al., 2020; Tseng et al., 2006). Treatment with BIO increased the expression of β-catenin (Figure 3a, b) and promoted the translocation of β-catenin into the nucleus (Figure 3c). As the promotion of NRCM proliferation induced by GSK-3I was inhibited by knockdown using siRNA for β-catenin (Figure 3d), GSK-3I promotes proliferation via the GSK-3/β-catenin pathway in NRCMs, as previously reported (Tseng et al., 2006). Knockdown of β-catenin did not influence YAP expression and the

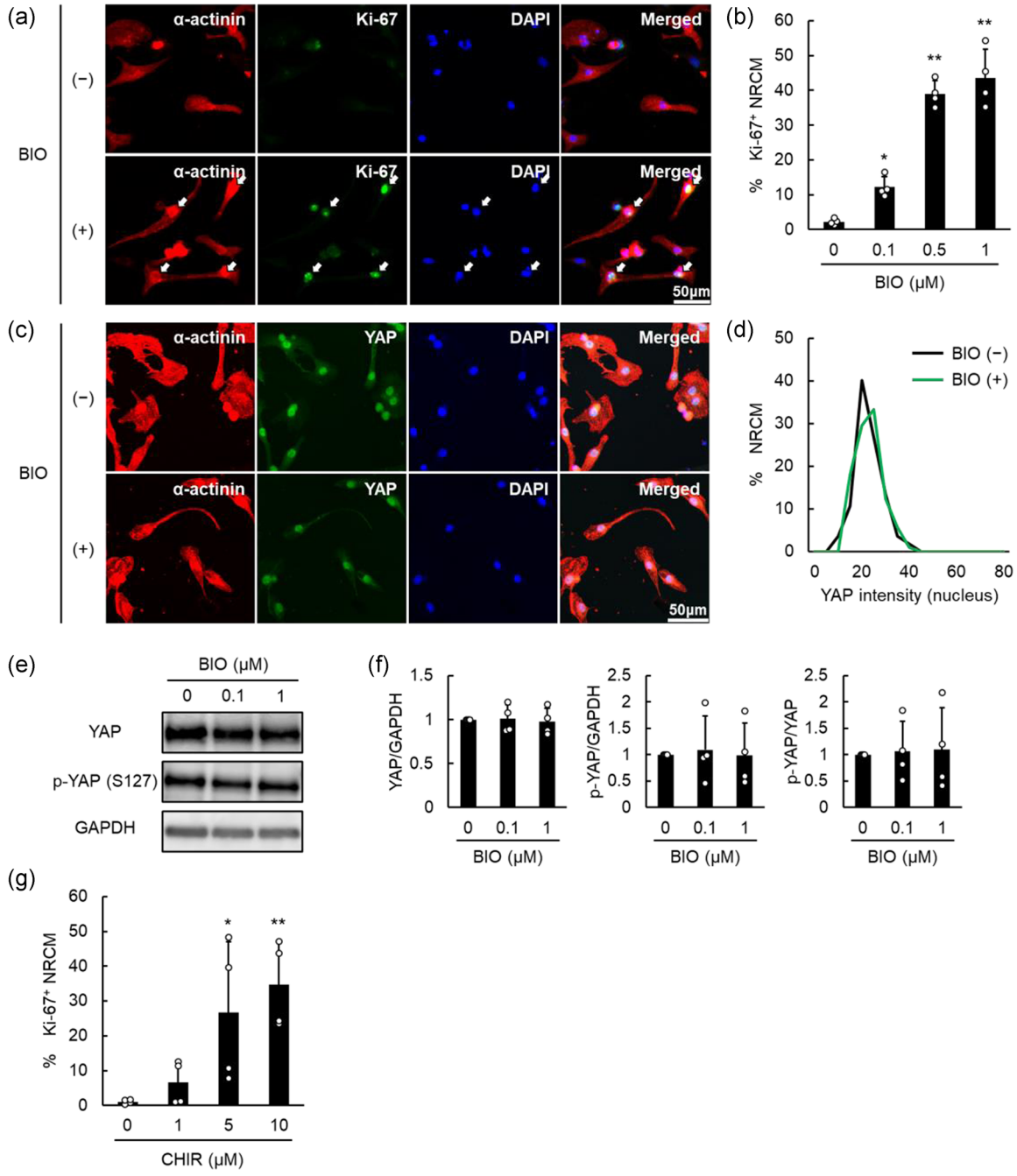


**FIGURE 1** GSK-3Is promoted the proliferation of NRCMs without activating YAP. (a–f) NRCMs were treated with BIO at the indicated concentrations for 24 h. (a, b) Cells were stained with anti-α-actinin (red) and anti-Ki-67 (green) antibodies. Nuclei were stained with DAPI. (a) Representative images (BIO: 1 μM). Arrows indicate Ki-67$^+$α-actinin$^+$ cells. (b) Ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. (c, d) Cells were stained with anti-α-actinin (red) and anti-YAP (green) antibodies. (c) Representative images. (d) Fluorescence intensity of nuclear YAP was measured ($n$ > 100 cells/group). (e, f) Immunoblot analysis was performed with anti-YAP, anti-p-YAP (S127), and anti-GAPDH antibodies. (e) Representative images. (f) Quantitative data are shown. (g) NRCMs were treated with CHIR-99021 (CHIR) at the indicated concentrations for 24 h. The ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N$ = 4), $^*p < 0.05$, $^{**}p < 0.01$ versus basal control by one-way ANOVA followed by Dunnett test.

expression of β-catenin was not altered by treatment with siRNAs for YAP (Figure 3e, f). Consistently, GSK-3I induced the nuclear localization of β-catenin in NRCMs transfected with either control or YAP siRNA (Figure 3g, h). Collectively, endogenous YAP activity potentiated GSK-3I-induced proliferation without modulating the β-catenin pathway.

### 3.3 | Inactivation of endogenous YAP inhibited GSK-3I-induced proliferation of hiPSC-CMs under sparse culture conditions

GSK-3Is promote the proliferation of hiPSC-CMs under sparse culture conditions (Buikema et al., 2020). Consistently, both BIO

