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Optimization of the treatment conditions with glycogen synthase kinase-3 inhibitor towards enhancing the proliferation of human induced pluripotent stem cells while maintaining an undifferentiated state under feeder-free conditions

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The small molecule inhibitor CHIR99021 (CHIR) is well known as a selective glycogen synthase kinase-3 inhibitor. The purpose of our study was to optimize the conditions of CHIR supplementation that will enhance the proliferation of human induced pluripotent stem cells (hiPSCs) while maintaining their undifferentiated state under feeder-free conditions in adherent cultures for 4 days. Our results revealed that both of the timing and concentration of CHIR affected cell behaviors of hiPSCs, such as colony formation, cell proliferation, and differentiation. The addition of 1–3 μM CHIR to hiPSCs cultures in the late 2-day period of a 4-day cultivation was effective in enhancing cell proliferation. Treatment with 3 μM CHIR significantly enhanced cell proliferation, but led to differentiation of hiPSCs when the treatment was carried out over 4 days. Treatment with higher concentration of CHIR was also conducive to deviating hiPSCs from their undifferentiated state. Treatment with 10 μM CHIR led to decreased expression of pluripotency-associated genes and increased level of mesoderm marker genes, but failed to provided any growth-promoting effect. Interestingly, when treatment with 1 μM CHIR was confined to the late 2-day period of a 4-day cultivation, cell proliferation was enhanced without detectable deviation from the undifferentiated state as evidenced by the expression levels of pluripotency-associated genes, e.g., OCT3/4, NANOG, SOX2, and REX1. Repeated use of 1 μM CHIR in subcultures provided no adverse effect on the proliferation of hiPSCs. Our results indicated that carefully designed CHIR treatment allows for enhanced proliferation of hiPSCs.

[**Key words:** Cell proliferation; CHIR99021; Human induced pluripotent stem cells; Undifferentiated state; Differentiation]

Induced pluripotent stem cells (iPSCs) present significant potential as a robust source of cells for both basic and applied research, with applications in the fields of regenerative medicine and drug development, because of their self-renewal properties and ability to differentiate into any cell types of the body (1). Human iPSCs (hiPSCs) are conventionally maintained in adherent culture systems using Petri dishes, with or without feeder cells, where the cells form flat and monolayer colonies similarly to human embryonic stem cells (hESCs) (2). The adherent culture system using Petri dishes is widely employed as a pre-culture method for preparing sufficient amount of hiPSCs for lab-scale experimental applications. The outcome of the pre-cultures will directly affect the overall experimental result. Therefore, an efficient and controlled pre-culture cell expansion system should be in place.

In recent years, the use of small molecule inhibitors, which inhibit specific signaling pathways, has represented a powerful approach towards controlling various cell behaviors in pluripotent stem cells (PSCs) (3–5). A selective glycogen synthase kinase-3 (GSK-3) inhibitor has been utilized on various types of PSCs. The inhibition of GSK-3 increases the accumulation of β-catenin that acts as an effector molecule in the Wnt/β-catenin signaling pathway (6,7). Wnt/β-catenin signaling pathway plays a central role in many developmental events, such as somatic cell reprogramming (8,9), cell proliferation (10,11) and cell differentiation (12–16). Sato et al. (3) reported that a GSK-3 inhibitor, 6-bromoindirubin-3′-oxime (BIO), is effective in maintaining the undifferentiated phenotype in both of human and mouse ESCs. Another GSK-3 inhibitor CHIR99021 (CHIR) is known as a key component to prepare 3i medium for sustaining the undifferentiated state (17,18) and also noted for promoting self-renewal of PSCs (10,11). In recent years, CHIR is more frequently employed than other GSK-3 inhibitors for the purpose of controlling self-renewal and differentiation of PSCs. Though CHIR has emerged as an important regulator of GSK-3 or Wnt/β-catenin signaling pathway, it is not fully understood how CHIR affects cell proliferation of hiPSCs in adherent culture systems. In previous studies, CHIR concentrations ranging from 0.1 to 15 μM have been used at different phases of cultivation and for various lengths of time (8–16). It was reported that large differences in the used concentration of CHIR could have opposite effect on self-renewal, pluripotency, and differentiation (9,15). However, it remains controversial whether and how CHIR affects self-renewal and differentiation of PSCs (19,20). In particular, only few studies have optimized the treatment period with CHIR and performed the growth-kinetically-based investigation in the pre-cultures of hiPSCs. Both efficient cell expansion and maintenance of undifferentiated state should be

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satisfied in the pre-cultures in order to prepare the required amount of hiPSCs. We believe that CHIR is a promising molecule to satisfy the requirements for hiPSC pre-cultures.

In the present study, we investigated the effect of the treatment period and concentration of CHIR on cell proliferation and differentiation of hiPSCs to establish an efficient and controlled pre-culture cell expansion system. We optimized the conditions of use of CHIR to promote the proliferation of hiPSCs while maintaining their undifferentiated state.

