

CXCR2 Inhibition in Human Pluripotent Stem Cells Induces Predominant Differentiation to Mesoderm and Endoderm Through Repression of mTOR, β -Catenin, and hTERT Activities

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On the basis of our previous report verifying that chemokine (C-X-C motif) receptor 2 (CXCR2) ligands in human placenta-derived cell conditioned medium (hPCCM) support human pluripotent stem cell (hPSC) propagation without exogenous basic fibroblast growth factor (bFGF), this study was designed to identify the effect of CXCR2 manipulation on the fate of hPSCs and the underlying mechanism, which had not been previously determined. We observed that CXCR2 inhibition in hPSCs induces predominant differentiation to mesoderm and endoderm with concomitant loss of hPSC characteristics and accompanying decreased expression of mammalian target of rapamycin (mTOR), β -catenin, and human telomerase reverse transcriptase (hTERT). These phenomena are recapitulated in hPSCs propagated in conventional culture conditions, including bFGF as well as those in hPCCM without exogenous bFGF, suggesting that the action of CXCR2 on hPSCs might not be associated with a bFGF-related mechanism. In addition, the specific CXCR2 ligand growth-related oncogene α (GRO α) markedly increased the expression of ectodermal markers in differentiation-committed embryoid bodies derived from hPSCs. This finding suggests that CXCR2 inhibition in hPSCs prohibits the propagation of hPSCs and leads to predominant differentiation to mesoderm and endoderm owing to the blockage of ectodermal differentiation. Taken together, our results indicate that CXCR2 preferentially supports the maintenance of hPSC characteristics as well as facilitates ectodermal differentiation after the commitment to differentiation, and the mechanism might be associated with mTOR, β -catenin, and hTERT activities.

Introduction

DESPITE CONSIDERABLE EFFORT by the global scientific community, potential applications for cell therapy and regenerative medicine using human pluripotent stem cells (hPSCs) are not yet fully realized. Although first established in 1998, the progress of human embryonic stem cell (hESC) research was confounded by ethical issues and immune rejection problems [1]. These issues have been largely overcome in the case of human induced pluripotent stem cells (iPSCs), which were first reported in 2007, and much progress has since been made in regenerative medical research [2]. However, several obstacles remain. One of the major problems has been the establishment of a safe and effective *in vitro* hPSC culture system for clinical application, which we have addressed in our previous studies [3–7]. The proper manipulation of hPSCs is not completely understood despite the fact that several essential factors have been identified.

Basic fibroblast growth factor (bFGF), in particular, is an essential hPSC-sustaining factor that has been added to all currently utilized media for hPSC propagation [8–10]. On the other hand, it is not clear whether other factors can support hPSC propagation in the absence of bFGF or other essential factors. We predicted the existence of pluripotency maintenance factors secreted by supportive feeder cells derived from human placenta after our successful propagation of hESCs without any supplements [6]. In our previous study, we developed a human placenta-derived cell conditioned medium (hPCCM) to exclude the exogenous addition of essential hPSC growth factors and prevent the risk of feeder-dependent conditioning. We demonstrated that the hPCCM could support feeder-free propagation of hPSCs through chemokine (C-X-C motif) receptor 2 (CXCR2) ligands, despite the absence of bFGF. Thus, we identified CXCR2 and its related ligands as novel and essential components for the maintenance of hPSC characteristics [11]. However, the

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internal signaling mechanism subsequent to CXCR2 activation in hPSCs has not yet been determined.

Another major hurdle for hPSC utilization is the lack of complete understanding of the underlying signaling pathways that might be exploited for manipulations before cell therapy. Even though several major signaling pathways associated with hPSC fate determination have been elucidated, variable and conflicting observations have been reported owing to culture in different microenvironments [12–16].

Previously, we identified that inhibition of CXCR2 by small interfering RNA (siRNA) knockdown in hPSCs resulted in their predominant differentiation to mesendoderm, which was similar to the results obtained following mammalian target of rapamycin (mTOR) inhibition in hESCs [12]. This observation suggested that there might be an association between CXCR2 signaling and mTOR. In general, the mTOR pathway is associated with human diseases such as diabetes, obesity, and certain cancers [17]. mTOR is known to be activated by the stimulation of various upstream pathways with insulin, growth factors, or amino acids [18]. It is also well established that the mTOR specific inhibitor, rapamycin, can inhibit mTORC1, which activates the translation of proteins that induce cellular growth and proliferation under conditions of adequate energy resources, nutrient availability, oxygen abundance, and proper growth factors so that protein translation can begin [19]. We hypothesized that CXCR2 association with mTOR might support the proliferation and maintenance of pluripotency in hPSCs. In addition, we also suspected the potential for a relationship between CXCR2 and mTOR with human telomerase reverse transcriptase (hTERT), which is more active in hPSCs and cancer cells than in normal differentiated cells [20,21]. In particular, recent studies have shown that hTERT suppression in hESCs promotes the induction of differentiation into all three germ layers and the β -catenin pathway regulates the activity of hTERT [22,23]. Considering these findings, we developed a hypothesis that CXCR2 activation in hPSCs is associated with mTOR signaling and hTERT activation through the β -catenin pathway. Furthermore, we supposed that the blockage of CXCR2, crucial for ectodermal development, results in predominant differentiation to mesoderm and endoderm by suppressing ectodermal differentiation.

In this study, we established the potential of CXCR2 manipulation to control the fate determination of hPSCs and verified the probable association of CXCR2 with mTOR, β -catenin, and hTERT to support hPSCs regardless of culture conditions. To the best of our knowledge, this is the first study demonstrating a novel CXCR2-dependent mechanism governing the lineage commitment of hPSCs.

Materials and Methods

Cell culture

All of the hPSC lines, including H1, H9, iPSC-1 (foreskin), and iPSC-2 (IISHi-BM1), were purchased from the WiCell Research Institute (Madison, WI) and handled according to supplier instruction. hPSCs in the experimental group were cultured on a gelatin-coated dish in hPCCM according to the protocol described in our previous article [11]. hPSCs in the control group were cultured on hESC-qualified Matrigel-coated dishes (No. 354277; BD Biosciences, San Jose, CA) with a mTeSRTM1 feeder-free culture medium (No. 05850;

StemCell Technologies, Inc.; Vancouver, BC, Canada), according to the manufacturer's protocol. Human mononuclear cells, human mesenchymal stem cells (hMSCs), WI-38s (lung fibroblasts), human dermal fibroblasts, and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to supplier protocol. We performed all the manipulations and cultivations in a clean germ-free facility at the Stem Cell Laboratory in Korea University Medical Center. The Institutional Review Board of Anam Hospital of Korea University Medical Center approved the experimental design and procedures associated with this study (AN12277-004).

Antibodies and reagents

Antibodies against phospho-mTOR (No. 5536), mTOR (No. 2983), phospho-p70S6K (No. 9208), p70S6K (No. 2708), β -catenin (No. 9562), OCT-4 (No. 2750), SOX-2 (No. 4900), NANOG (No. 4893), and SSEA-4 (No. 4755) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). FGF2 (sc-74412) and β -actin (sc-69879) antibodies were from Santa Cruz Biotechnology (Dallas, TX). Human CXCR2 antibody was purchased from Abcam (ab21641; Cambridge, UK). The source of recombinant human growth-related oncogene α (GRO α) was R&D Systems, Inc. (P09341; Minneapolis, MN). Alexa488 (No. A11034) and 4',6-diamidino-2-phenylindole (DAPI) solutions (No. D1306) were from Invitrogen (Carlsbad, CA). The small molecule inhibitors SB225002 (No. 2725) and SB265610 (No. 2724) were purchased from Tocris Bioscience (Bristol, UK). PNU 74654 (P0052), FK506 (F4679), and rapamycin (R8781) were from Sigma-Aldrich Corporation (St. Louis, MO). The mTOR inhibitor RAD001 (everolimus) was purchased from InvivoGen (San Diego, CA). BC2059 (A14381) was obtained from AdooQ Bioscience LLC (Irwin, CA).

