

ORIGINAL ARTICLE

cP1P Maintains Long-Term Pluripotency in Human Pluripotent Stem Cells

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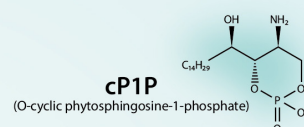
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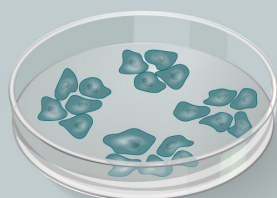
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Long Term Culture

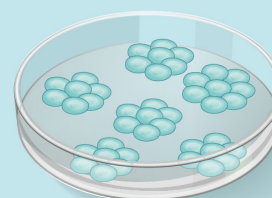
-cP1P



Aged hPSC colonies

1. Colony collapse & heterogeneous morphology
2. Spontaneous differentiation ↑
3. Genomic/epigenetic instability
4. Self-renewal & proliferative potential ↓

+cP1P



Naïve hPSC colonies

1. Intact and well-defined colony morphology
2. Maintenance of pluripotency ↑
3. Genome stability ↑
4. Sustained self-renewal and proliferative potential
5. Preservation of tri-lineage differentiation potential

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Long-term culture of human pluripotent stem cells (hPSCs) can lead to spontaneous mutations, genomic abnormalities, and alterations in gene expression, thereby compromising their self-renewal and pluripotency. Thus, optimizing the long-term culture conditions of hPSCs is crucial. In this study, we introduce O-cyclic phytosphingosine-1-phosphate (cP1P, Axceso Biopharma Co. Ltd.), a novel culture additive structurally analogous to S1P, which markedly enhances hPSC self-renewal and survival. Our results demonstrate that cP1P supplementation promotes long-term proliferation of hPSCs by upregulating pluripotency markers and maintaining their ability to differentiate into cell types derived from the three germ layers. Furthermore, RNA-seq analysis reveals that cP1P alleviates long-term culture-induced upregulation of apoptosis- and chordate embryonic development-related genes, while preventing the downregulation of stem cell maintenance pathways. Collectively, these findings suggest that cP1P effectively supports the proliferation, pluripotency, and differentiation potential of hPSCs during both short- and long-term cultures.

Keywords: Human pluripotent stem cells, Long-term culture, Cyclic phytosphingosine-1-phosphate, Pluripotency maintenance, Transcriptomic profiling

Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), possess unique cellular properties such as unlimited self-renewal, extensive proliferation, and the capacity to differentiate into all cell types. hESCs are derived from the inner cell mass of preimplantation blastocysts and can be established *in vitro* (1). iPSCs also can be established using exogenous genes (Yamanaka's factors) transduction into various types of cells isolated from tissues and body fluids (2). These hPSCs have demonstrated significant potential for clinical applications, including the replacement of degenerative cell types, drug

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discovery, and studies on human embryogenesis and disease mechanisms (3-5).

For the clinical use of hPSCs, it is essential to establish protocols that enable large-scale and cost-effective cultivation of hPSCs under xeno-free conditions while maintaining their pluripotency. Culture conditions critically influence several parameters related to the pluripotent state of hPSCs. Numerous xeno-free culture protocols involving media formulations, matrices, and environmental conditions have been developed (6). However, several studies have reported that prolonged culture of hPSCs can lead to spontaneous mutations, genomic abnormalities, or changes in gene expression, which in turn affect the purity, consistency, potency, and functional activity of hPSCs (7, 8). Such alterations may cause misleading results in disease modeling, cell replacement therapy, and drug-screening studies, raising substantial safety concerns for clinical applications.

Traditionally, hPSCs were maintained on mitotically inactivated feeder cells in serum-containing media (1). However, these culture systems have issues with undefined factors from feeder cells and serum, as well as lot-to-lot variability, making them unsuitable for clinical-grade applications. To overcome these issues, defined culture systems have been developed by identifying serum components associated with hPSC pluripotency and by establishing extracellular matrices as alternatives to feeder cells (9, 10). Components such as DMEM/F12, β FGF, TGF β , insulin, and transferrin were shown to support hPSC pluripotency during prolonged culture (6, 9). Selenium and ascorbic acid have also been reported to contribute to the maintenance of pluripotency. Moreover, Matrigel, composed of laminin, entactin, collagen IV, and heparin sulfate, was developed as a feeder-free alternative (4, 6). Surface coatings using laminin-511, fibronectin, or vitronectin were further shown to minimize xenogeneic contamination induced by animal-derived Matrigel (4, 6).