MATERIALS AND METHODS

Human pluripotent stem cells Human iPS cell lines 201B7 (1) and 253G1 (21) were provided by the RIKEN BRC through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. hiPSCs were maintained on mitomycin C-inactivated SNL76/7 feeder cells (ECACC 07032801, DS Pharma Biomedical Co., Osaka, Japan) in medium consisting of DMEM-Ham's F-12 basal medium (Nacalai Tesque, Kyoto, Japan) supplemented with 20% knockout serum replacement (KSR, Gibco, Grand Island, NY, USA), 1% GLUTAMAX supplement (Gibco), 0.1 mM non-essential amino acids (Life Technologies, Carlsbad, CA, USA), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA), 25 units/ml penicillin and 25 μg/ml streptomycin (Gibco), and 4 ng/ml Recombinant human fibroblast growth factor 2 (FGF2, Oriental Yeast Co., Ltd., Tokyo, Japan). The cells were maintained at 37°C in a humidified 3% CO_2 atmosphere and were grown to form colonies. The medium was changed daily.

Addition of CHIR onto adherent cultures of hiPSCs hiPSCs were cultured in NutriStem hESC XF medium (Biological Industries, Kibbutz Beit-Haemek, Israel) under feeder-free conditions. hiPSC colonies were enzymatically dissociated using Accutase solution (Innovative Cell Technologies, San Diego, CA, USA) and broken into a single cell suspension by gentle pipetting. Y-27632 (ROCK inhibitor, Nacalai Tesque) was used to reduce dissociation-induced apoptosis. Single cell-dissociated hiPSCs were subsequently seeded at 1.0×10^4 cells/cm^2 on a 6-well plate precoated with vitronection (Life Technologies) at a concentration of 0.5 mg/cm^2 and cultured for 4 days. The medium was changed on a daily basis. CHIR99021 (CHIR, Fujifilm Wako, Osaka, Japan) was selected as a typical GSK-3 inhibitor, because CHIR99021 has been most widely used in stem cell researches. Incubation with CHIR was carried out by replacing the medium with NutriStem hESC XF containing varied concentrations of CHIR. As schematically shown in Fig. 1A, CHIR treatment was conducted under different conditions with varying onset periods and CHIR concentrations. Treatment with 3 μM CHIR was performed in three different periods of a 4-day cultivation, i.e., over the entire 4-day culture period, over the early 2-day period, and over the late 2-day period. In parallel, the cultures were also exposed to at various CHIR concentrations (1, 3, or 10 μM) during the late 2-day period.

RNA extraction and qRT-PCR analysis Total RNA extraction and cDNA synthesis were carried out using NucleoSpin RNA (Takara Bio, Otsu, Japan) and ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan), respectively, following the manufacturer's instructions. The qRT-PCR reaction and analysis were performed on a Thermal Cycler Dice (Takara Bio) using Thunderbird SYBR qPCR Mix (Toyobo). The primers used for qRT-PCR were as follows: *Homo sapiens* actin, beta (ACT-β), 5′-TGGCACCCAGCACAATGAA-3′ sense and 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′ antisense; *Homo sapiens* POU class 5 homeobox 1 (POU5F1, OCT3/4), 5′-TGAAGCTGGAGAAGGAGAAGCTG-3′ sense and 5′-GCAGATGGTCGTTTGGCTGA-3′ antisense; *Homo sapiens* Nanog homeobox (NANOG), 5′-TCCAACATCCTGAACCTCAGCTA-3′ sense and 5′-AGTCGGGTTCACCAGGCATC-3′ antisense; *Homo sapiens* SRY (sex determining region Y)-box 2 (SOX2), 5′-GTGAGCGCCCTGCAGTACAA-3′ sense and 5′-GCGAGTAGGACATGCTGTAGGTG-3′ antisense; *Homo sapiens* zinc finger protein 42 (ZFP42, REX1), 5′-GGCCTTCACTCTAGTAGTGCTCA-3′ sense and 5′-CTCCAGGCAGTAGTGATCTGAGT-3′ antisense; *Homo sapiens* T, brachyury homolog (mouse) (T), 5′-TGTGACAGGTACCCAACCCTGA-3′ sense and 5′-ATGGGATTGCAGCATGGATAAAC-3′ antisense; *Homo sapiens* GATA binding protein 4 (GATA4), 5′-TGGTCAGATGGCAGCCAGAG-3′ sense and 5′-TGCTTCGAATTCGTGTTGCAG-3′ antisense; *Homo sapiens* wingless-type MMTV integration site family, member 3A (WNT3A), 5′-TCTACGACGTGCACACCTG-3′ sense and 5′-GAACCTTACAGGGGGTTGG-3′ antisense; *Homo sapiens* Wnt family member 8A (WNT8A), 5′-GTGATGGGTCAAACAATGGA-3′ sense and 5′-ATCCTTTCCCCAAATTCCAC-3′ antisense; *Homo sapiens* SRY (sex determining region Y)-box 17 (SOX17), 5′-CTGCAGGCCAGAAGCAGTGTTA-3′ sense and 5′-CCCAAACTGTTCAAGTGGCAGA-3′ antisense; *Homo sapiens* alpha-fetoprotein (AFP), 5′-CAGCCACTTGTTGCCAACTCA-3′ sense and 5′-GGACATATGTTTCATCCACCACCA-3′ antisense; *Homo sapiens* SRY (sex determining region Y)-box 1 (SOX1), 5′- CAGCAGTGTCGCTCCAATTCA-3′ sense and 5′-GCCAAGCACCGAATTCACAG-3′ antisense; *Homo sapiens* paired box 6 (PAX6), 5′-AATTGATTGCAGAGTGTCGCTTC-3′ sense and 5′-GCTCAGGTGCTCGGGTTCTAA-3′ antisense. qRT-PCR amplification was carried out for 40 cycles, with the denaturation step conducted at 95°C for 5 s. Annealing and elongation were carried out at 60°C for 10 s and at 72°C for 20 s, respectively. Relative gene expression levels were calculated using the comparative Ct method after normalization to an endogenous gene control (ACT-β).

