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Original Article

Selective effects of collagen-derived peptides Pro-Hyp and Hyp-Gly on the proliferation and differentiation of SSEA3-positive human dental pulp stem cells

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ABSTRACT

Introduction: Collagen-derived peptides demonstrate diverse biological activities, but their effects on human dental pulp stem cells (hDPSCs) remain unclear. This study investigated the influence of prolyl-hydroxyproline (Pro-Hyp) and hydroxyproline-glycine (Hyp-Gly) on stage-specific embryonic antigen 3 (SSEA3)-positive hDPSCs.
Methods: SSEA3-positive and SSEA3-negative hDPSCs were isolated and cultured with Pro-Hyp or Hyp-Gly (200 μg/ml) under stem cell medium or differentiation-inducing conditions. Cell proliferation was assessed using the MTT assay. The expression of the chondrogenic and osteogenic markers was analyzed by real-time PCR. Matrix production was evaluated using Alcian blue and Alizarin red S staining.
Results: Pro-Hyp and Hyp-Gly enhanced the proliferation of SSEA3-positive hDPSCs compared with SSEA3-negative cells. Pro-Hyp promoted chondrogenic differentiation through early upregulation of Col II followed by sustained SOX9 expression, with enhanced matrix production after 2 weeks in chondrogenic medium. Hyp-Gly specifically enhanced the osteogenic differentiation of SSEA3-positive cells through the temporal regulation of gene expression, progressing from early transcription factors (RUNX2 and RANKL) to matrix proteins (ALPL, Col I, BSP, and OCN), resulting in increased mineralization under osteogenic conditions.
Conclusions: This study demonstrated that Pro-Hyp and Hyp-Gly selectively influenced SSEA3-positive hDPSC fate through the distinct temporal regulation of differentiation pathways. These findings provide new insights into the development of targeted regenerative strategies using collagen-derived peptides in dental tissue engineering.

1. Introduction

Collagen, the most abundant protein in the human body, contains unique amino acid sequences with hydroxyproline (Hyp) residues that contribute to its structural integrity and biological functions [1]. During collagen metabolism, specific peptides containing Hyp are generated and can be detected in the circulation, with prolyl-hydroxyproline (Pro-Hyp) and hydroxyproline-glycine (Hyp-Gly) being particularly prevalent [2,3]. These collagen-derived peptides demonstrate diverse biological activities, including enhancement of cell proliferation, regulation of differentiation, and modulation of extracellular matrix production across various cell types [4,5]. Recent studies have revealed that Pro-Hyp can be detected at concentrations of several hundred μM in human

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blood after collagen hydrolysate ingestion, suggesting its potential role as a bioactive signaling molecule [6,7].

The biological mechanisms underlying the effects of collagen-derived peptides, particularly in stem cell differentiation and tissue regeneration, remain incompletely understood. Although previous studies have demonstrated that Pro-Hyp promotes osteoblast differentiation through Foxg1-mediated regulation of Runx2 [8,9] and enhances chondrogenic differentiation under hypoxic conditions [5], the specific effects of these peptides on human dental pulp stem cells (hDPSCs) have not been explored. Progenitor and stem cells within the dental pulp reside in distinct niches, including the subodontoblastic layer, central pulp, and apical pulp, where they contribute to tissue homeostasis and repair [10,11]. Previous studies suggest that SSEA3-positive cells, a subpopulation with high regenerative potential, may preferentially localize to these areas, particularly in response to tissue injury [12,13]. However, their precise *in vivo* distribution remains unclear, necessitating further histological investigations to confirm their spatial arrangement in dental pulp tissues.

Pro-Hyp has also been shown to influence various cellular processes including myogenic differentiation [14], brown adipocyte differentiation [15], and wound healing [16]. Furthermore, the relationship between stage-specific embryonic antigen 3 (SSEA3) expression and cellular response to collagen-derived peptides remains unknown, despite SSEA3 being recognized as a marker for cells with enhanced regenerative potential [13,17]. Understanding these interactions is crucial for developing more effective regenerative therapies, particularly in the context of dental tissue engineering, where the manipulation of stem cell fate determination is essential.