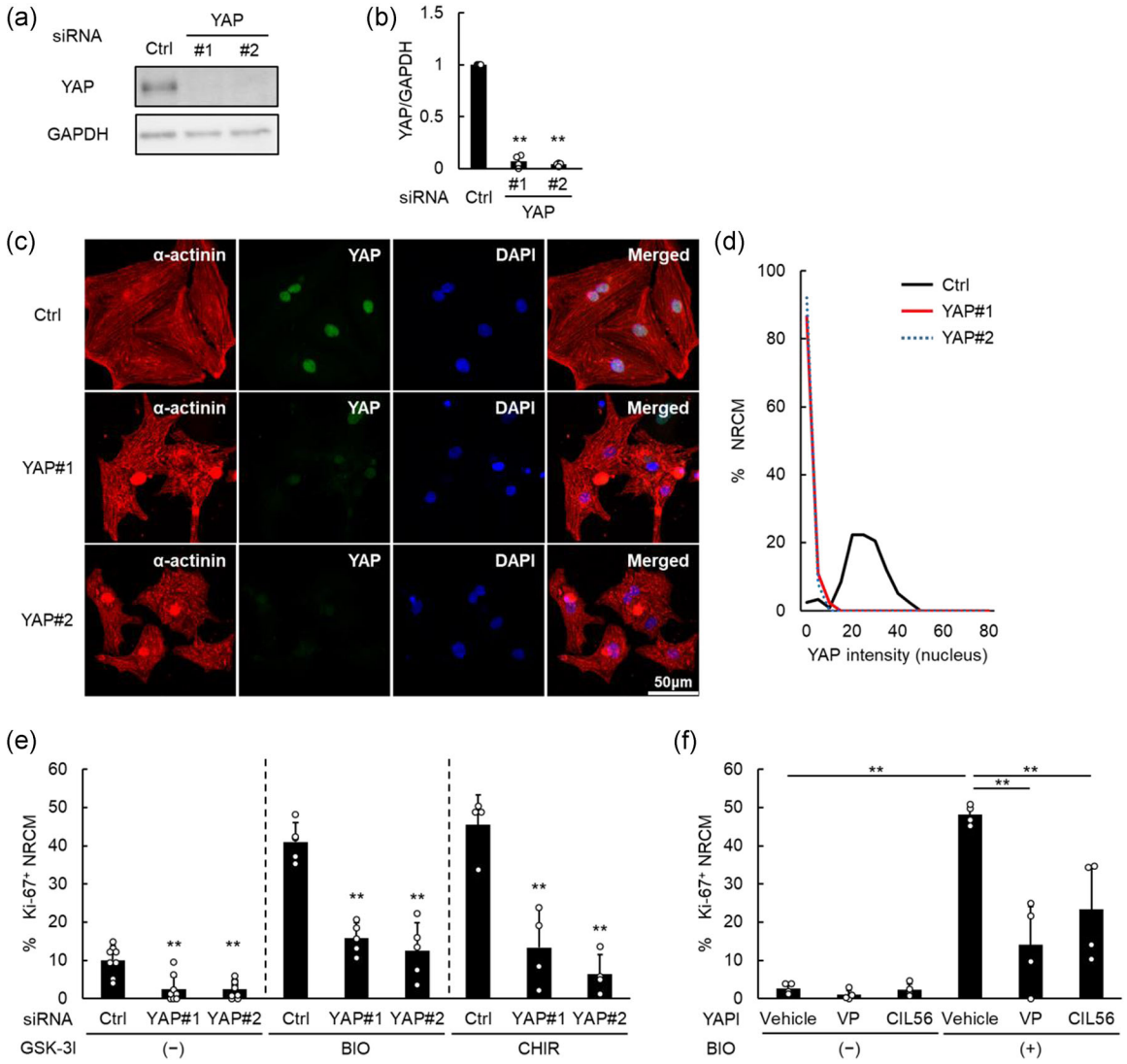


**FIGURE 2** Inactivation of endogenous YAP inhibited the BIO-induced proliferation of NRCM. (a–e) NRCMs were transfected with control siRNA (Ctrl) or siRNAs for YAP (YAP#1 and YAP#2) at 10 nM. (a, b) Immunoblot analysis was performed with anti-YAP and anti-GAPDH antibodies. (a) Representative images. (b) Data are quantitatively shown as mean ± SD ($N = 4$), $^{**}p < 0.01$ versus control by one-way ANOVA followed by Dunnett test. (c, d) Cells were stained with anti-α-actinin (red) and anti-YAP (green) antibodies. (c) Representative images. (d) Fluorescence intensity of nuclear YAP was measured ($n > 100$ cells/group). (e) NRCMs, transfected with siRNA, were treated with BIO (1 μM) for 24 h. The ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N = 4$–9), $^{**}p < 0.01$ versus control by one-way ANOVA followed by Dunnett test. (f) NRCMs, pretreated with YAP inhibitors (verteporfin [VP, 0.5 μM] and CIL56 [1 μM]) for 24 h, were cultured with BIO (1 μM) and YAP inhibitors for 24 h. The ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N = 4$), $^{**}p < 0.01$ versus control by one-way ANOVA followed by Tukey–Kramer test.

and CHIR strongly promoted hiPSC-CM proliferation (Figure 4a, b). To clarify whether endogenous YAP was activated in hiPSC-CMs, immunofluorescence microscopic analysis was performed. YAP was mainly localized in the nuclei of hiPSC-CMs either in the presence or absence of CHIR under sparse conditions (Figure 4c, d). Furthermore, CHIR did not affect the expression of YAP or p-YAP in hiPSC-CMs (Figure 4e, f). Notably, the pharmacological inhibition of YAP significantly suppressed the hiPSC-CM proliferation induced by GSK-3I (Figure 4g), indicating that endogenous YAP potentiated the GSK-3I-induced proliferation of hiPSC-CMs.

### 3.4 | Activation of YAP by the knockdown of αT-catenin restored the GSK-3I-induced proliferation of hiPSC-CMs under dense culture condition

As CHIR does not increase the proliferation of hiPSC-CMs under dense culture conditions (Buikema et al., 2020), we examined whether YAP activity was endogenously activated in hiPSC-CMs under dense culture conditions. Immunofluorescence microscopic analysis demonstrated that the fluorescence intensities of YAP were lower in hiPSC-CMs under dense culture conditions than under sparse conditions (Figure 5a, b). Because the cardiac-specific ablation