Cell count, and calculation of specific growth rate and doubling time The number of hiPSCs was counted every 24 h as follows: hiPSC colonies were enzymatically dissociated using Accutase solution and broken down into single cells by gentle pipetting. Cell suspension was centrifuged at 180 ×*g* in at 5°C for 5 min, and supernatant was discarded. Cell pellet was gently re-suspended with the same amount of 0.4% trypan blue solution. The number of cells excluding trypan blue was counted as viable cells using a hemocytometer. The number of viable cells per culture area was referred to as cell yield (y_c). Specific growth rate (μ) and doubling time (t_d) were calculated from the variations in cell number based on the following equations. The growth rate is proportional to the cell number (X):

$$\frac{dX}{dt} = \mu \cdot X \tag{1}$$

where μ and t are the specific growth rate and culture time, respectively. Therefore,

$$\mu = \frac{ln(X_2/X_1)}{t_2 - t_1} \tag{2}$$

t_d is the period when $X_2 = 2X_1$, therefore:

$$t_d = \frac{ln(2X_1/X_1)}{\mu} = \frac{ln2}{\mu} \tag{3}$$

Effect of CHIR on population doublings in subcultures Human iPS cell lines 201B7 and 253G1 were serially cultured to passage 10. Each passage was started with the initial cell density of 1.0×10^4 cells/cm^2 on a 6-well plate. Treatments with CHIR at various concentrations of 1, 3, and 10 μM were performed during the late 2-day period in a 4 day-cultivation. Viable cell number was counted on day 4. The total number of cells harvested from each subculture on day 4 was calculated and the population doubling (PD) per passage determined using the following equation:

$$PD = \frac{ln(A/B)}{ln2} \tag{4}$$

where A is the number of harvested viable cells and B is the number of plated cells (11). Each experiment was repeated three times.

Statistical analysis At least three independent experiments were conducted. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test and values of a p-value < 0.05 was considered statistically significant.

RESULTS

CHIR treatment period affects colony morphology, cell yields, and mRNA expression levels of pluripotency-associated genes First, we investigated the period for CHIR treatment at the concentration of 3 μM, because 3 μM CHIR is considered as a standard concentration in stem cell researches (10,17,18). hiPS cell line of 201B7 was subjected to 3 μM of CHIR following three treatment periods in a 4 day-cultivation (Fig. 1A). Fig. 1B shows the phase-contrast images of hiPSC colonies in adherent cultures between day 1 and 4. When 3 μM of CHIR was applied to the cultures in the late 2-day period of a 4 day-cultivation, hiPSCs formed typical colonies with defined boundaries. These colonies appeared to be more swollen than colonies formed in the absence of CHIR (control). When 3 μM of CHIR was applied to the cultures over the entire 4-day cultivation, hiPSC colonies took an atypical form with poorly defined boundaries on day 4. When the use of CHIR was limited to the early 2-day period, the atypical colonies emerged as well as over the entire 4-day cultivation.

Fig. 2 shows the effect of CHIR treatment period on cell yield and pluripotency–associated gene expression level. All CHIR treatment periods led to an increase in cell yield (Fig. 2A). When CHIR treatment was carried out over the entire 4-day cultivation, the highest cell yield was obtained, however the expression levels of pluripotency–associated genes of OCT3/4 and NANOG were significantly lower compared to control (Fig. 2B). When CHIR