Knockdown studies

CXCR2 expression was silenced using short hairpin RNA (shRNA)-containing lentiviral particles (SC-40028-V; Santa Cruz Biotechnology). The shRNA lentiviral particles were a pool of three different shRNA plasmids: sc-40028-VA, hairpin sequence: GATCCGTCTACTCATCCAATGTTAT TCAAGAGATAACATTGGATGAGTAGACTTTTT, corresponding siRNA sequences (sc-40028A): sense: GUCUA CUCAUCCA AUGUUAtt, antisense: UAACAUUGGAUGA GUAGACTt; sc-40028-VB, hairpin sequence: GATCCCCT CAAGATTCTAGCTATATTCAAGAGATATAGCTAGA ATCTT GAGGTTTT, corresponding siRNA sequences (sc-40028B): sense: CCUCAAGAUUCUAGCUAUAtt, antisense: UAUAGCUAGAAUCUUGAGGt; and sc-40028-VC, hairpin sequence: GATCCGCCACTAAATTGACACTTATT CAAGAGATAAGTGTCAATTTAGTGGCTTTTT, corresponding siRNA sequences (sc-40028C): sense: GCCACUAA AUUGACACUUAtt, antisense: UAAGUGUCAUUUAGUG GCt. All sequences are provided in 5' \rightarrow 3' orientation.

For viral infections, hPSCs (100–200/clump) were seeded into a 24-well culture dish and cultured overnight. The next day, hPSCs were incubated in 500 μ L prewarmed mTeSR1 medium containing 6 μ g/mL polybrene for 15 min, and then 10 μ L 1×10^6 TU/mL viral particles were added to the culture medium. Secondary infection was induced after 24 h with 30 μ L 1×10^6 TU/mL viral particles following the same

protocol. After 24 h, the infection medium was replaced with fresh medium without polybrene. Cells were then cultured in 1 $\mu\text{g}/\text{mL}$ puromycin for the next 4–7 days to isolate the population of cells exhibiting shRNA expression.

Detection of alkaline phosphatase activity

The alkaline phosphatase (AP) activity was measured using an ES Cell Characterization kit (No. SCR001; Chemicon International, Inc., Temecula, CA), according to the manufacturer's protocol. Stained cells were imaged using an Olympus microscope (IX71; Olympus, Tokyo, Japan).

Immunofluorescence

For immunofluorescence staining, hPSCs were cultured and fixed in a two-well culture dish (Corning, Inc., Corning, NY) with 4% (w/v) paraformaldehyde, permeabilized with 0.1% (v/v) Triton X-100, and blocked for 1 h with 3% (v/v) normal horse serum (Gibco, Grand Island, NY) in phosphate-buffered saline (PBS). Fixed cells were incubated with the primary antibody (diluted 1:500) overnight at 4°C and the secondary antibody (diluted 1:1,000) for 1 h at room temperature. Between incubations, the cells were washed three to five times with PBS. Before mounting, the cells were incubated with DAPI for 5 min in the dark before mounting with fluorescence mounting medium (Vector Labs, Burlingame, CA). We observed the stained cells under a fluorescence microscope (Olympus). Fluorescence intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD).

Proliferation assays

Cell proliferation measurements were performed after plating at 5×10^3 cells/well (somatic cells) or 1×10^4 cells/well (hPSCs) in 96-well plates using the CCK-8 assay (No. CK04-05; Dojindo Laboratories, Kumamoto, Japan). After seeding, 10 μL CCK-8 was added and incubated for 3 h ($n=4$) at each time point (24–144 h), and then the absorbance was measured at 450 nm. Each experiment was repeated in triplicate.

Embryoid body formation

For embryoid body (EB) formation assays, hPSCs were transferred to low-attachment plates and allowed to undergo spontaneous EB formation in the DMEM-F12 medium supplemented with 20% knockout serum replacement (KOSR), 1% nonessential amino acids, and 0.1 mM β -mercaptoethanol. The medium was changed every 2–3 days. After 2 weeks in suspension, mRNA was isolated from formed EBs and analyzed by real-time quantitative polymerase chain reaction (qPCR), as described below.

Western blotting

Cells were washed with cold PBS and then proteins were extracted with the lysis buffer (20 mM KCl, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM DTT, 1 mM EGTA, 1 \times protease inhibitor, 10% glycerol, and 50 mM Tris-HCl, pH 7.5) for 15 min on ice; the lysed cells were then centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration in the supernatant was determined by the Bradford assay (Bio-

Rad Laboratories, Hercules, CA). Total protein (30 μg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were blocked and probed overnight with primary antibodies (diluted 1:1,000) at 4°C, followed by incubation with HRP-conjugated secondary antibodies (diluted 1:2,000) for 1 h. We detected the signals using an ECL reagent (GE Healthcare Life Sciences).

Real-time qPCR

Total RNA was isolated from cells using a Qiagen RNeasy kit (No. 74106; Qiagen, Hilden, Germany) and quantified using a Nano Drop Spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA). cDNA was synthesized by adding 2 μg total RNA to a 20 μL reaction mixture containing oligo (dT) primers and Superscript II reverse transcriptase (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Synthesized cDNA was amplified using a Bio-Rad iCycler iQ system with the iQ SYBR Green qPCR Master Mix (No. 1708882; Bio-Rad). The primers used for qPCR analysis are described in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). Comparative threshold cycle values were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); relative expression levels were calculated using the control.

Statistical analysis

Statistical significance was determined using two-tailed Student's *t*-tests or two-way analysis of variance (two-way ANOVA) for multiple comparisons, followed by Tukey's testing. All experiments were performed in triplicate. *P* values less than 0.05 were considered statistically significant.

Results

CXCR2 knockdown in hPSCs induces marked differentiation to mesendodermal lineage commitment

All hPSC experiments were performed in mTeSR1 (with supplements and bFGF) on Matrigel, as well as in the developed conditioned medium hPCCM (without supplements and bFGF) on 0.1% gelatin to determine whether the presence of bFGF or other growth factors would influence the results. We used various hPSC lines, including H1, H9, iPSC-1 (foreskin), and iPSC-2 (IISH1i-BM1). To better assess the effect of *CXCR2* repression in hPSCs, we utilized a lentivirus system. By 10 days after the knockdown of *CXCR2* in H9 and iPSC-2 cells, majority of the hPSC population was greatly differentiated and showed significant loss of expression of pluripotency markers such as OCT4, SOX2, and SSEA4 (Fig. 1A, B) compared to the shControl group. Previously, we had observed that transfection with si*CXCR2* resulted in a significant reduction in extracellular signal-regulated kinase (ERK) phosphorylation and proliferative capacity [11]. Likewise, cell proliferation capacity in the sh*CXCR2* group was markedly decreased (>50%) compared to the shControl group (Fig. 1C). To determine whether these events promoted differentiation to a specific lineage, we performed real-time qPCR to analyze the gene