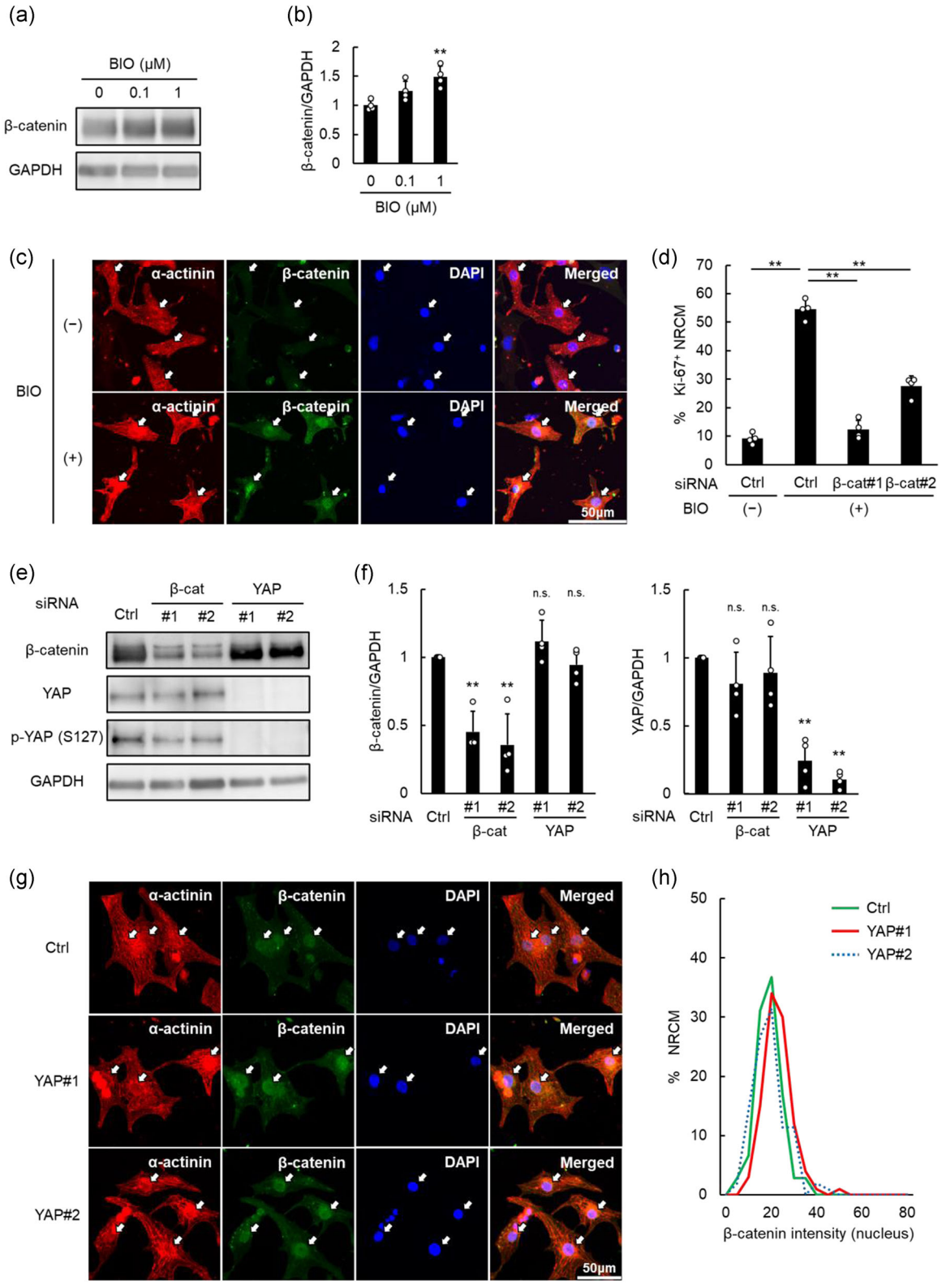


**FIGURE 3** (See caption on next page)

of α-catenin activates YAP (Li et al., 2015; Vite et al., 2018), we focused on α-catenin and observed that αT-catenin accumulated during the cell–cell adhesion of hiPSC-CMs under dense culture conditions (Figure 5c). Using siRNA against αT-catenin, we examined the activity of YAP (Figure 5d, e). Knockdown of αT-catenin increased the expression of YAP without altering that of p-YAP/YAP (Figure 5f, g), indicating that αT-catenin knockdown increases the expression of nonphosphorylated, activated YAP under dense culture conditions. Finally, the knockdown of αT-catenin enhanced the ratio of Ki-67-positive cardiomyocytes in the presence of CHIR but not in the absence of CHIR (Figure 5h), indicating that YAP activation potentiates the proliferative activity induced by GSK-3I under dense conditions.