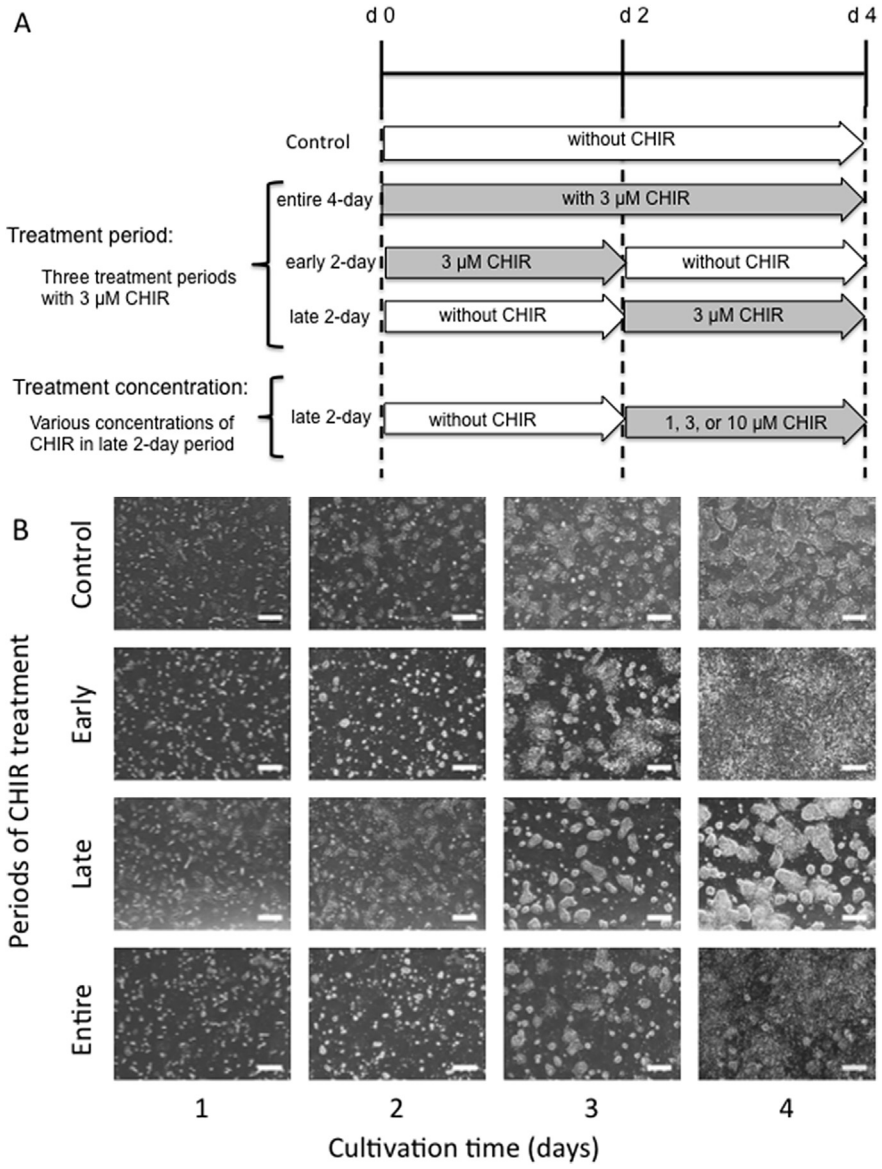

FIG. 1. Schematic representation of the various CHIR treatment conditions and colony morphology of the hiPSC line 201B7. (A) Schematic of CHIR treatment conditions. Treatment period: Treatment with 3 μM CHIR was performed in three different periods of a 4-day cultivation, i.e., over the entire 4-day culture period, over the early 2-day period, and over the late 2-day period. Treatment concentration: Various concentrations of CHIR (1, 3, or 10 μM) were applied for late 2-day period of a 4-day cultivation. (B) Effect of CHIR treatment period on colony morphology. Phase-contrast images of adherent cultures of single cell-dissociated hiPSCs between day 1 and day 4. Scale bar: 500 μm.

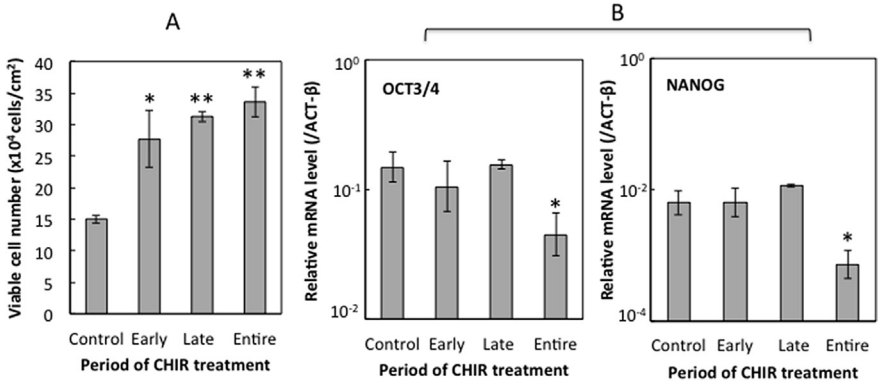

FIG. 2. Effect of CHIR treatment period on cell yields and mRNA expression levels of pluripotency–associated genes of hiPSC line 201B7. (A) Cell yields: y_c, i.e., viable cell number per area of culture dish on day 4. Data are represented as mean ± SD (n = 3). $^*P < 0.05$, $^{**}P < 0.01$, significance versus control calculated by Student's *t*-test. (B) Pluripotency–associated genes: OCT3/4 and NANOG. Total RNA was extracted from hiPSC cultures on day 4. Data were normalized to *Homo sapiens* actin, beta (ACT-β) expression as an endogenous control. Data are represented as mean ± SD (n = 3). $^*P < 0.05$, significance versus control calculated by Student's *t*-test.

treatment was carried out over the late 2-day period, the cell yield was enhanced to the same extent as that obtained when cells were exposed to CHIR over the entire 4-day cultivation and maintained the expression levels of OCT3/4 and NANOG. When CHIR treatment was carried out in the early 2-day period, the effects were almost similar to those obtained when CHIR treatment was applied over the late 2-day period. However, the experimental data obtained upon the early 2-day period varied widely compared to those obtained in the late 2-day period. The above results suggest that CHIR treatment during the late 2-day period can enhance the proliferation of hiPSCs without deviation from their undifferentiated state.