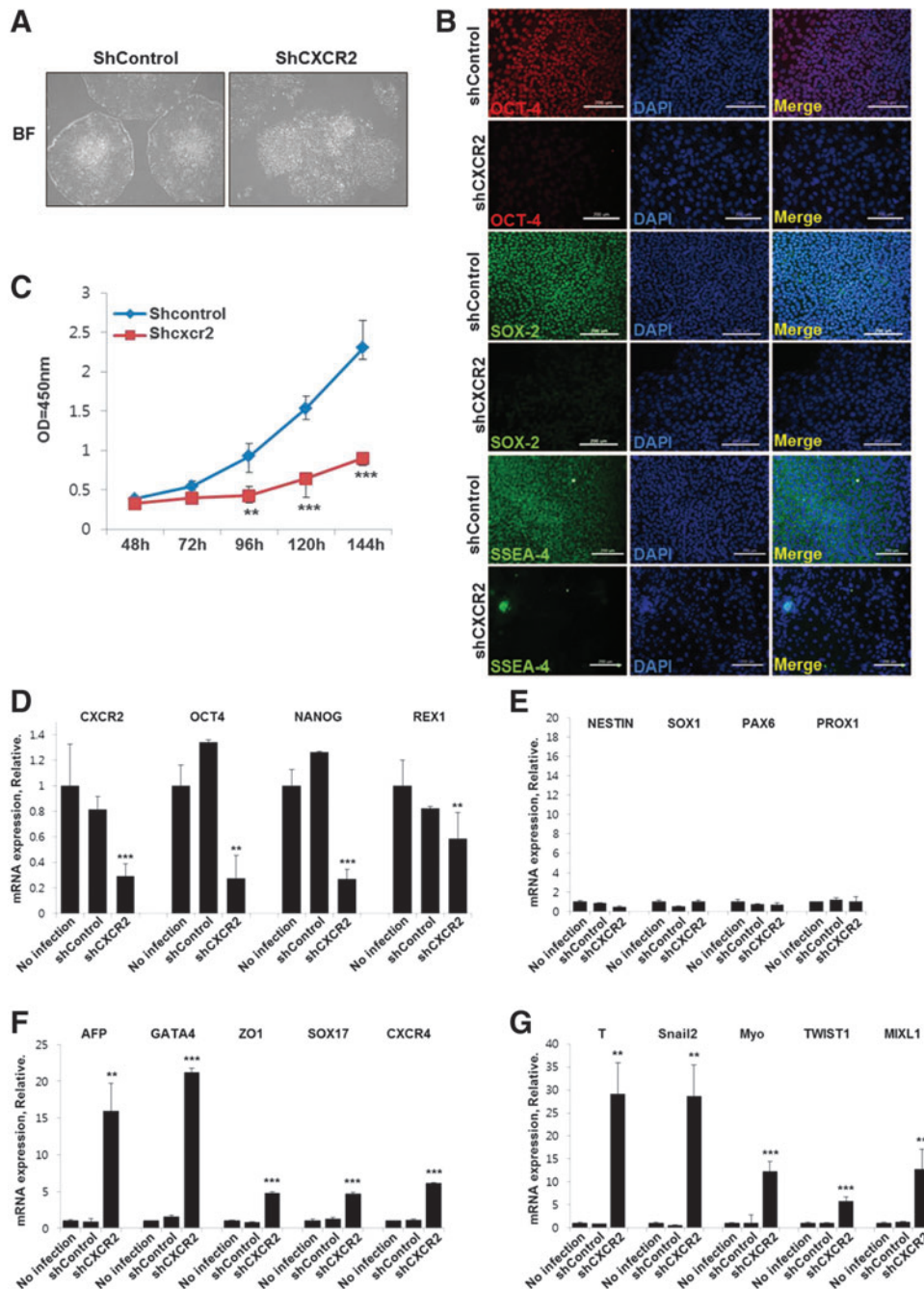


FIG. 1. *CXCR2* knockdown attenuates pluripotency and promotes mesoderm and endoderm differentiation. **(A)** iPSC-2 morphology was visible 2 weeks after infection with an shRNA lentivirus. Magnification: 40 \times , BF: bright field. **(B)** The expression of pluripotency markers was examined by immunofluorescence. Scale bars: 200 μ m. **(C)** Cell proliferation capacity was assessed in shCXCR2-iPSCs and shControl-iPSCs. The data represent the means \pm SEM from three independent experiments. **(D)** The mRNA expression of pluripotency markers was analyzed by real-time qPCR, including **(E)** ectodermal markers, **(F)** endodermal markers, and **(G)** mesodermal markers. The data represent the means \pm SD from three independent experiments after normalization to *GAPDH*. $**P < 0.01$, and $***P < 0.001$. *CXCR2*, chemokine (C-X-C motif) receptor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; iPSC, induced pluripotent stem cell; qPCR, quantitative polymerase chain reaction; shRNA, short hairpin RNA.

expression of pluripotency markers and various markers of the three germ layers (ectoderm, endoderm, and mesoderm). We found that the relative expression level of the *CXCR2* gene (>70%) was significantly decreased after the *CXCR2* knockdown in hPSCs. Subsequently, we determined that the relative expression of the pluripotency genes *OCT4*, *NANOG*, and *REX1* was reduced in the shCXCR2 group (Fig. 1D). Notably, little to no difference was observed for ectodermal lineage gene expression (Fig. 1E), whereas significant increases in the expression of mesodermal and endodermal lineage genes were found in the shCXCR2 group compared to the shControl group (Fig. 1F, G). These results were consistent with a previous report describing the endodermal and mesodermal differentiation of hESCs in response to the

inhibition of mTOR signaling [12]. Hence, these data indicate that the repression of *CXCR2* in hPSCs not only facilitates a loss of pluripotency but also leads to mesodermal lineage commitment.

CXCR2 regulates mTOR, β -catenin, and hTERT activities

Next, we investigated the underlying signaling of *CXCR2* to define its role in hPSCs. According to the above results, *CXCR2* might be involved in the process of cell fate decision. We speculated that hPSCs were maintained in a pluripotent state with normal cell division, in which *CXCR2* supported the self-renewal and pluripotency. As mentioned