Among serum components, bioactive lipids such as lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) have been found to play crucial roles in maintaining hESCs in an undifferentiated state under feeder-free conditions (11). In particular, S1P, which is supplied by platelets, erythrocytes, and endothelial cells (12-14), acts as a key regulator of stem cell maintenance, survival, and differentiation (15-17) with increased genome stability (18). Recently, we demonstrated that a novel defined culture additive, O-cyclic phytosphingosine-1-phosphate (cP1P; Axceso Biopharma Co. Ltd.), which is structurally similar to S1P, enhances the therapeutic potential of mesenchymal stem cells and promotes endothelial cell differ-

entiation (19, 20).

However, the effect of cP1P on the pluripotency of hPSCs during long-term cultivation remains unknown. Therefore, in this study, we examined the effects of cP1P on the characteristics of hPSCs during long-term culture and investigated the underlying molecular mechanisms using RNA-seq analysis.

Materials and Methods

Cell culture and treatment conditions

Human induced pluripotent stem cells (hiPSCs-NT4, CHA Biotech) were used at early (P-1) and late (P-15) passages. For passaging, 35-mm culture plates (SPL Life Sciences; Cat. No. 10035) were coated with vitronectin (STEMCELL Technologies; Cat. No. 07180), and cells were maintained using the TeSRTM-E8TM Kit (STEMCELL Technologies; Cat. No. 05990). Cultures were maintained at 37°C in a humidified incubator with 5% CO₂. Dimethyl sulfoxide (DMSO; control, Sigma-Aldrich; Cat. No. D2650) or cP1P (AXCESO Biopharma) was added to the culture medium at a final concentration of 10 nM as experimental treatment. This concentration was selected based on preliminary dose-response optimization showing maximal enhancement of pluripotency marker expression without cytotoxicity. All procedures were approved by and conducted in accordance with the guidelines of the Institutional Review Board of Hanyang University (IRB Approval No. HYI-17-137-17).

Colony formation assay

Cells were seeded in 6-well plates (SPL Life Sciences; Cat. No. 30006) at a density of 500~1,000 cells per well and cultured for 10~14 days. Colonies were fixed with 4% paraformaldehyde (Wako; Cat. No. 163-20145) for 15 minutes and stained with 0.1% crystal violet (Sigma-Aldrich; Cat. No. C3886) for 30 minutes. After washing with distilled water, colonies containing more than 50 cells were manually counted under a light microscope (Olympus CX23). Images were captured using a digital camera mounted on the microscope. Colony numbers were compared between the control (CTL) and cP1P-treated groups at P-1 and P-15 passages.

Immunofluorescence staining

Immunofluorescence staining was performed to assess the expression of pluripotency markers including OCT4, SOX2, and SSEA-4. Cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Cat. No. X100) in phos-

phate-buffered saline (PBS). Primary antibodies against OCT3/4 (Santa Cruz Biotechnology; Cat. No. sc-5279), SOX2 (Santa Cruz Biotechnology; Cat. No. sc-365823), and SSEA-4 (Chemicon; Cat. No. 90231) were incubated overnight at 4°C. After washing, cells were incubated for 1 hour with Alexa Fluor 488-conjugated secondary antibody (Invitrogen; Cat. No. A11001). Fluorescence images were captured using a confocal microscope (Leica TCS-SP5) under identical imaging settings to ensure comparability between groups.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from hPSCs using TRIzol reagent (Favorgen; Cat. No. FATRR001). Reverse transcription was performed using the iScriptTM cDNA Synthesis Kit (Bio-Rad; Cat. No. 1708891). qRT-PCR reactions were carried out using the SensiFASTTM SYBR Kit (Bioline; Cat. No. BIO-98005). Gene-specific primers were designed for *OCT4*, *SOX2*, *SSEA-4*, and *GAPDH* (internal control). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and all experiments were performed in triplicate.

RNA-seq and bioinformatics analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen; Cat. No. 74104). RNA quality and integrity were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies; Cat. No. G2939BA). Sequencing libraries were prepared and analyzed on an Illumina NovaSeq platform. Differential gene expression analysis was performed using DESeq2, and functional enrichment analysis was carried out using Gene Ontology (GO) and protein-protein interaction network analyses.