## 4 | DISCUSSION

In this study, we addressed the involvement of endogenous YAP activity in the GSK-3I-induced proliferation of NRCMs and hiPSC-CMs. As previously reported, GSK-3Is promote the proliferation of NRCMs through β-catenin activation (Tseng et al., 2006). Interestingly, more than 95% of the nuclei were positively stained with anti-YAP antibodies, either in the presence or absence of GSK-3Is. The pharmacological inhibition or knockdown of YAP dramatically reduced GSK-3I-induced cardiomyocyte proliferation without altering β-catenin expression. β-Catenin knockdown suppressed GSK-3I-induced NRCM proliferation without influencing YAP activity. Importantly, although the inhibition of GSK-3 promoted the proliferation of hiPSC-CMs under sparse culture conditions, GSK-3I-induced proliferation was limited under dense culture conditions. However, the activation of YAP by the knockdown of αT-catenin restored the proproliferative activity of GSK-3Is. These results indicate that the endogenous activity of YAP potentiates the effects of GSK-3Is on the proliferation of NRCMs and hiPSC-CMs.

Previously, we demonstrated that adult mammalian cardiomyocytes exhibit proliferative activity during myocarditis (Miyawaki et al., 2017). To develop a novel strategy to enhance the proliferative activity of cardiomyocytes, we performed library screening using a fluorescent ubiquitination-based cell cycle indicator system (Sakaue-Sawano et al., 2008) and immunofluorescence microscopic analysis using an anti-Ki-67 antibody. Among the 1360 compounds tested, BIO exhibited the most potent proliferative activity against NRCMs. Therefore, we focused on GSK-3Is in this study.

GSK-3Is promote the proliferation of NRCMs and hiPSC-CMs; however, the relationship between the proliferative effects of GSK-3Is and YAP activity remains unclear. In the absence of GSK-3Is, 10.1 ± 3.8% of cardiomyocytes were positively stained with an anti-Ki-67 antibody in NRCMs, although YAP was activated in more than 95% of NRCMs. Therefore, YAP only weakly promoted cardiomyocyte proliferation. Importantly, the inhibition of YAP reduced cardiomyocyte proliferation more remarkably in the presence of GSK-3Is than in their absence. Considering that GSK-3Is failed to alter YAP activity and that the blockade of YAP did not influence β-catenin activation by GSK-3Is, YAP synergistically enhanced cardiomyocyte proliferation with β-catenin. This finding is consistent with a previous report that the nuclear interaction between YAP and β-catenin regulates cardiomyocyte proliferation and controls heart size in vivo (Heallen et al., 2011).

Recently, Buikema et al. demonstrated that GSK-3Is induce a massive expansion of sparsely cultured hiPSC-CMs but that the induction of hiPSC-CM proliferation is independent of YAP activity (Buikema et al., 2020). In our study, we also observed that GSK-3Is promoted the proliferation of hiPSC-CMs; however, the pharmacological inhibition of YAP partially suppressed GSK-3I-induced hiPSC-CM proliferation. The difference between these studies could be explained by the culture conditions, especially the status of YAP activity. Under these assay conditions, the ratio of nuclear YAP-positive hiPSC-CMs to total hiPSC-CMs was less than 10% under dense culture conditions, whereas that of nuclear YAP-positive hiPSC-CMs was more than 95% under sparse culture conditions in our experiments.

The data presented here explain how GSK-3I fails to induce the proliferation of hiPSC-CMs under dense culture conditions. It has been previously reported that YAP is inactivated by α-catenins, and the ablation of α-catenin genes results in YAP activation, leading to cardiomyocyte proliferation (Li et al., 2015; Vite et al., 2018). In contrast, αT-catenin knockdown failed to induce cardiomyocyte proliferation in the absence of GSK-3I in our study. This difference might be owing to the differences in the experimental conditions. In their studies, cells were cultured in medium containing FBS, whereas