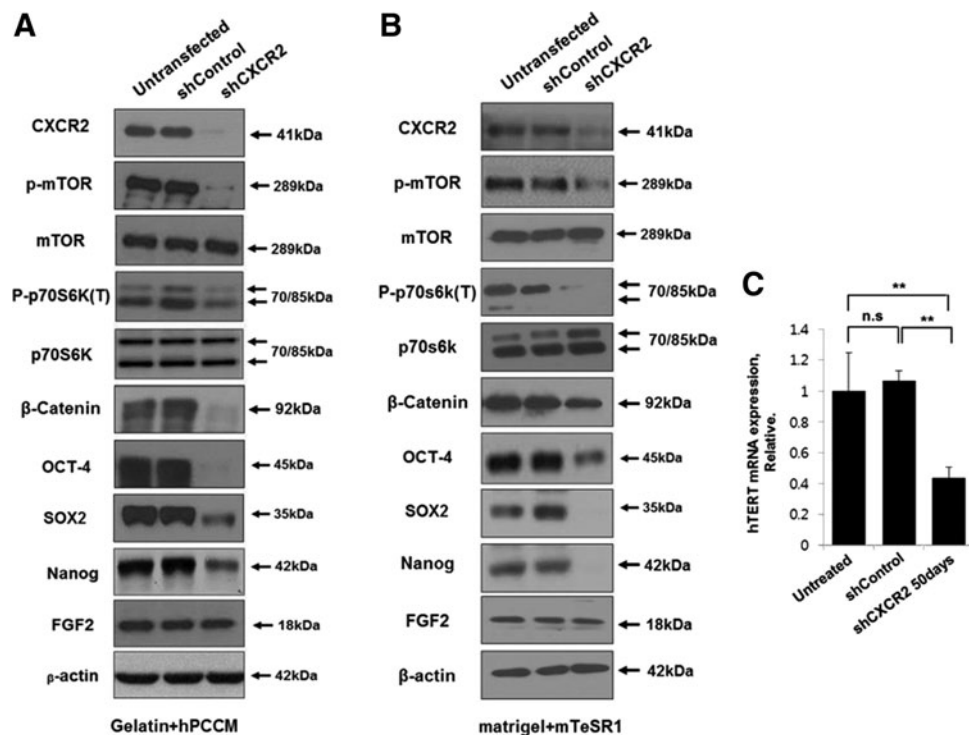
previously, Zhou et al. found that mTOR signaling supported self-renewal and pluripotency in hESCs and suppressed mesoderm and endoderm activities [12]. To further verify the consequence of CXCR2 suppression in hPSCs, we directly measured the sequential effects on underlying signaling. We performed the consistent knockdown of CXCR2 in two hPSC lines [H9 and iPSC-2 (IISH1i-BM1)] using a shRNA-mediated system. Western blot analysis revealed markedly the knockdown of CXCR2 and weakened phosphorylation of both mTOR and p70S6K, regardless of the presence of bFGF. In addition, the expression levels of β -catenin, pluripotency markers, and *hTERT* in CXCR2-silenced hPSCs were considerably lower than those in nonsilenced hPSCs (Fig. 2A–C). To directly elucidate the mechanism by which active CXCR2 regulates mTOR phosphorylation in hPSCs, we measured phospho-mTOR protein levels using western blotting. The hPSCs were cultured in a basal medium (DMEM-F12+20% KOSR) without any supplement to avoid confusion from the effects caused by other factors. The sample was treated with a CXCR2-specific ligand, GRO α , for 24 h. We observed the increased levels of phospho-mTOR and phospho-p70S6K proteins after CXCR2 stimulation by GRO α (Supplementary Fig. S1). These data provide the evidence that CXCR2 regulates the activity of mTOR signaling to maintain hPSC characteristics. In turn, the mTOR activity in hPSCs raised the question of whether mTOR suppression could affect downstream molecules. Propagating hPSCs with (mTeSR1 control) and without (hPCCM) bFGF were treated with CXCR2 antagonists (SB225002, SB265610) or mTOR inhibitors (rapamycin, RAD001). After 5 days of treatment, the phosphorylation levels of mTOR and p70S6K were found to be attenuated in CXCR2 or mTOR-repressed cells. Furthermore, we observed that hPSCs treated with CXCR2 antagonists or mTOR inhibitors exhibited a significant decrease in

OCT4 and β -catenin protein levels, respectively (Fig. 3A, B). We then analyzed the mRNA expression levels of *hTERT* in both groups following hPSC treatment with mTOR inhibitors and found that the expression levels of *hTERT* were significantly reduced compared to those in the vehicle groups (Fig. 3C). Indeed, as several studies have shown a relationship between *hTERT* and β -catenin involvement in ESCs and cancers [22–24], we assumed that these molecules might work cooperatively with underlying mTOR mechanisms. We therefore checked the endogenous expression levels of the *hTERT* gene in various human cells and confirmed the high relative expression level of *hTERT* in hPSCs. In contrast, hMSCs and terminally differentiated cells showed low levels of *hTERT* expression (Supplementary Fig. S2).

CXCR2 and mTOR act together in supporting the pluripotency and proliferation of hPSCs

To test whether CXCR2 downstream signaling networks and mTOR signaling act in concert to affect the features of hPSCs, we treated hPSCs with mTOR inhibitors (rapamycin or RAD001). To specifically determine the effect of mTOR itself on hPSCs, H1 cells propagated in feeder-free conditions in the presence of bFGF (mTeSR1 control) or its absence (hPCCM) were treated with low dosages of mTOR inhibitors. A significant differentiation of hPSCs (60–70%) concomitant with the loss of AP activities in the rapamycin-treated group was observed after 2 weeks of treatment in both culture conditions. No defects in AP activities were visible following treatment with vehicle (DMSO) or FK506, a structural analog of rapamycin (Fig. 4A, B). We then directly examined the effects of mTOR signaling on CXCR2 by measuring the proliferation ability and CXCR2 expression on the cell surface during the treatment of hPSCs with

FIG. 2. CXCR2 regulates the mTOR signaling pathway activity. H9 cells were transfected with lentiviral particles and protein expression in shControl and shCXCR2 cells was measured by western blotting for cells cultured in (A) hPCCM or (B) mTeSR1. (C) *hTERT* mRNA expression was detected by qPCR. After shRNA transfection, we selected against noninfected cells using puromycin for 1 week. Cells exhibited stable knockdown status for 50 days posttransfection. The data represent the means \pm SD from three independent experiments. n.s., nonsignificant, $**P < 0.01$. hPCCM, human placenta-derived cell conditioned medium; *hTERT*, human telomerase reverse transcriptase; mTOR, mammalian target of rapamycin.