Effect of CHIR concentrations on proliferation of hiPSCs There is the possibility that cellular behaviors towards CHIR may be different depending on hiPS cell lines. Therefore, we investigated the effect of CHIR concentration on the proliferation of both hiPS cell lines 201B7 (B7-hiPSCs) and 253G1 (G1-hiPSCs). As shown in Fig. 1A, various concentrations of CHIR at 1, 3, and 10 μM were added to hiPSC cultures 2 days after cell seeding, and the cultivation was continued in the presence of CHIR for a further 2 days. As an index of growth activity, doubling time (t_d) was calculated based on the changes in cell numbers between day 2 and day 3 of growth curves shown in Fig. 3A using Eqs. 1–3. Fig. 3B shows cell yield (y_c) in viable cell number per culture area on day 4. Cell growth-related data of t_d and y_c are summarized in Table 1. The colony morphologies observed on day 4 at passage 1 are shown in the upper part of Fig. 4A.

In the absence of CHIR (control), both B7- and G1-hiPSCs formed colonies with poorly defined boundaries, and the cell growth-related data were as follows: t_d, 18.2 ± 7.0 h (B7), 12.4 ± 2.5 h (G1); y_c, 17.8 ± 4.0 × 10^4 cells/cm^2 (B7), 25.4 ± 4.5 × 10^4 cells/cm^2 (G1). The addition of 1 or 3 μM CHIR led to enhanced cell proliferation, and both B7- and G1-hiPSCs were able to form colonies with distinct edges. Treatment with 3 μM CHIR significantly shortened t_d and increased y_c: t_d, 11.0 ± 1.7 h (B7), 10.2 ± 1.4 h (G1); y_c, 36.1 ± 4.0 × 10^4 cells/cm^2 (B7), 51.6 ± 11.8 × 10^4 cells/cm^2 (G1). Treatment with 1 μM CHIR moderately promoted cell proliferation. On the other hand, treatment with 10 μM CHIR failed to provided any growth-promoting effect, and led to the formation of colonies with ill-defined boundaries in both B7- and G1-hiPSC cultures as shown in the upper part of Fig. 4A. These results suggest that the addition of 1–3 μM CHIR in the late 2-day period were the most appropriate conditions to enhance the proliferation of both B7- and G1-hiPSCs.

TABLE 1. Cell growth-related data of t_d and y_c in both B7- and G1-hiPSC cultures.

CHIR concn. (μM)		0	1	3	10
Doubling time: t_d (h)	201B7	18.2 (±7.0)	12.3 (±2.0)	11.0 (±1.7)	16.3 (±3.0)
	253G1	12.4 (±2.5)	11.1 (±1.3)	10.2 (±1.4)	21.1 (±0.6)
Cell yield: y_c ($\times 10^4$ cells/cm^2)	201B7	17.8 (±4.0)	24.8 (±5.4)	36.1 (±4.0)	21.5 (±5.3)
	253G1	25.4 (±4.5)	40.3 (±15.8)	51.6 (±11.8)	27.1 (±5.0)

Data are represented as mean ± SD (n = 3).

Effect of CHIR concentrations on cell proliferation in subcultures The effect of CHIR (0, 1, 3, and 10 μM) on the proliferation of hiPSCs was investigated in serial subcultures. Cumulative PDs of B7- and G1-hiPSCs during 10 passages are shown in Fig. 4B. The rate of increase in cumulative PDs was used as an index of proliferation potential of hiPSCs. The increase in cumulative PDs was proportional to the number of passage in cultures without CHIR treatment (control) and with 1 μM CHIR treatment. Cumulative PDs in the cultures with 1 μM CHIR treatment were higher than those in controls in both the cell lines during 10 passages. In contrast, treatment with 10 μM CHIR caused a drastic cell death in both the cell lines, and the