This study aimed to investigate the effects of collagen-derived peptides, specifically Pro-Hyp and Hyp-Gly, on SSEA3-positive and SSEA3-negative hDPSC populations. Building on previous findings demonstrating the diverse effects of these peptides on cellular behavior [18,19], we evaluated the impact of these peptides on cell proliferation and assessed their influence on lineage-specific differentiation pathways, focusing particularly on chondrogenic and osteogenic differentiation. Through comprehensive analysis of gene expression patterns, protein markers, and functional outcomes [20,21], we sought to elucidate the mechanisms by which these peptides modulate stem cell behavior and differentiation potential. Understanding these interactions could provide valuable insights for optimizing tissue engineering strategies and developing more effective regenerative therapies in dental medicine [22].

2. Materials and methods

2.1. Cell source and isolation

Impacted third molars were collected from healthy donors (age range: 16–30 years) at the Department of Dental and Oral Surgery, Ryukyu University Hospital. The study protocol received approval from the University's Ethics Committee (approval no.: 1907), and written informed consent was obtained from all participants or their legal guardians for those under 20 years of age.

Patients were excluded if they presented with: infectious diseases (Hepatitis B, Hepatitis C, HIV, HTLV-1, or syphilis), diabetic complications, osteomyelitis, jaw bone cysts, malignant tumors, apical periodontitis, pulp gangrene, or pulpitis. Additional exclusion criteria were applied based on the attending dentist's clinical assessment.

The extracted teeth were immediately placed in perfusion buffer (Perfusion Solution MOD. #1; Mediatech, Inc., USA) containing 1 % penicillin–streptomycin and transported to the laboratory on ice. The dental pulp tissue was exposed by vertical sectioning of the teeth after careful removal of the surrounding tissues. The isolated pulp tissue was mechanically minced and enzymatically digested using Liberase (Roche, Switzerland) at 37 °C for 90 min with gentle agitation, following previously described protocols [13].

2.2. Cell culture

The isolated cells were expanded in ADSC growth medium (ADSC-BulletKit™; Lonza, Basel, Switzerland) at 37 °C in a humidified atmosphere containing 5 % CO2. The culture medium was replenished every 48 h until cells reached 80%–90 % confluence. Primary cultures (P0) were passaged to generate first-passage (P1) cells. Subsequent passages were performed according to standardized cell density parameters to maintain optimal growth conditions.

2.3. Flow cytometric sorting of the SSEA3+ and SSEA3-hDPSC populations

SSEA3-expressing cell populations were isolated using a Sony SH800S Cell Sorter (Sony Biotechnology Inc., Tokyo, Japan) equipped with a 130-μm sorting chip. Cells were stained with rat anti-human SSEA3 monoclonal antibody (Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by goat anti-rat IgM (FITC) secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) according to the manufacturer's protocol. Before sorting, the cell suspensions were filtered through a 35-μm nylon mesh to remove aggregates. The sorted SSEA3+ and SSEA3-populations were subsequently analyzed for cell proliferation, gene expression, and immunostaining characteristics.

2.4. Peptide preparation and treatment

Hydroxyproline (Hyp)-containing dipeptides (Hyp-Gly and Pro-Hyp; Bachem AG, Bubendorf, Switzerland) were reconstituted to a final concentration of 200 μg/ml. For long-term experiments (≥3 days), peptides were replenished every 72 h. The control groups were maintained under identical conditions without peptide supplementation.

The peptide concentration of 200 μg/ml was chosen based on previous studies showing that Pro-Hyp and Hyp-Gly exhibit optimal biological activity within this range. Yazaki et al. (2017) demonstrated that Pro-Hyp can reach micromolar concentrations in human plasma after the oral administration of collagen hydrolysates [6]. This concentration is consistent with the physiological levels and is therefore relevant for regenerative applications. Additionally, previous *in vitro* studies reported that Pro-Hyp at 200 μg/ml significantly enhances the proliferation and differentiation of fibroblasts and osteoblast-like cells [2,8]. Thus, this concentration was selected to ensure both biological relevance and consistency with previous research.