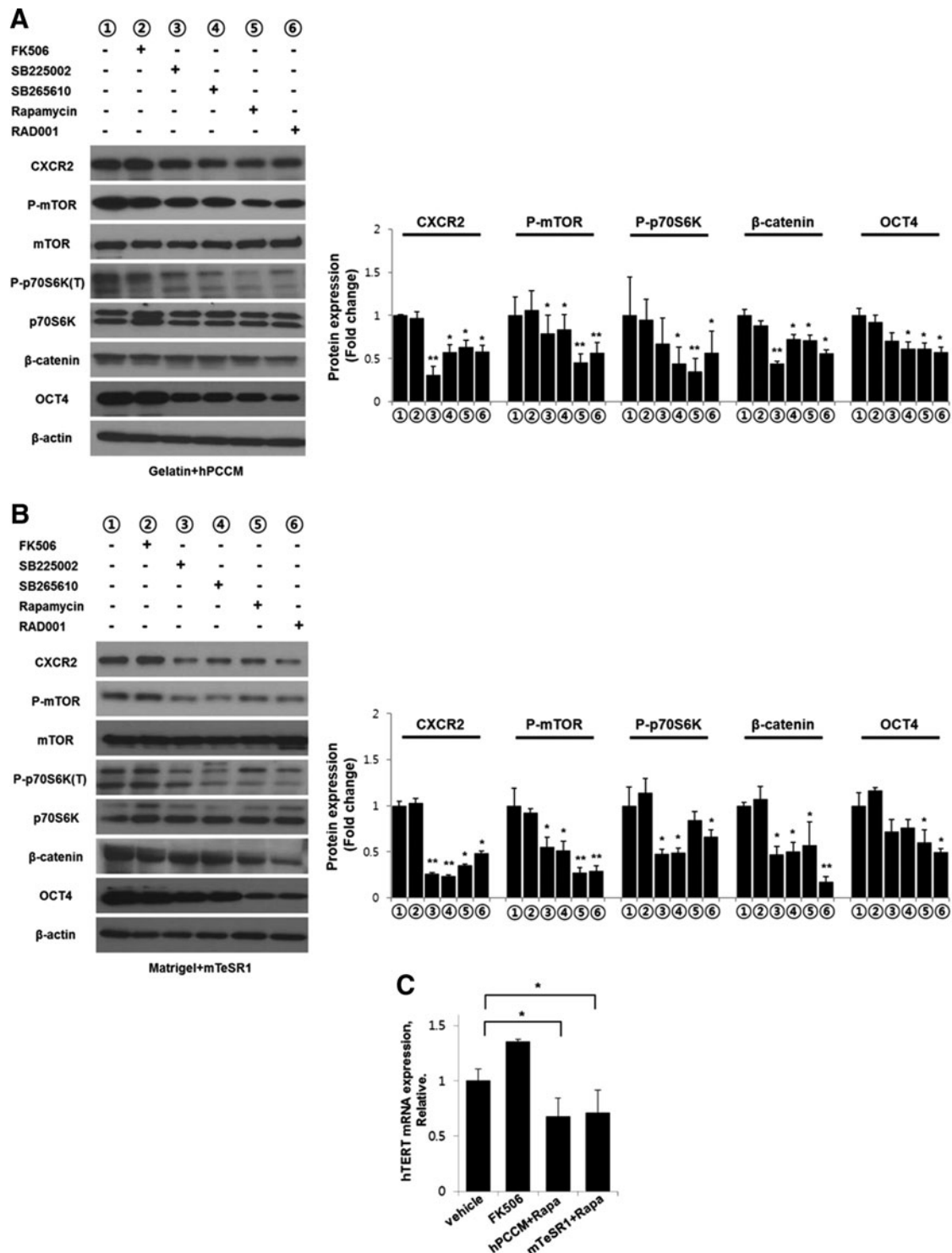


FIG. 3. CXCR2/mTOR cascade influences β -catenin and hTERT expression. H1 cells were treated with 1 μ M FK506, 200 nM SB225002, SB265610, rapamycin, or RAD001 for 5 days. After 5 days, cells were harvested and relative protein expression levels were assessed by western blotting of hPSCs propagated in (A) hPCCM or (B) mTeSR1. Measured results are shown relative to the control and quantified using Image J software. The graph shows values normalized to β -actin (phospho/total). Western blotting for each protein was performed at least twice. (C) qPCR analysis was used to measure *hTERT* expression following mTOR inhibitor treatment. The graph shows values normalized to *GAPDH*. The error bars represent the means \pm SD. * P < 0.05, ** P < 0.01. hPSC, human pluripotent stem cell.

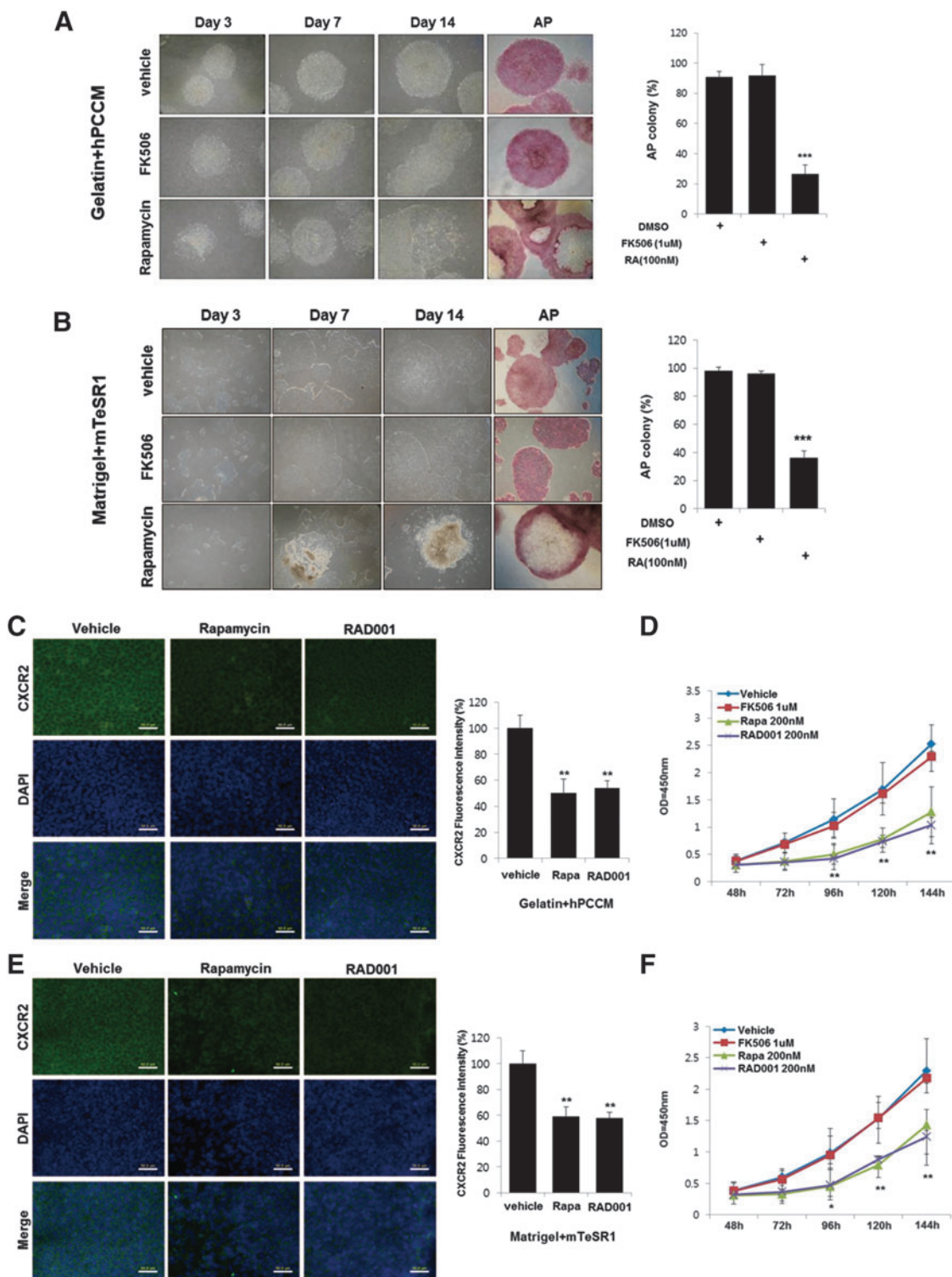


FIG. 4. mTOR inhibitors suppressed hPSC pluripotency and proliferation. (A) H1 cells were cultured in the hPCCM medium containing rapamycin (100 nM) or FK506 (1 μ M) for 2 weeks. Undifferentiated colonies were measured using AP staining. (B) H1 cells were cultured in mTeSR1 with rapamycin or FK506. AP-positive colonies were counted for statistical analysis. The data represent the means \pm SD from three independent experiments. *** P < 0.001. Magnification: 40 \times . (C, E) H1 cells were treated with the mTOR inhibitors rapamycin or RAD001 (200 nM) for 5 days and propagated in hPCCM on gelatin-coated plates or in mTeSR1 media on Matrigel-coated plates. After 5 days of treatment, cells were stained for immunofluorescence using a CXCR2 antibody. (D, F) Cell proliferation was detected in a time-dependent manner using a CCK-8 assay following treatment with FK506 (1 μ M), rapamycin, or RAD001. Fluorescence intensity was quantified using Image J software. Cell images were captured from 10 random locations and measured for statistical analysis. The data represent the means \pm SEM from three independent experiments. Statistical significance relative to the vehicle-treated control group is indicated as * P < 0.05, ** P < 0.01. Scale bars: 50.0 μ m. AP, alkaline phosphatase.

mTOR inhibitors for 5 days of culture. From this, we observed a significant decrease of CXCR2 expression in hPSCs and cell proliferation to about 40–50% compared to cells cultured with the vehicle controls (Fig. 4C–F). The results were unaffected by whether the hPSCs were propagated in mTeSR1 or hPCCM, suggesting that mTOR signaling is closely associated with CXCR2 and independent of the bFGF pathway. In addition, mTOR inhibitor decreased CXCR2 expression by 50–60% as well as the proliferation capacity of terminally differentiated somatic cells such as human primary dermal fibroblasts and primary HUVECs (Supplementary Fig. S3). These data indicated that CXCR2 and mTOR might be intimately related with each other in supporting the proliferation and pluripotency of hPSCs, as well as the proliferation of differentiated somatic cells, independent of culture environments.