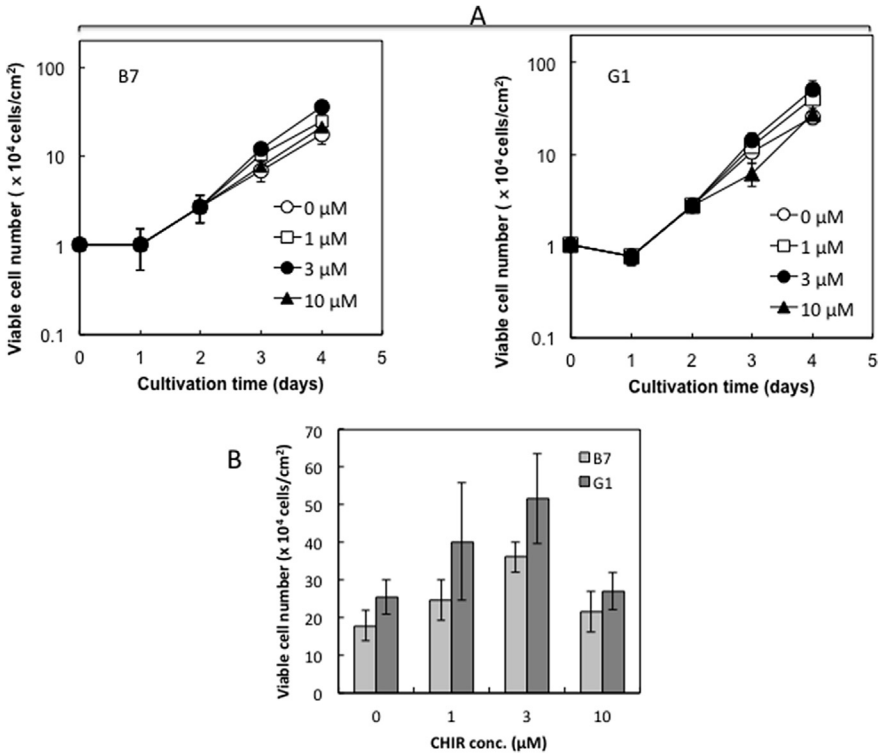

FIG. 3. Effect of CHIR concentrations on cell proliferation and cell yield of hiPSC lines 201B7 and 253G1. (A) Growth curves. (B) Cell yields: y_c, i.e., viable cell number per area of culture dish on day 4. Data are represented as mean ± SD (n = 3).

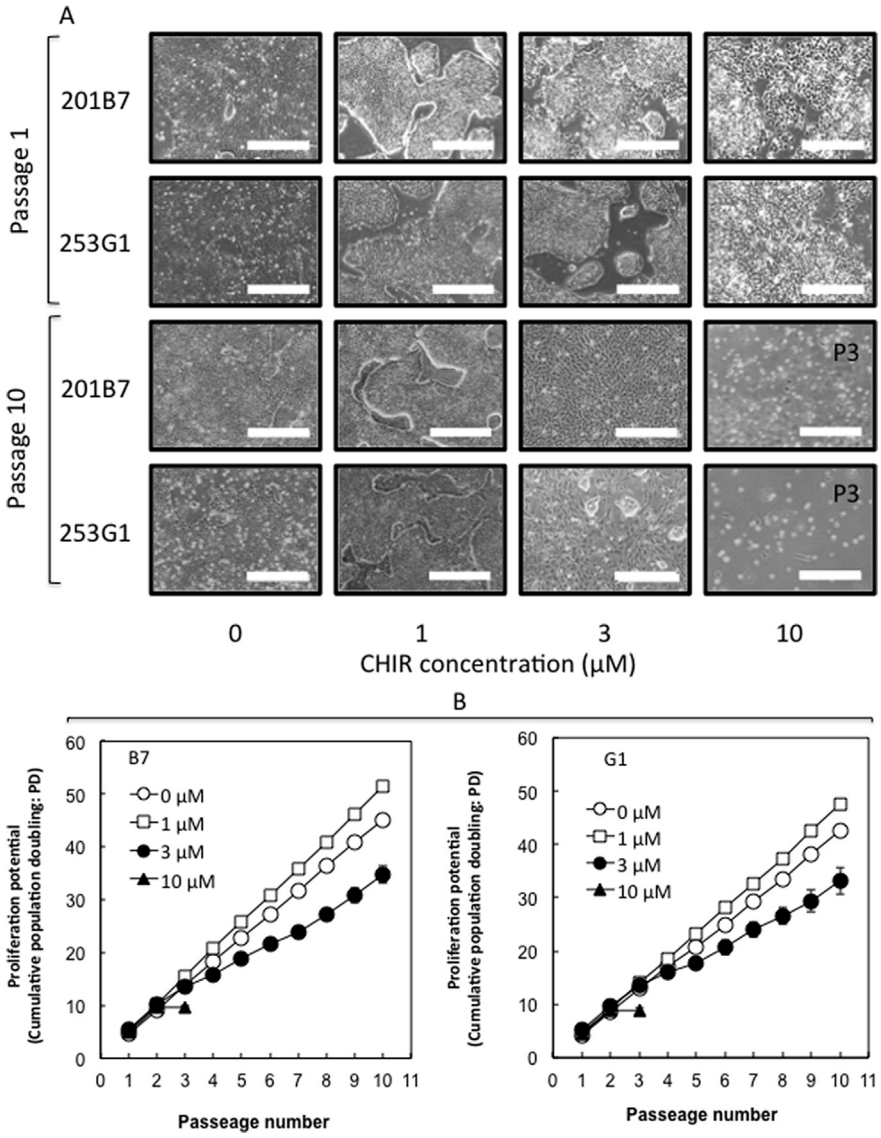

FIG. 4. Effect of CHIR concentrations on colony morphology and proliferation of hiPSC lines 201B7 and 253G1 in serial subcultures. (A) Phase-contrast images of hiPSCs in adherent cultures on day 4 at passage 1 (upper) and passage 10 (lower). P3 means that the images were derived from the cultures on day 4 at passage 3. Scale bar: 500 μm. (B) Cumulative PDs of hiPSCs treated with various concentrations CHIR (0, 1, 3, and 10 μM) during 10 passages. Each experiment was repeated three times. Data are represented as mean ± SD (n = 3).

subcultures could not continue to be more than passage 3. Treatment with 3 μM CHIR reduced the rate of increase in cumulative PDs in both the cell lines to lower level than controls after passage 3. As shown in the lower part of Fig. 4A, colonies with distinct edges were observed in the cultures treated with 1 μM CHIR during 10 passages. On the other hand, the formation of colonies distinct edges was gradually reduced with increasing passage number when treated with 3 μM CHIR. These results suggest that the addition of 1 μM CHIR to serial subcultures provided no adverse effect on the proliferation of both B7- and G1-hiPSCs.