2.5. Cell proliferation assay

The proliferation rates of SSEA3+ and SSEA3-hDPSCs in response to Hyp-containing peptides were evaluated using an MTT Cell Proliferation Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Sorted hDPSCs were seeded in 96-well plates at a density of 3×10^3 cells/cm^2 and cultured in ADSC-BM supplemented with Hyp-Gly or Pro-Hyp (200 μg/ml). After 72 h of incubation, 20 μL of MTT solution (5 mg/ml) was added to each well, followed by incubation for 90 min at 37 °C. The absorbance was measured at 570 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Lineage-specific differentiation

SSEA3+ and SSEA3-hDPSCs were induced to undergo lineage-specific differentiation following previously established protocols [23]. For chondrogenic differentiation, sorted cells were cultured in 48-well plates with chondrocyte differentiation-inducing medium (CDIM; Funakoshi Co., Ltd., Tokyo, Japan). Osteogenic differentiation was induced by maintaining cells in osteoblast differentiation-inducing medium (ODIM) consisting of ADSC-BM supplemented with 10 % fetal bovine serum (FBS), 1 % antibiotics, 10 nM dexamethasone, 0.1 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate. ADSC-BM served as the stem cell medium (SCM) for the control cultures. The experimental time points (2, 4, and 8 days) were chosen to capture the distinct phases of differentiation in SSEA3-positive hDPSCs. Early time points (2 and 4 days) were selected to monitor the initial activation of transcription factors (e.g., RUNX2, SOX9) and early matrix proteins (e.g., Col II). The 8-day time point was included to assess the progression from transcriptional activation to extracellular matrix production and mineralization. These time points align with previous studies on stem cell differentiation, which highlighted the temporal dynamics of lineage-specific marker expression and functional outcomes [5,13]. All differentiation media were replenished every 72 h throughout the experimental period. Differentiation was confirmed through lineage-specific gene expression analysis and histological staining.

2.7. Gene expression analysis

Total RNA was extracted from SSEA3+ and SSEA3-hDPSCs using TRI Reagent (Merck KGaA, Darmstadt, Germany) and a PureLink RNA Mini kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Complementary DNA was synthesized using a PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions.

Gene expression was analyzed using a Rotor-Gene Q 2plex System (Qiagen GmbH, Hilden, Germany) with FAM/ZEN/IBFQ probes (Integrated DNA Technologies, Inc., Coralville, IA, USA). The following genes were assessed: alkaline phosphatase (ALPL; Hs. PT.56a.40555206), bone sialoprotein (BSP; Hs. PT.58.4556745), type I collagen (Col I; Hs. PT.58.15517795), type II collagen (Col II; Hs. PT.58.4107778), osteocalcin (OCN; Hs. PT.56a.39318706.g), receptor activator of nuclear factor-kappa B ligand (RANKL; Hs. PT.58.23324760), runt-related transcription factor 2 (RUNX2; Hs. PT.56a.19568141), and SRY-box transcription factor 9 (SOX9; Hs. PT.58.38984663).

PCR reactions were performed using 10 ng of cDNA under the following conditions: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Data were analyzed using the Rotor-Gene Q software version 2.3.1 (Qiagen).

Eight reference genes were evaluated for stability: Hypoxanthine Phosphoribosyltransferase 1 (Hs. PT.58v.45621572), beta 2-microglobulin (Hs. PT.58v.18759587), TATA-Box Binding Protein (Hs. PT.58v.39858774), Glucuronidase Beta (Hs. PT.58v.27737538), Actin Beta (ACTB; Hs. PT.39a.22214847), RNA Polymerase II Subunit A (Hs. PT.39a.19639531), Ribosomal Protein Lateral Stalk Subunit P0 (Hs. PT.39a.22214824), and Peptidylprolyl Isomerase A (Hs. PT.58v.38887593.g). Based on the validation experiments, ACTB was selected as the internal control using the HEX/ZEN/IBFQ probes. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [24], with the results expressed as fold changes relative to the mean of three experimental measurements.

2.8. Histological analysis

To detect acid mucopolysaccharides in differentiated chondrocytes, SSEA3+ and SSEA3-hDPSCs were fixed with 2 % paraformaldehyde (PFA) for 10 min at room temperature after washing with PBS. After three rinses with tap water, the cells were treated with 3 % acetic acid for 5 min. The cells were then incubated with Alcian blue solution (Sigma-Aldrich, St. Louis, MO, USA) for 2 h, followed by a second 5-min treatment with 3 % acetic acid. Images of the blue-stained matrices were captured under microscopic examination. To visualize calcium deposition in differentiated osteoblasts, cells were fixed with 2 % PFA for 10 min at room temperature after washing with PBS. Following three rinses with tap water, the cells were stained with Alizarin red S solution (Sigma-Aldrich) for 5 min at room temperature. After three additional tap water rinses, the red-stained matrices were photographed under microscopic examination. The staining intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) from the captured microscopic images.