β -catenin influence on hPSC fate with the correlation of mTOR

One of the most important roles of β -catenin is in the pluripotency and differentiation of ESCs [25], although the actual data therefrom has been controversial. β -catenin is associated with cell growth, proliferation, and differentiation through the regulation of target gene transcriptional levels [26–28]. In addition, several studies have shown that mTOR/ β -catenin signaling is correlated in human cancers [29,30]. Therefore, we treated hPSCs with β -catenin inhibitors (PNU-74654 and BC2059) to define the functional role of β -catenin in undifferentiated cells. PNU-74654 is an inhibitor of the Wnt/ β -catenin pathway and functions by blocking the interaction between β -catenin and T-cell factor [31]. BC2059 disrupts the binding of β -catenin to TBL-1 and promotes β -catenin degradation, attenuating nuclear and cytoplasmic β -catenin levels [32]. After 48-h treatment of hPSCs with PNU-74654, the expression of pluripotency markers, including the β -catenin target genes *OCT4* and *hTERT*, was decreased compared to cells treated with the vehicle (Fig. 5A, B). Furthermore, the proliferation capacity of hPSCs was significantly attenuated in the PNU-74654-treated group compared to the vehicle group as well, regardless of culture condition (Fig. 5C, D). Notably, β -catenin repression by BC2059 led to decreased expression of stemness markers (*OCT4*, *NANOG*, and *REX1*) and *hTERT* (Fig. 5E, F). We next measured the protein expression of associated downstream molecules by western blot. After 24-h treatment of hPSCs by BC2059, we observed significant decreases in the expression of β -catenin as well as phosphorylated mTOR, and phosphorylated p70s6k (Fig. 5G). Contrarily, the change of protein level of CXCR2 was equivocal despite the moderately decreased mRNA level (data not shown). Taken together, these results indicate that β -catenin expression is related to the capacity of proliferation and maintenance of pluripotency of hPSCs. These data led us to postulate that all of these molecules and signaling pathways are closely related and exert systematic effects on the others. However, the ordering of the entire mechanistic process is not yet clear and will be examined further in future studies.

CXCR2 stimulation in EBs governs the differentiation to ectoderm

Next, we investigated how these signaling pathways, as a network, contribute to influence hPSC fate. We speculated

that the CXCR2-induced mTOR activity plays a role in hPSC differentiation to the ectodermal lineage after the commitment to differentiation, as both are essential for the formation of ectoderm-derived neurons [33]. To further explore this hypothesis, we sought to confirm these results in a second model system; thus, we used the two hPSC lines: H1 and iPSC-1 (foreskin). We verified the proposed role of CXCR2 in hPSCs using a CXCR2-specific ligand, GRO α , as the only stimulation input. IL-8 is the one popular ligand of CXCR2, exhibits not only high affinity to the CXCR2 but also strongly binds to the CXCR1; we ruled out treatment with IL-8 in this set of experiments. To examine whether the results of this experiment were valid for hPSCs and not experimental artifacts, we tested cultured hPSCs in both group conditions (mTeSR control and hPCCM). We treated EBs derived from hPSCs from both group conditions with a low concentration of GRO α to activate CXCR2 (Fig. 6A). After 2 weeks of differentiation, mRNA was isolated from EBs and the expression of germ layer markers analyzed by qPCR. While 2 weeks was an insufficient period to effect terminal differentiation, we were able to observe the distinct induction of ectodermal differentiation markers in both groups of hPSCs. These CXCR2-activated hPSCs tended toward differentiation to ectoderm compared to other lineages. We analyzed mRNA expression levels of several ectodermal lineage genes (*Nestin*, *SOX1*, *PAX6*, and *PROX1*). The relative mRNA expression levels prominently increased in the activated EB groups compared to the nontreated control EBs (Fig. 6B). In contrast, the overall results of mesodermal (*T*, *SNAIL2*, *MIXL1*, *TWIST1*, *MYO*, and *FLT1*) and endodermal (*AFP*, *GATA4*, *CXCR4*, *ZO1*, *SOX17*, and *FOXA2*) lineage gene expression levels were much reduced compared to those of the ectodermal lineage genes (Fig. 6C, D). These specific differentiation features of hPSCs generated by differing mechanisms provided strong evidence implying that a balance of mutual relationships between network mechanisms was maintained under normal conditions, whereas active target signaling led to or inhibited particular lineages. Thus, we theorize that mesodermal and endodermal differentiation of shCXCR2-hPSCs is likely due to interruptions in one or more pathways that promote ectoderm differentiation after the commitment to differentiation.

Discussion

CXCR2 is a G protein-coupled receptor that primarily couples to G₁ and is rapidly internalized following receptor activation [34]. Presently, the function of CXCR2 signaling is twofold, both reinforcing senescence in normal senescent cells and enhancing cancer progression and metastasis [35–38]. However, the effect of CXCR2 on stem cells was little realized until we recently identified its role in hPSC pluripotency and proliferation [11]. In this study, we performed a comparative analysis of hPSC propagation and proliferation in hPCCM (without bFGF) and in conventional media with bFGF (mTeSR1) to more fully characterize the CXCR2 regulatory mechanism in hPSCs. It is somewhat counterintuitive that CXCR2 functions both to promote hPSC proliferation and potentiate senescence in normal senescent cells. To resolve this discrepancy, we induced the suppression of CXCR2 expression in normal differentiated human cells and hPSCs using mTOR inhibitors and found a