Annexin V staining for apoptosis detection

Apoptosis was evaluated using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences; Cat. No. 556547). Cells were harvested, washed twice with cold PBS, and resuspended in Annexin V binding buffer (BD Biosciences; Cat. No. 51-66121E). Annexin V-FITC (5 μ L) and PI (5 μ L) were added, and samples were incubated for 15 minutes at room temperature in the dark. Fluorescence signals were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences). Early apoptotic cells were identified as Annexin V⁺/PI⁻, and late apoptotic or necrotic cells as Annexin V⁺/PI⁺. Flow cytometric data were processed using FlowJo software (v10).

TUNEL assay for apoptosis detection

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche; Cat. No. 11684795910). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. The TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and labeled dUTP was added and incubated at 37°C for 1 hour in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 μ g/mL; Thermo Fisher Scientific; Cat. No. D1306) for 5 minutes. Apoptotic nuclei appeared as red fluorescence (TUNEL-positive), whereas total nuclei were visualized as blue fluorescence (DAPI) under a confocal microscope (Leica TCS-SP5).

Flow cytometry analysis of differentiation potential

hPSCs were induced to differentiate into ectoderm, mesoderm, and endoderm lineages using established protocols. Cells were stained with Alexa Fluor 488-conjugated antibodies specific for MAP2 (ectoderm; Abcam; Cat. No. ab32454), Snail1 (mesoderm; Cell Signaling Technology; Cat. No. 3879), and AFP (endoderm; R&D Systems; Cat. No. MAB1368). Samples were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo software.

Statistics

All data are presented as mean $\pm$ standard error of the mean from at least three independent experiments. Statistical analyses were performed using SPSS software (v16.0, IBM Corp.). Group comparisons were conducted using one-way ANOVA. A p-value < 0.05 was considered statistically significant. For all quantitative analyses, n=3 independent biological replicates were used for colony formation, qRT-PCR, and apoptosis assays. Technical replicates were averaged within each biological replicate before statistical analysis.

Results

cP1P enhances stemness and colony formation efficiency in hPSCs during long-term culture

Before initiating the long-term culture experiments, we first optimized the concentration of cP1P to identify the most effective dose for maintaining hPSC morphology and stemness. Treatment with 10 nM cP1P resulted in compact, well-defined colony morphology similar to the control, whereas higher concentrations ($\geq 1,000$ nM) induced partial differentiation and irregular colony edges (Supplementary Fig. 1A). Based on these observations, 10 nM cP1P was selected as the fixed concentration for all sub-

Fig. 1. Long-term culture scheme of hiPSCs treated with cP1P. Schematic representation of long-term culture conditions for hiPSCs over a period of 60 days, spanning 15 passages (P-1 to P-15). Two groups were compared: the control (CTL) group cultured in TeSR-E8 medium supplemented with DMSO, and the experimental (cP1P) group cultured in TeSR-E8 medium supplemented with 10 nM cP1P. Cells were passaged every 4 days on average, and the effect of chronic exposure to cP1P was assessed throughout the duration of the culture. cP1P: O-cyclic phytosphingosine-1-phosphate, hiPSCs: human induced pluripotent stem cells.