Effect of CHIR concentration on pluripotency and differentiation We investigated the effect of CHIR concentration, when applied in the late 2-day period, on the expression of pluripotency- and differentiation-associated genes in both B7- and G1-hiPSCs. Fig. 5A shows the expression levels of the pluripotency-associated genes OCT3/4, NANOG, SOX2, and REX1. When treated with 10 μM CHIR, the expression levels of NANOG and SOX2 significantly decreased in both cell lines. There was the difference in the effect of 10 μM CHIR on OCT3/4 gene expression between B7- and G1-hiPSCs. Treatment with 10 μM CHIR significantly decreased the expression level of OCT3/4 gene in B7-hiPSCs, but that was not decreased in G1-hiPSCs. We observed no decrease in the expression levels of REX1 with any of the tested concentrations of CHIR. Altogether, our results indicated that the expression levels of most pluripotency-associated genes are maintained when both B7- and G1-hiPSCs cultures are treated with CHIR in the concentrations ranging from 1 to 3 μM.

As shown in Fig. 5B, the expression levels of early mesoderm marker genes T, GATA4, WNT3A, and WNT8A were significantly increased when treated with 3 μM CHIR and 10 μM CHIR. On the other hand, treatment with 1 μM CHIR provided little effect on the expression levels of these same genes.

Fig. 5C shows the expression levels of early endoderm marker genes SOX17 and AFP, and early ectoderm marker genes SOX1 and PAX6. There was no effect of CHIR on the expression levels of SOX1 and PAX6 genes regardless of the CHIR concentration used. The expression levels of the AFP gene decreased with increasing concentrations of CHIR. The expression levels of SOX17 were markedly