**FIGURE 3** GSK-3 inhibition promoted the proliferation of NRCMs through the activation of β-catenin. (a, b) NRCMs were treated with BIO at the indicated concentrations for 24 h. Immunoblot analysis was performed with anti-β-catenin and anti-GAPDH antibodies. (a) Representative images. (b) Quantitative analysis. Data are shown as mean ± SD ($N = 4$), $^{**}p < 0.01$ versus basal control by one-way ANOVA followed by Dunnett test. (c) NRCMs were treated with BIO (1 μM) for 24 h and stained with anti-α-actinin (red) and anti-β-catenin (green) antibodies. Representative images. Arrows indicate α-actinin$^+$ cells. (d) NRCMs were transfected with control siRNA (Ctrl) or siRNA for β-catenin (β-cat#1 and β-cat#2) at 50 nM, followed by treatment with BIO (1 μM) for 24 h. The ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N = 4$), $^{**}p < 0.01$ versus other conditions by one-way ANOVA followed by Tukey–Kramer test. (e–h) NRCMs, transfected with control siRNA (Ctrl), or siRNA for β-catenin (β-cat #1 and #2) or YAP (YAP #1 and #2), were treated with BIO (1 μM) for 24 h. (e, f) Immunoblot analysis was performed with anti-β-catenin, anti-YAP, anti-p-YAP (S127), and anti-GAPDH antibodies. (e) Representative images. (f) Quantitative data are shown as mean ± SD ($N = 4$). $^{**}p < 0.01$ versus control by one-way ANOVA followed by Dunnett test. n.s., not significant. (g, h) Cells were stained with anti-α-actinin and anti-β-catenin antibodies. (g) Representative images. Arrows indicate α-actinin$^+$ cells. (h) Fluorescence intensity of nuclear β-catenin was measured ($n > 100$ cells/group).