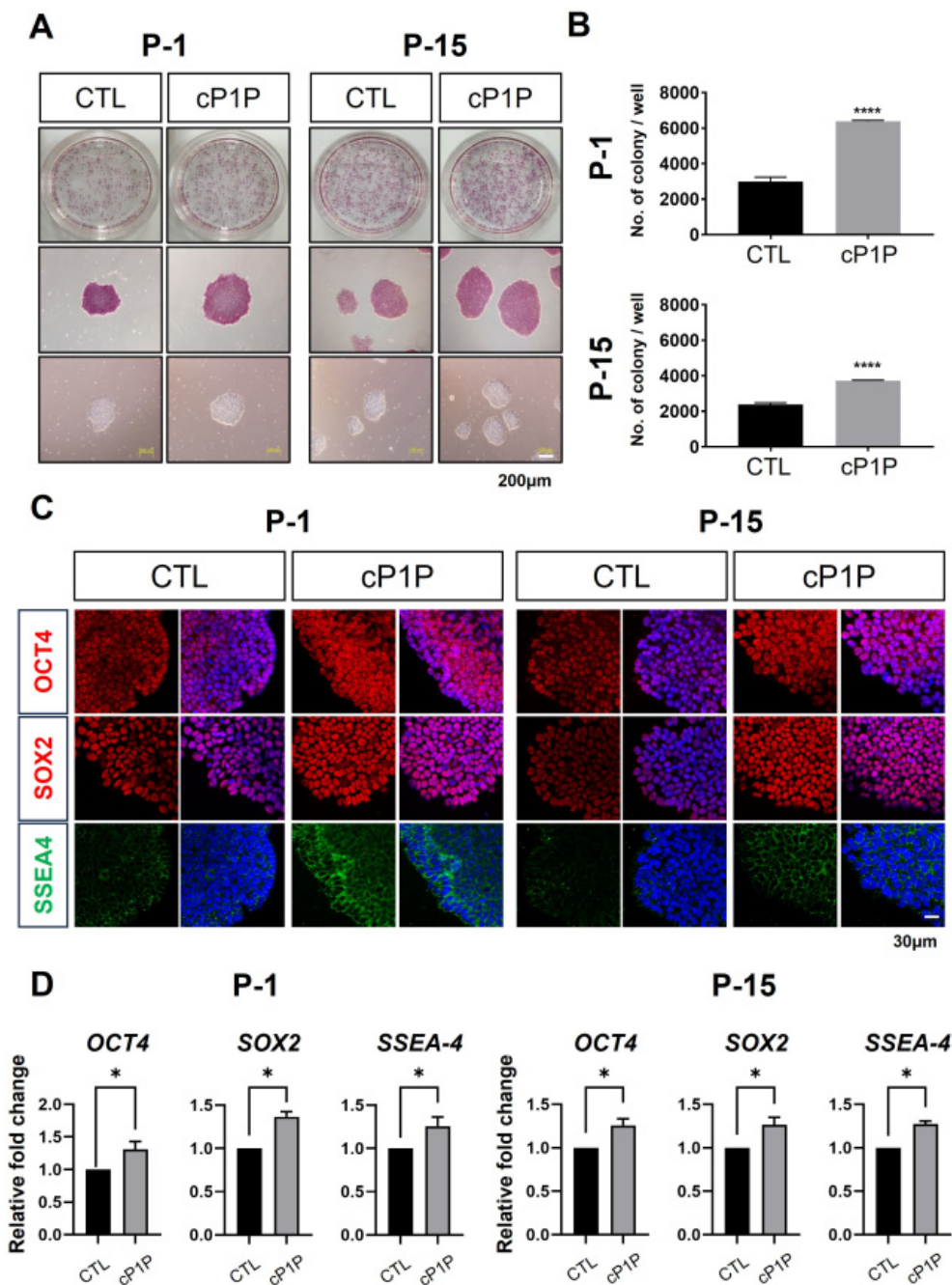


Fig. 2. cP1P enhances the stemness efficacy in hPSCs during both short-term (P-1) and long-term (P-15) passaging. (A) Colony formation assay in hPSCs. Representative images of colonies formed by hPSCs at passage 1 (P-1) and passage 15 (P-15) under control (CTL) and cP1P treatment conditions. The top row shows whole-well images of colonies, the middle row shows close-up images stained with alkaline phosphatase, and the bottom row shows phase-contrast images. Scale bar=200 μm. (B) Quantification of the number of colonies formed under CTL and cP1P treatment conditions. Data are presented as mean±standard deviation (SD). (C) Immunofluorescence staining of OCT4, SOX2, and SSEA-4 in hPSCs at P-1 and P-15. Representative images show the expression patterns of these stemness markers under CTL and cP1P treatment conditions. Scale bar=30 μm. (D) Quantitative polymerase chain reaction analysis of OCT4, SOX2, and SSEA-4 expression in hPSCs at P-1 and P-15. The bar graphs show the relative fold change in expression levels under CTL and cP1P treatment conditions. Data are presented as mean±SD. cP1P: O-cyclic phytosphingosine-1-phosphate, hPSCs: human pluripotent stem cells. * $p < 0.05$, **** $p < 0.0001$.