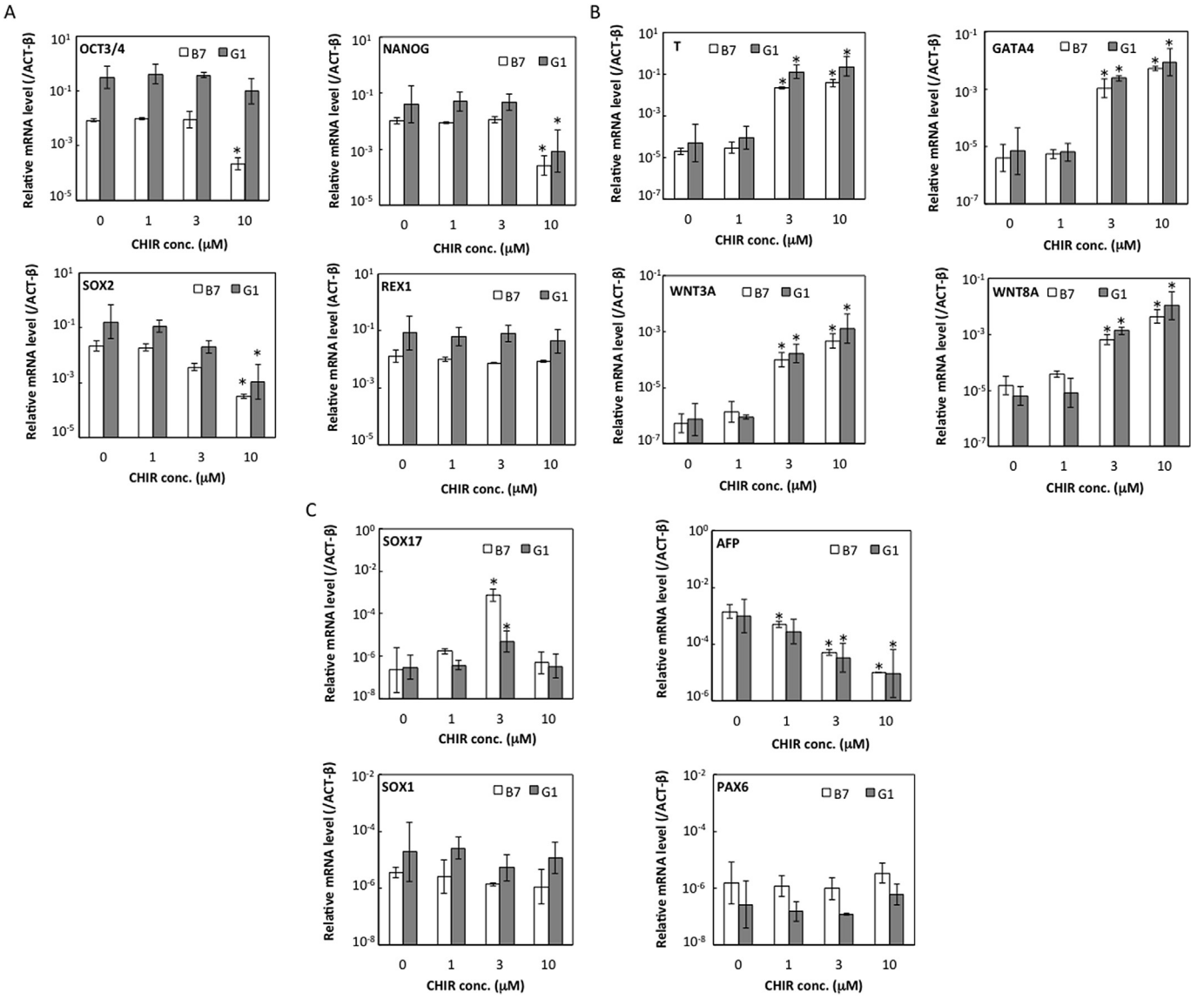

FIG. 5. Effect of CHIR concentrations on the expression levels of pluripotency-associated and differentiation marker genes. (A) Pluripotency–associated genes: OCT3/4, NANOG, SOX2, and REX1. (B) Early mesoderm marker genes: T and GATA4, WNT3A, and WNT8A. (C) Early endoderm marker genes: SOX17 and AFP, and early ectoderm marker genes: SOX1 and PAX6. Total RNA was extracted from hiPSC cultures on day 4. Data were normalized to *Homo sapiens* actin, beta (ACT-β) expression as an endogenous control. Data are represented as mean ± SD (n = 3). $^*P < 0.05$, significance versus control (0 μM CHIR) calculated by Student's *t*-test.

enhanced when treated with 3 μM CHIR only. Overall, the expression levels of SOX17, AFP, SOX1, and PAX6 genes were similar to those in the control cultures, when treated with 1 μM CHIR.

DISCUSSION

In the present study, we investigated the effect of the treatment period and concentration of CHIR, a GSK-3 small inhibitor, on the cell proliferation and differentiation of hiPSCs. Our results revealed that the treatment period and concentration of CHIR affected the cell behaviors of hiPSCs, such as colony formation, cell proliferation, and differentiation. The addition of 1–3 μM CHIR to hiPSCs cultures in the late 2-day period of a 4-day cultivation was effective in enhancing cell proliferation, and hiPSC colonies presented a swelled morphology with defined boundaries. Treatment with 3 μM CHIR significantly enhanced cell proliferation, but deviated hiPSCs from their undifferentiated state when treated over the entire 4-day cultivation. There was no growth-promoting effect when cells were treated with 10 μM CHIR. It has previously been reported that a moderate level of GSK-3 inhibition was necessary to support an effective growth of rat ESCs (22). From the above results, it is considered that the application of 1–3 μM CHIR in the late 2-day period may be corresponding to a moderate level of GSK-3 inhibition to enhance cell proliferation. There was no obvious cytotoxicity in a single treatment with 3 μM CHIR. However, performing serial subculture with 3 μM CHIR, proliferation potential was decreased after passage 3. Repeated use of CHIR in subcultures may enhance the toxic action of CHIR to hiPSCs. Previous study reported that GSK-3 inhibitors concentration-dependently reduced the viability of mouse embryonic stem cells (23). Therefore, 1 μM CHIR will be a preferable concentration in serial subcultures.

The expression levels of pluripotency-associated genes, such as OCT3/4, NANOG, SOX2, and REX1, were maintained at levels that were similar to those of the control cultures, when the cells were treated with 1 μM or 3 μM CHIR in the late 2-day period (Fig. 5A). However, when treated with 3 μM CHIR, the expression levels of T, GATA4, WNT3A, and WNT8A genes were significantly enhanced. In other words, the expression levels of T, GATA4, WNT3A, and

WNT8A genes increased with increasing CHIR concentrations (Fig. 5B). When treated with 10 μM CHIR, a decrease in the level of pluripotency-associated genes and an increase in the level of mesoderm marker genes were observed. In an exceptional case, the expression level of OCT3/4 gene was not decreased in G1-hiPSCs, even when treated with 10 μM CHIR. We attribute the difference in OCT3/4 gene expression between B7- and G1-hiPSCs to the establishment conditions (24). G1-hiPSC line was established without transcription factor c-Myc (21), while the other was established by four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (1). As shown in Fig. 5B, however, there was no difference in the expression levels of early mesoderm marker genes between B7- and G1-hiPSCs. It was also reported that high concentrations of CHIR99021 could promote the differentiation of hESCs toward a mesodermal cell fate (15). These findings imply that CHIR applied in high concentrations promotes the mesodermal-differentiation of hESCs and hiPSCs. Therefore, it was considered that 3 μM CHIR may be a boundary concentration between maintaining pluripotency and promoting differentiation.

In the present study, we determined that treatment of hiPSCs with 1 μM CHIR for the last 2 days of a 4-day cultivation period resulted in enhanced cell proliferation without causing deviation from the undifferentiated state, and did not result in any increased in the expression of early mesoderm marker genes (T, GATA4, WNT3A, and WNT8A).

In conclusion, CHIR is a multifunctional molecule affecting both cell proliferation and differentiation in a dose- and time-dependent manner. Our results indicated that carefully tested CHIR stimulation conditions allow for enhancement of cell proliferation of hiPSCs without causing deviation from the undifferentiated state in conventional adherent cultures. Further investigations are required towards the design of well-controlled culture conditions for the production of hiPSCs that will fulfill varied applications in the field of basic and applied studies.

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