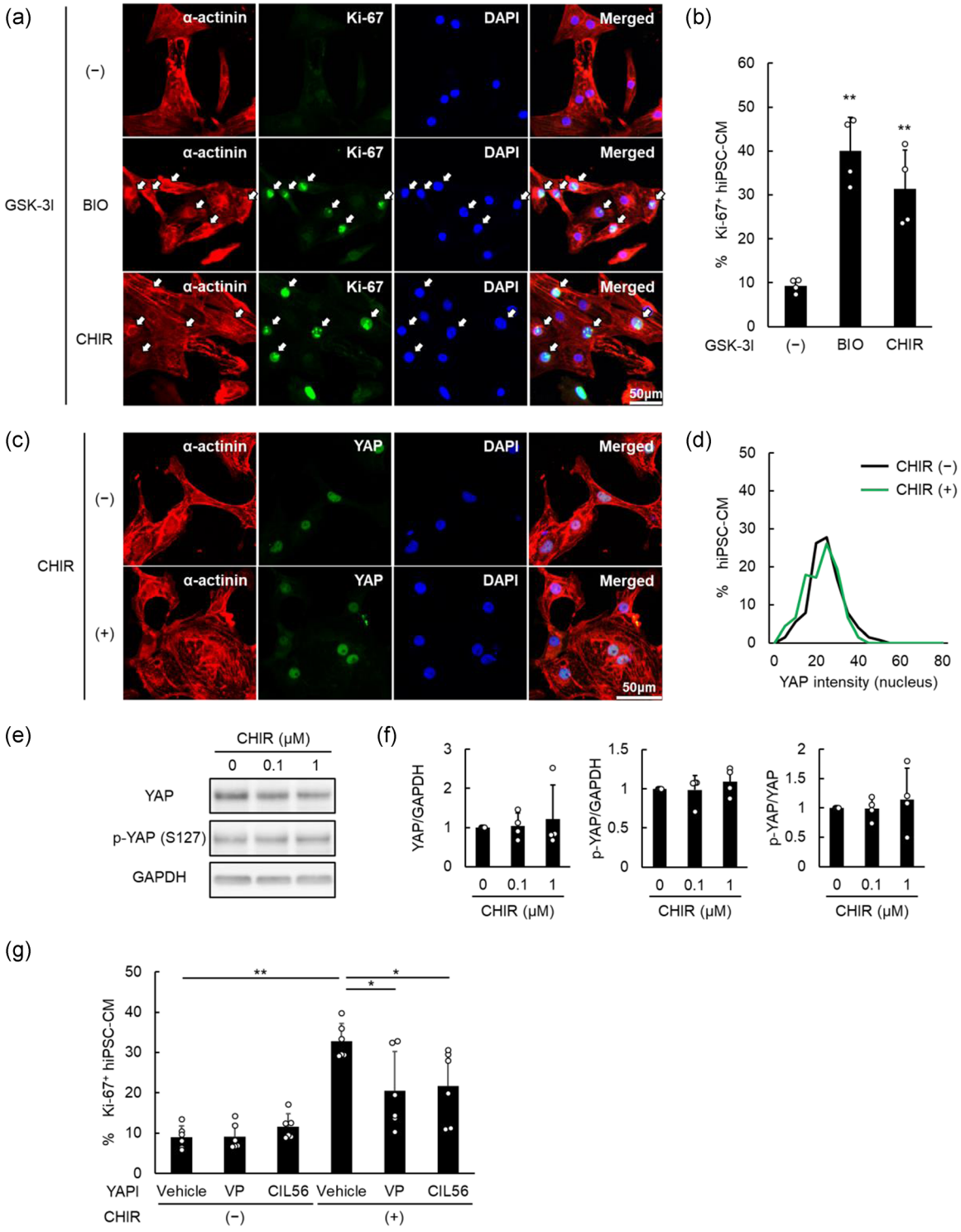


**FIGURE 4** Inactivation of endogenous YAP inhibited the GSK-3I-induced proliferation of hiPSC-CMs under sparse culture condition. (a, b) hiPSC-CMs, cultured under sparse condition (~$3.0 \times 10^4$ cells/cm$^2$), were treated with BIO (0.1 μM) or CHIR (1 μM) for 24 h. Cells were stained with anti-α-actinin (red) and anti-Ki-67 (green) antibodies. (a) Representative images. Arrows indicate Ki-67$^+$α-actinin$^+$ cells. (b) Ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N = 4$), $^{**}p < 0.01$ versus basal control by one-way ANOVA followed by Dunnett test. (c, d) hiPSC-CMs were treated with CHIR (1 μM) for 24 h. Immunofluorescence staining was performed with anti-α-actinin (red) and anti-YAP (green) antibodies. (c) Representative images. (d) Fluorescence intensity of nuclear YAP. (e, f) hiPSC-CMs were treated with CHIR at the indicated concentrations for 24 h. Immunoblot analysis was performed with anti-YAP, anti-p-YAP (S127), and anti-GAPDH antibodies. (e) Representative images. (f) Quantitative data are shown as mean ± SD ($N = 4$). (g) hiPSC-CMs were pretreated with verteporfin (VP, 0.5 μM) or CIL56 (1 μM) for 24 h, followed by treatment with CHIR (1 μM) for 24 h. The ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N = 6$), $^*p < 0.05$ versus other conditions by one-way ANOVA followed by Tukey–Kramer test.