Table 1. Oligonucleotide sequences used for reverse transcription polymerase chain reaction

Gene	Oligonucleotides	
	Forward (5' ~3')	Reverse (5' ~3')
<i>OCT4</i>	GCTGGATGTCAGGGCTCTTT	TCAAGAGATTTATCGAGCACCTTCT
<i>SOX2</i>	AGTCTCCAAGCGACGAAAAA	TTTCACGTTTGCAACTGTCC
<i>SSEA-4</i>	TGGACGGGCACAACTTCATC	GGGCAGGTTCTTGGCACTCT
<i>APLNR</i>	GGGACACACTGAGGCAATGA	CTTCGCTCCTAAAGCACGGA
<i>APLN</i>	CTGAAGCAGGACTGAAGGGG	AGGAAGATGGACTGGACGGA
<i>FOSL1</i>	CCTCTGACCTACCCTCAGTACA	CTAGGCGTCCTTCTGCTT
<i>FOSL2</i>	CGGGAACCTTGACACCTCGT	GATGAATGCACTGCCTGAGC
<i>JUN</i>	GAGCTGGAGCGCCTGATAAT	CCCTCCTGCTCATCTGTAC
<i>JUND</i>	ATCGACATGGACACGCAGGA	AGCTCCGTGTTCTGACTCTTG
<i>VDR</i>	ACCTCTGAGGACCAGATCGT	GTCACCTGACGCGGTACTTGT
<i>ESRRB</i>	CAGGGATCAGAGCAACTCCC	ACATGGTGAGCCAGAGATGC
<i>PRDM14</i>	CCGCCCCGAAGTCTACCG	GCGGGTAGCACCTTGTC
<i>TBX3</i>	CTTCAACTGCTTCGCCAG	ATGCTCCTCTTTGCTCTCGG
<i>OTX2</i>	AAATCTCCCTGAGAGCGGGAC	TAGATGTGGCGAGTGAGGGC
<i>GAPDH</i>	GTCATCCCTGAGCTGAACGG	CCACCTGGTGCTCAGTGTAG

groups (Fig. 3B).

GO enrichment analysis revealed that DEGs in the cP1P-treated group were enriched for biological processes associated with apoptosis, cell proliferation, and development (Fig. 3C, 3D). Specifically, cP1P down-regulated genes involved in apoptotic processes and embryonic development, while up-regulated genes critical for stem cell maintenance and cell adhesion, suggesting that cP1P preserves the functional integrity of hPSCs during long-term culture.

Comparative analysis of gene expression changes

To validate the transcriptomic findings, we performed qRT-PCR analysis of key genes identified in the RNA-seq analysis. qRT-PCR results confirmed that stress response and apoptosis-related genes (e.g., *APLNR*, *APLN*, *FOSL1*, *FOSL2*, *JUN*, *JUND*, *VDR*, and *ESRRB*) were significantly upregulated in P-15 hPSCs under CTL conditions but downregulated in the cP1P-treated group (Fig. 3E), validating the protective effect of cP1P against stress-induced gene expression changes. Similarly, stemness-associated genes (e.g., *SOX2*, *PRDM14*, *TBX3*, and *OTX2*) were better maintained in cP1P-treated hPSCs compared to CTL (Fig. 3F), indicating the role of cP1P in preserving the pluripotent state.

cP1P mitigates apoptosis and promotes cell survival in long-term cultured hPSCs

Based on the transcriptomic evidence of reduced apoptotic signaling, we assessed apoptosis levels using flow cytometry and TUNEL assays. Flow cytometry analysis demonstrated a significant decrease in Annexin-V positive

apoptotic cells in cP1P-treated hPSCs at both P-1 and P-15 compared to CTL (Fig. 4A), with quantification revealing a statistically significant reduction in apoptosis ($p < 0.01$). TUNEL assay results also confirmed fewer TUNEL-positive cells in the cP1P-treated group (Fig. 4B), indicating reduced DNA fragmentation and cell death.

Additionally, growth curve analysis showed that cP1P-treated hPSCs exhibited significantly enhanced proliferation compared to CTL cells, particularly at P-15 (Fig. 4C), with a marked increase in total cell count.

Discussion

The prolonged culture of hPSCs poses significant challenges due to the potential accumulation of spontaneous mutations, genomic abnormalities, and alterations in gene expression. These changes can compromise the self-renewal capacity and pluripotency of hPSCs, making it essential to evaluate the impacts of long-term culture on these cells. In this study, we introduced cP1P, a novel culture additive developed by Axceso Biopharma Co. Ltd., which is structurally analogous to S1P. Our findings demonstrate that cP1P significantly enhances the self-renewal and survival of hPSCs, thereby addressing key challenges associated with prolonged culture.