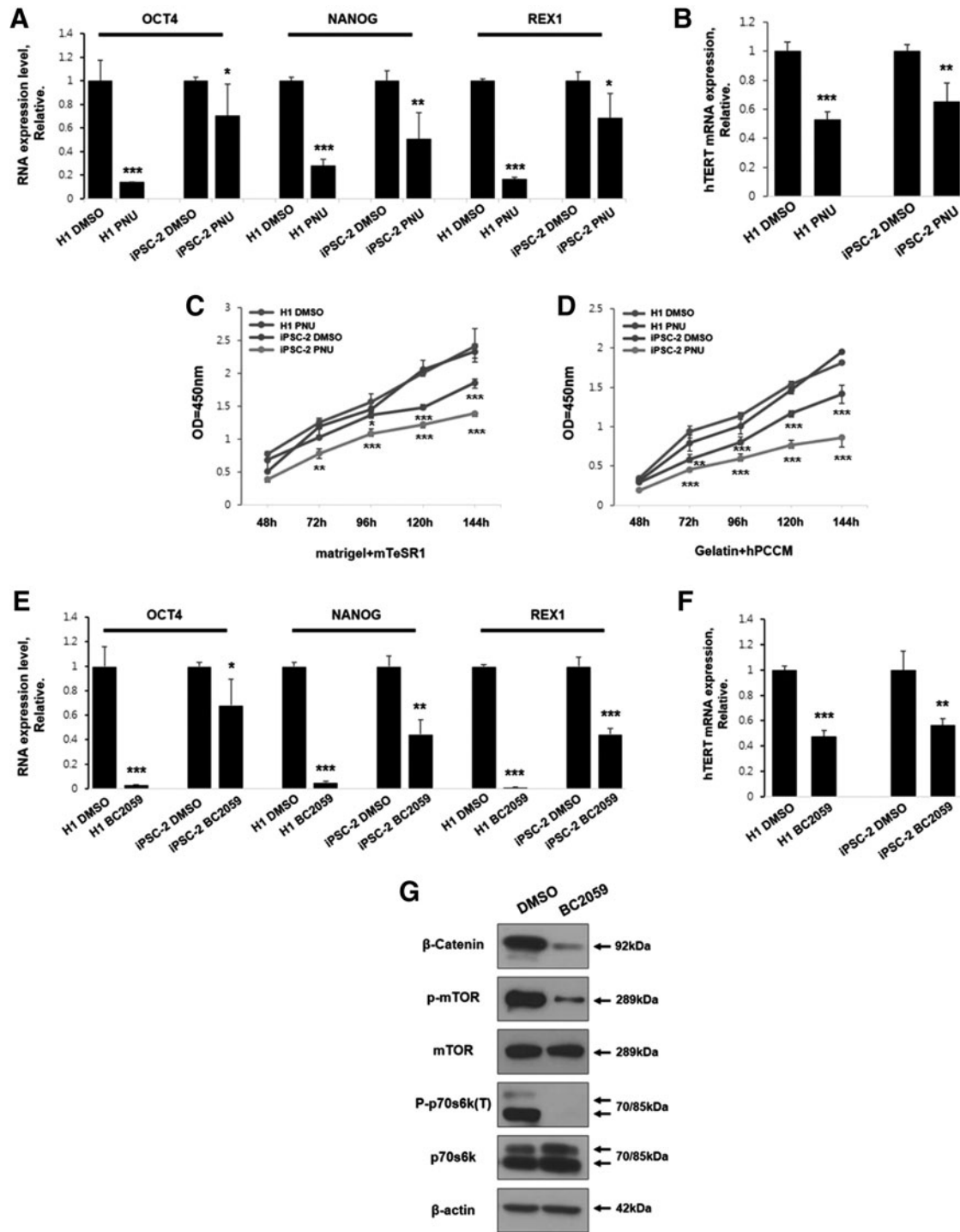


FIG. 5. Effect of β -catenin repression by PNU-74654 and BC2059 on the proliferation and pluripotency of hPSCs. (**A**, **B**) hPSCs were treated with the β -catenin inhibitor, 20 μ M PNU-74654, for 48 h and the relative expression of pluripotency markers and *hTERT* genes was measured by qPCR. (**C**, **D**) Proliferation capacity of hPSCs measured by the CCK-8 assay. The hPSCs were plated in 96-well plates at 1×10^4 cells per well and treated with PNU-74654 (20 μ M). Proliferation rates were measured at the indicated time points using a spectrophotometer to detect the A450 values. Cell numbers in quadruplicate wells were measured. The data represent the means \pm SEM from three independent experiments. (**E**, **F**) β -catenin repression in hPSCs following treatment with 100 nM BC2059 for 24 h. The relative gene expression levels of pluripotency markers and *hTERT* were measured by qPCR. (**G**) Protein expression levels of β -catenin in the repressed hPSCs were detected by western blot. hPSCs were treated with BC2059 (100 nM) for 24 h and harvested. Prepared whole lysate samples (30 μ g) were loaded into each lane. The data represent the means \pm SD after normalization to *GAPDH*. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

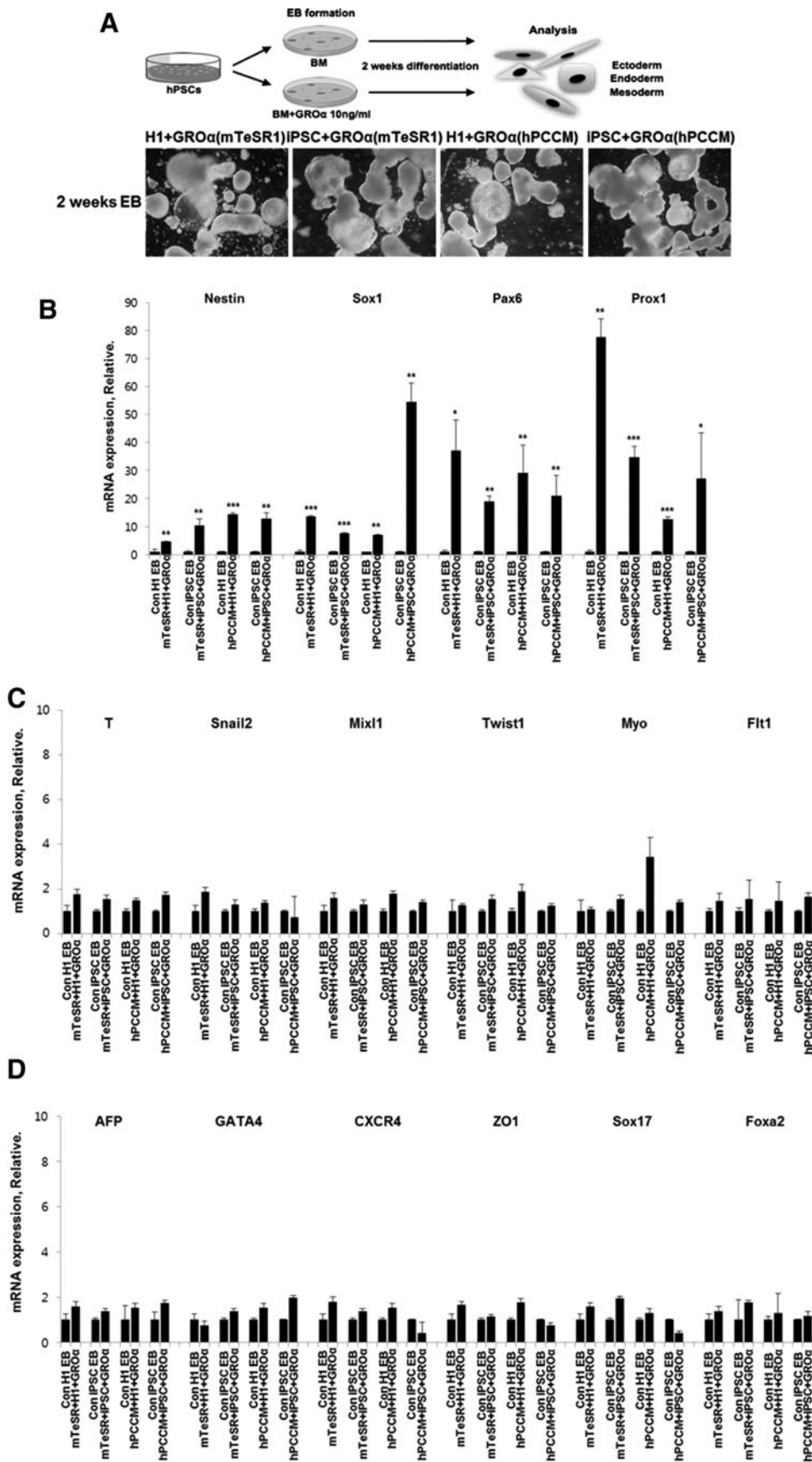


FIG. 6. CXCR2 activation-induced differentiation of ectodermal lineages. (A) Schematic of the experimental procedure (*upper panel*). hPSCs (H1 and iPSC-1) from two culture conditions (mTeSR1 and hPCCM) were induced to differentiate in the presence or absence of GRO α (10 ng/mL) on low-attachment plates for 2 weeks to promote the formation of EBs (*lower panel*). After 2 weeks of differentiation, germ layer expression of (B) ectoderm, (C) mesoderm, and (D) endoderm lineage genes in EBs was analyzed by qPCR. The data represent the means \pm SD after normalization to *GAPDH*. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. GRO α , growth-related oncogene α ; EBs, embryoid bodies.