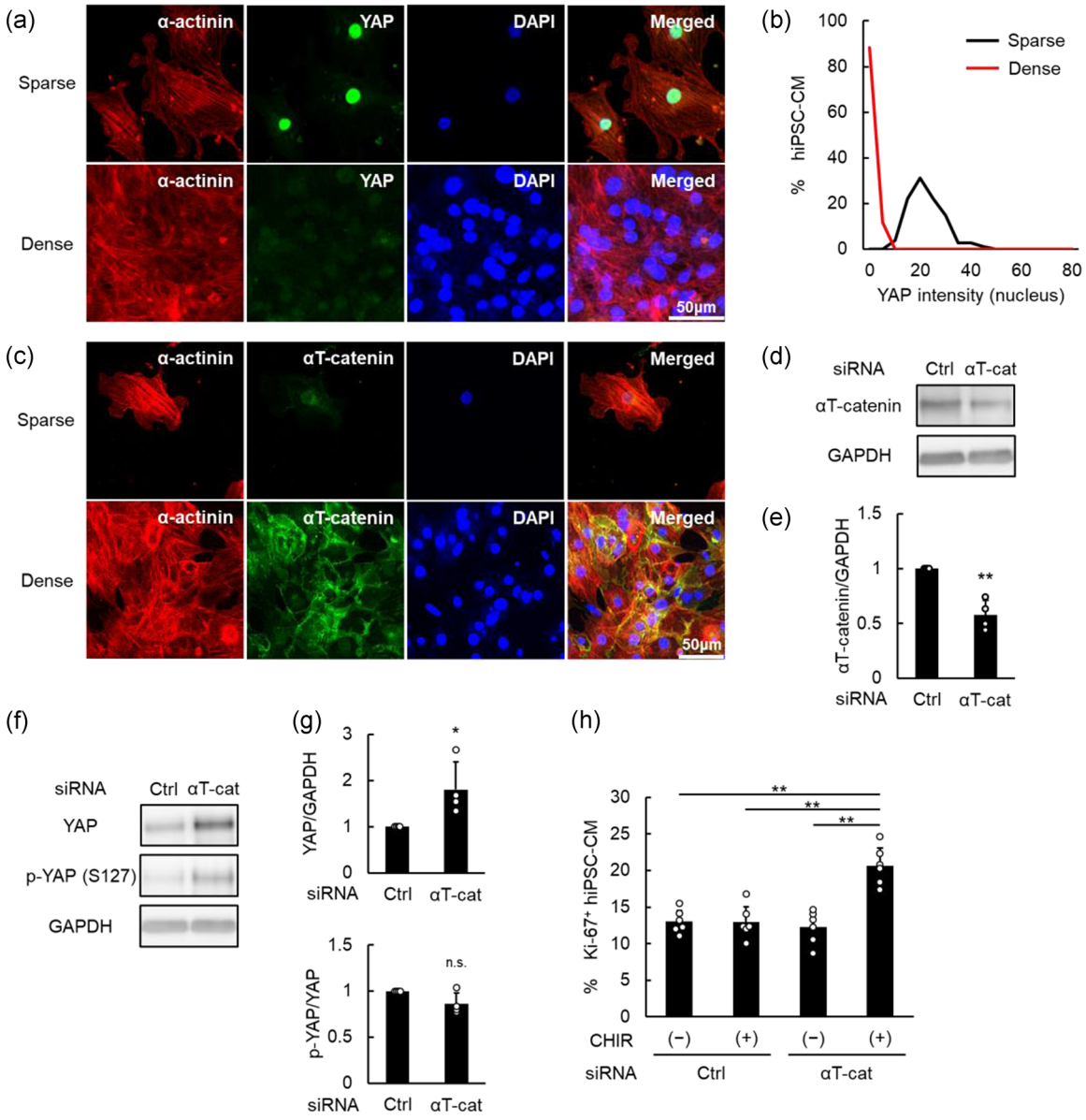


**FIGURE 5** Activation of YAP by knockdown of αT-catenin restored the proliferative effects of GSK-3Is in hiPSC-CMs under dense culture condition. (a–c) hiPSC-CMs were cultured under sparse ($1.1 \times 10^4$ cells/cm$^2$) or dense (~$1.1 \times 10^5$ cells/cm$^2$) conditions and stained with anti-α-actinin (red) and anti-YAP or αT-catenin (green) antibodies. (a, b) Representative images of double staining for α-actinin (red) and YAP (green) and fluorescence intensity of nuclear YAP. (c) Representative images of double staining for α-actinin (red) and αT-catenin (green). (d–h) hiPSC-CMs were cultured under dense (~$1.1 \times 10^5$ cells/cm$^2$) condition and transfected with control siRNA (Ctrl) or siRNA for αT-catenin (αT-cat) at 50 nM. Immunoblot analysis was performed with anti-αT-catenin and anti-GAPDH antibodies (d, e) and anti-YAP, anti-p-YAP (S127), and anti-GAPDH antibodies (f, g). Data are quantitatively shown as mean ± SD ($N = 4$). $^*p < 0.05$, $^{**}p < 0.01$ versus control by Student's $t$ test. n.s., not significant. (h) hiPSC-CMs were treated with CHIR (1 μM) for 24 h. The ratio of Ki-67$^+$α-actinin$^+$ cells to α-actinin$^+$ cells was evaluated. Data are shown as mean ± SD ($N = 6$), $^{**}p < 0.01$ versus other conditions by one-way ANOVA followed by Tukey–Kramer test.

in our experiments, they were cultured in FBS-free medium. Therefore, we performed αT-catenin knockdown experiments in the presence and absence of FBS. Consistent with these results, αT-catenin knockdown resulted in a significant increase in Ki-67$^+$ cells in the presence of FBS, even in the absence of GSK-3Is (Supporting Information Figure). Considering that the ablation of α-catenins enhances the proliferative activities of GSK-3Is even under dense culture conditions, treatment with GSK-3Is in combination with YAP activation is beneficial for the amplification of cardiomyocytes.

In conclusion, we demonstrated that YAP activity potentiated the GSK-3I-induced proliferation of NRCMs and hiPSC-CMs. These results contribute to the understanding of the molecular regulation of cardiomyocyte proliferation, leading to the development of a novel therapeutic strategy that produces new cardiomyocytes in vitro.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

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