Our results indicate that supplementation with cP1P not only improves the long-term proliferation of hPSCs but also sustains the expression of key pluripotency markers, such as *OCT4*, *SOX2*, and *SSEA-4*. The enhanced expression of these markers suggests that cP1P helps preserve the stemness and functionality of hPSCs even after extended periods

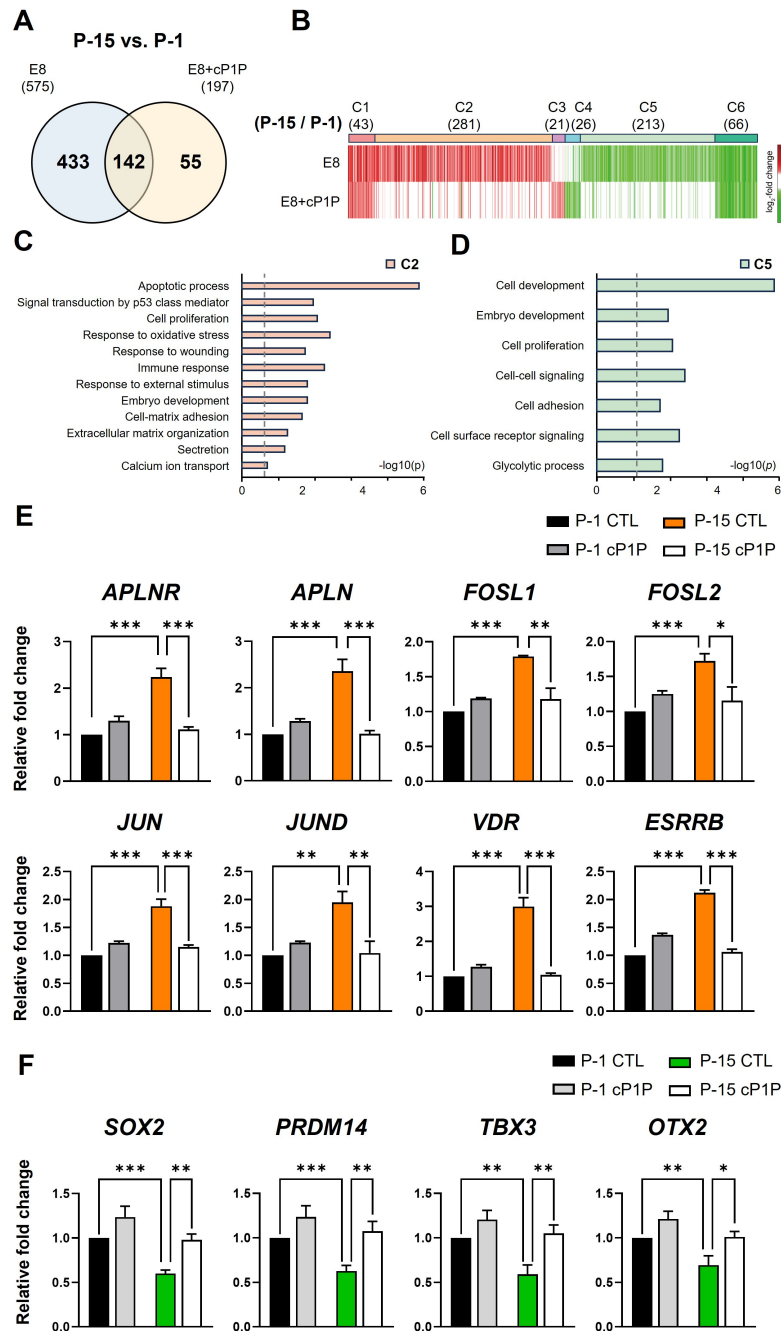


Fig. 3. Transcriptomic analysis of long-term cultured hPSCs reveals distinct gene expression patterns and biological processes modulated by cP1P. (A) Venn diagram showing the overlap of DEGs between passage 15 (P-15) vs. passage 1 (P-1) in hPSCs cultured in E8 medium alone (blue) and E8 medium with cP1P treatment (yellow). (B) Heatmap representing the clustering of DEGs in hPSCs between P-15 and P-1 under E8 and E8+cP1P conditions. The clusters C1 to C6 indicate groups of genes with similar expression patterns. (C) GO enrichment analysis of DEGs in cluster C2, highlighting processes such as apoptosis, oxidative stress response, and extracellular matrix organization. (D) GO enrichment analysis of DEGs in cluster C5, showing enrichment in cell development, adhesion, and glycolytic processes. (E) Bar graphs depicting the relative fold change in gene expression of *APLNR*, *APLN*, *FOSL1*, *FOSL2*, *JUN*, *JUND*, *VDR*, and *ESRRB* under four experimental conditions: P-1 CTL (black), P-15 CTL (orange), P-1 cP1P (gray), and P-15 cP1P (white). (F) Graphs showing the relative fold changes in gene expression of *SOX2*, *PRDM14*, *TBX3*, and *OTX2* across the same treatment conditions as in (A). P-15 CTL (green bars) demonstrates notable differences in gene expression compared to other conditions. cP1P: O-cyclic phytosphingosine-1-phosphate, hPSCs: human pluripotent stem cells, DEGs: differentially expressed genes, GO: Gene Ontology, CTL: control. The genes show variable expression profiles across different treatment conditions, with statistically significant differences denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