significant decrease of proliferation and CXCR2 expression in both types of cells. This result suggests that the principle role of CXCR2 might be to support the proliferation of normal cells, including both hPSCs, and terminally differentiated human cells. Nevertheless, CXCR2 has also been shown to activate a self-amplifying secretory program that reinforces growth arrest in normal senescent cells through multiple pathways, including the activation of NF- κ B, C/EBP β , and p53, as well as through increased reactive oxygen species (ROS) production [39]. Ruan et al. found that the production of ROS in response to CXCR2 activation occurred after the induction of senescence, and therefore, p21 activation concomitant with DNA damage was essential for the commencement of senescence [40].

mTOR inhibition has been shown to decelerate cellular senescence through the suppression of ROS production in normal senescent cells [41,42]. In cancers, mTOR is thought to play a critical role in tumor cell growth and proliferation as the dysregulation of the PI3K/Akt/mTOR signaling pathway occurs in many human cancers [43–49]. We found that CXCR2 was related to mTOR for the regulation of hPSC fate and mTOR inhibitors attenuated CXCR2 expression as well as the proliferation of hPSCs and differentiated normal cells providing additional insight into the action of mTOR. Moreover, our study first provided the direct evidence for the close relationship of CXCR2 and mTOR by showing that GRO α , CXCR2 ligand, increased the mTOR activity in hPSCs. In addition, several studies have shown that mTOR and β -catenin signaling is known to be correlated. For example, mTOR levels in colon cancer are reported to be regulated by β -catenin [29]. Furthermore, β -catenin and phosphorylated mTOR expression are not only closely related to tumor size and metastasis but data also suggested that β -catenin might be a target of mTOR in hepatocellular carcinoma [30]. In our study, both CXCR2 suppression and mTOR inhibition resulted in a decreased hTERT activity, implying that hPSCs maintain their characteristics not only through CXCR2 and mTOR function but also through the hTERT activity. The high telomerase activity in hPSCs overcomes telomere shortening by binding a telomerase RNA component (TERC) that aligns telomerase with the chromosomal ends and acts as a template for the addition of telomeric DNA [22]. Recently, Hoffmeyer et al. reported that β -catenin regulates TERT expression in mouse ESCs through interaction with Klf4, a core component of the pluripotency transcriptional network [24]. In hPSCs, β -catenin regulates hTERT by binding to the hTERT promoter region and inducing hTERT expression, which subsequently increases the rate of telomere lengthening through the activation of alternative lengthening of telomere pathway [23]. Based on this study, we cautiously suggest that the suppression of CXCR2 or mTOR induces the attenuation of β -catenin and hTERT activities in hPSCs, resulting in differentiation. In addition to the telomeric DNA synthesis activity, hTERT functions in cancer development and biological processes, including the regulation of gene expression, cellular proliferation, and mitochondrial function [50]. In cancer cells, rapamycin causes the rapid dissociation of the hTERT/mTOR/RAPTOR (regulatory-associated protein of mTOR) complex, which leads to a reduced telomerase activity and hinders hTERT mRNA expression independent of telomere length [20]. Radan et al. reported that post-

transcriptional regulation of hTERT under varying O₂ microenvironments might help regulate hESC survival, self-renewal, and differentiation capabilities through expression of extratelomeric telomerase isoforms [51]. In this situation, hTERT acts independent of TERC in the Wnt- β -catenin signaling pathway. The mechanism through which hTERT suppression induces the differentiation of hPSCs is not clearly defined. Yang et al. suspected that uncapped telomeres resulting from the low telomerase activity might be recognized as DNA damage, consequently activating a p53-dependent mechanism that induces hESC differentiation [22]. As we found that the suppression of CXCR2 or mTOR inhibited β -catenin and hTERT activities, TERC in the Wnt- β -catenin pathway might be a target of CXCR2 or mTOR, which would support the above suspicion.

If our hypothesis regarding the CXCR2/mTOR/ β -catenin/hTERT axis is correct, then CXCR2 blockage would be expected to suppress hTERT and thus result in the differentiation to all three germ layers. However, our study demonstrated that CXCR2 suppression led to predominant differentiation into mesoderm and endoderm. To investigate this discrepancy, we hypothesized that mTOR and CXCR2 promote proliferation in hPSCs, but induce differentiation to ectoderm, subsequent to the commitment to differentiation. In support of this theory, Zhou et al. reported that rapamycin treatment was associated with mesodermal and endodermal differentiation and the loss of pluripotency markers [12]. In addition, disruption of mTOR signaling causes neuronal degeneration and abnormal neural development [33]. Furthermore, hESCs passaged as single cells and cultured in the presence of the CXCR2 ligand, GRO α , were shown to lose apical–basal polarity and undergo neuronal differentiation [52]. However, these studies did not assess the direct effect

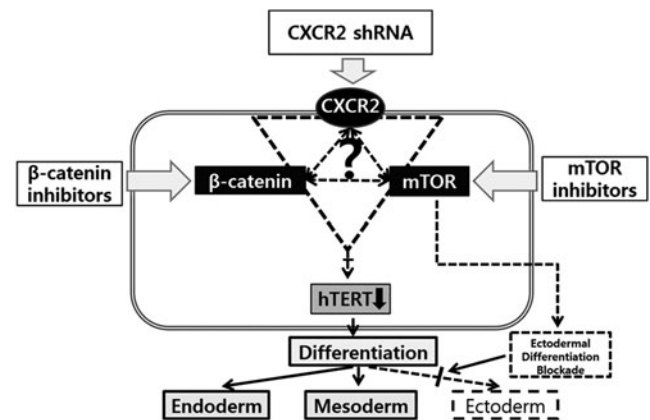


FIG. 7. A hypothetical model for how the CXCR2/mTOR/ β -catenin/hTERT axis regulate hPSC fate. CXCR2, mTOR, and β -catenin are closely related by unknown mechanism influencing each other reciprocally. CXCR2 centrally controls the fate of hPSCs by regulating mTOR, β -catenin, and hTERT. After the commitment to differentiation in hPSCs, the ectodermal differentiation may be inhibited by mTOR blockade because mTOR is crucial for the differentiation of ectodermal lineage. Therefore, CXCR2 inhibition hinders the phosphorylation of downstream pathway effectors for ectoderm differentiation and subsequently leads to mesodermal and endodermal differentiation.

of CXCR2 or mTOR on hPSC differentiation. Therefore, we treated EBs that had committed to differentiation from hPSCs with the selective CXCR2 ligand, GRO α , and confirmed the predominant differentiation thereof into ectoderm. This hypothesis, then, explained why only mesodermal and endodermal differentiation occurred in response to mTOR repression and CXCR2 shRNA treatment.

Together, these data suggest that CXCR2 signaling not only supports pluripotency but also influences the fate decision of hPSCs. Therefore, we cautiously propose a novel mechanism by which CXCR2 regulates the fate decision of hPSCs, as shown in Fig. 7. Therein, the CXCR2 of hPSCs repressed by specific inhibitors or shRNA regulates downstream signaling of proliferation and differentiation. Inhibition of CXCR2 downregulates mTOR and β -catenin activities, which leads to prominent differentiation of mesendoderm lineages. It also results in a loss of pluripotency and initiates lineage-committed differentiation simultaneously by reducing the hTERT transcriptional activity. Furthermore, the finding wherein CXCR2 blockade of hPSCs in the mTeSR1 control group resulted in their differentiation rather than proliferation suggests that this axis might be crucial in maintaining hPSC characteristics and might function through an innate mechanism.

This is the first study to investigate a CXCR2-associated mechanism for regulating the fate determination of hPSCs. Accordingly, we were able to obtain preliminary evidence in support of this model; further detailed investigation will be required for its confirmation. Nevertheless, this study might represent an important contribution to the advancement of our understanding of hPSC fate decisions in addition to suggesting novel targets for stem cell manipulation.

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Author Disclosure Statement

No competing financial interests exist.

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