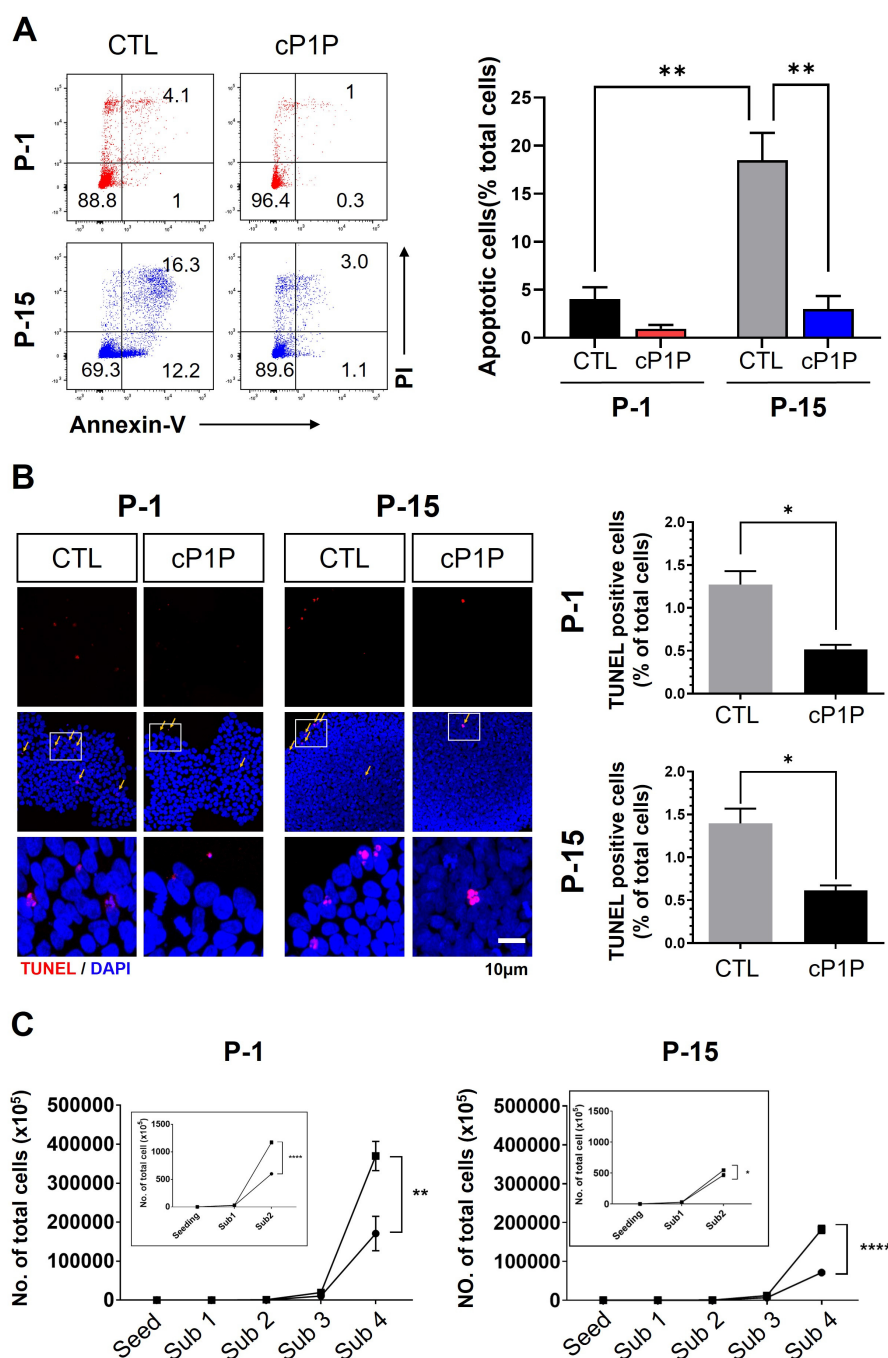


Fig. 4. Cellular apoptosis and proliferation under treatment conditions. (A) Flow cytometry analysis (left panels) and quantification (right panel) of apoptotic cells stained with Annexin-V in P-1 and P-15 cells treated with CTL and cP1P, showing significantly reduced apoptosis in P-15 cP1P cells. (B) TUNEL assay images and quantification of TUNEL-positive cells in P-1 and P-15 under CTL and cP1P conditions, demonstrating increased apoptosis. Scale bar=10 μ m. (C) Growth curves representing the total number of cells at different subcultures for P-1 and P-15 cells. cP1P: O-cyclic phytosphingosine-1-phosphate, CTL: control, DAPI: 4',6-diamidino-2-phenylindole. Insets show cell number at each subculture phase, with significant growth differences noted (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

in culture. Moreover, hPSCs treated with cP1P exhibited a robust capacity to differentiate into cell types representing all three germ layers, indicating that cP1P maintains the differentiation potential of these cells without com-

promising lineage specification. This is a critical finding, as the ability to generate diverse cell types is fundamental to the research and therapeutic utility of hPSCs.

Further supporting the beneficial effects of cP1P, our

RNA-seq analysis revealed that cPIP supplementation downregulated genes associated with apoptosis and chor-date embryonic development, which are typically induced by long-term culture. Additionally, cPIP upregulated genes crucial for stem cell maintenance. These transcriptional changes suggest that cPIP exerts a protective effect on hPSCs by protecting them against detrimental transcriptional and functional changes induced by prolonged culture and thereby preserving their functional integrity.

Independent of the current investigation, we observed that the cPIP which is structurally similar to S1P exerts its biological effects through activation of canonical S1P receptor-mediated signaling pathways. In mesenchymal stem cells and endothelial cells, cPIP has been shown to activate S1PR1 and S1PR3, thereby stimulating downstream Akt, ERK, and YAP/TAZ signaling that enhances cell survival, migration, and angiogenic potential (unpublished data). Consistent with these findings, our transcriptomic results showing suppression of apoptotic genes and enrichment of stemness-related pathways suggest that cPIP may also maintain hPSC pluripotency through similar S1PR-Akt/ERK-YAP/TAZ signaling mechanisms to preserve cell viability and self-renewal during long-term culture.

Collectively, our study suggests that cPIP is an effective additive for enhancing the proliferation, pluripotency, and differentiation potential of hPSCs during both short- and long-term culture. These findings have significant implications for the use of hPSCs in various research and clinical settings, where maintaining the quality and functionality of these cells over time is essential. By mitigating adverse effects associated with extended culture, cPIP could become a valuable tool for improving both the reliability and scalability of hPSC-based applications.

While our results are promising, further studies are needed to fully elucidate the molecular mechanisms by which cPIP exerts its effects on hPSCs. Additionally, long-term investigations assessing the genomic stability and *in vivo* differentiation potential of cPIP-treated hPSCs are warranted. Such investigations will be crucial for validating the safety, efficacy, and stability of cPIP as a culture additive for hPSCs, ultimately paving the way for its broader application in stem cell research and regenerative medicine.

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Potential Conflict of Interest

Two of the co-authors, Young Jun Park and Myeong-Jun Choi, have financial interests in Axcreso Biopharma Co., Ltd., which holds product and method-of-use patents pending for cPIP.

Patent title: Composition for stimulating stem cell proliferation comprising cPIP or a pharmaceutically acceptable salt thereof as an active ingredient

Patent application number: KR10-2019-0079547

Control number: KR2024-10-2276781

All other authors declare no competing financial or personal interests.

Authors' Contribution

Conceptualization: SHW, BHR. Data curation: BHR, SC, VM, DH. Formal analysis: BHR, SC, DH. Funding acquisition: KSK. Investigation: SHW, KSK. Methodology: SHW. Project administration: SHW, KSK. Resources: YJP, MJC. Supervision: KSK. Validation: SHW, BHR. Visualization: SHW. Technical assistance: BHR, WJJ. Writing – original draft: SHW, SR. Writing – review & editing: SHW, BHR, WJJ, VM, SR, KSK.

Supplementary Materials

Supplementary data including one figure can be found with this article online at <https://doi.org/10.15283/ijsc25107>

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