2.9. Statistical analysis

Statistical analyses were performed using EZR software version 2.13.0 (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing) (Kanda, 2013). Normality of the data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated with Levene's test. If normality and variance assumptions were met, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple group comparisons. For non-normally distributed data, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied for more than two groups, while the Mann-Whitney *U* test was used for two-group comparisons. Exact P-values are reported for all statistical comparisons.

3. Results

3.1. Enhanced proliferation of SSEA3-positive hDPSCs with hydroxyproline-containing peptides

The effects of hydroxyproline (Hyp)-containing peptides on cell proliferation were evaluated in SSEA3-positive and SSEA3-negative human dental pulp stem cells (hDPSCs) (Fig. 1). MTT assays revealed

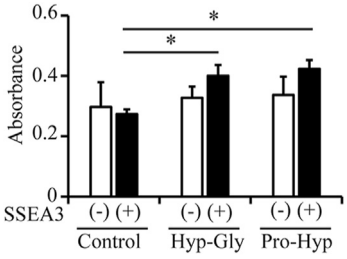

Fig. 1. Effects of Hyp-containing peptides on cell proliferation in SSEA3-positive and SSEA3-negative hDPSCs
Cell proliferation activity was assessed using MTT assays in SSEA3-positive (+) and SSEA3-negative (−) cells sorted from hDPSCs after 72 h of culture with Hyp-containing peptides. Cells were treated with either control medium, Pro-Hyp (200 μg/ml), or Hyp-Gly (200 μg/ml). Data are presented as mean absorbance values measured at 570 nm ± standard deviation (n = 3). Statistical analysis: One-way ANOVA followed by Tukey's post hoc test; *$p < 0.05$ was considered significant. White bars represent SSEA3-negative cells; black bars represent SSEA3-positive cells. Significant differences compared with the SSEA3-positive control group are marked as *$p < 0.05$.

that Hyp-Gly and Pro-Hyp peptides at 200 μg/ml significantly enhanced the proliferation of SSEA3-positive cells compared with the control group without peptide supplementation (Fig. 1). The proliferation-promoting effect was particularly evident in SSEA3-positive cells, which demonstrated higher absorbance values at 570 nm compared with their SSEA3-negative counterparts under peptide treatment conditions (Fig. 1). Specifically, SSEA3-positive cells treated with Hyp-Gly showed increased absorbance (0.410 ± 0.036) compared with untreated controls (0.273 ± 0.015) ($P = 0.0435$). Similarly, the Pro-Hyp treatment resulted in enhanced proliferation (0.423 ± 0.029) compared with the controls ($P = 0.0234$). In contrast, SSEA3-negative cells maintained consistent proliferation rates across all conditions (Control: 0.297 ± 0.081; Hyp-Gly: 0.327 ± 0.038; Pro-Hyp: 0.337 ± 0.060). These findings suggest that Hyp-containing peptides selectively stimulate the proliferation of SSEA3-positive hDPSCs.

3.2. Pro-hyp promotes chondrogenic differentiation and matrix production in SSEA3-positive hDPSCs

The chondrogenic differentiation potential of SSEA3-positive and SSEA3-negative hDPSCs in response to hydroxyproline (Hyp)-containing peptides was evaluated through analysis of gene expression and matrix production under SCM and CDIM conditions (Fig. 2). After 2 days of culture, Pro-Hyp treatment significantly increased Col II expression in SSEA3-positive cells under SCM (2.801 ± 0.557) and CDIM (4.270 ± 0.889) conditions compared with their respective controls (SCM: 1.167 ± 0.402; CDIM: 2.120 ± 0.361) (SCM: $P = 0.0469$; CDIM: $P = 0.0310$), while SOX9 expression showed only modest increases (Fig. 2A).

After 4 days of culture, a distinct temporal regulation of chondrogenic markers emerged, particularly for SOX9 expression (Fig. 2B). SSEA3-positive cells treated with Pro-Hyp demonstrated significantly elevated SOX9 levels in SCM (9.602 ± 3.547) and CDIM (6.820 ± 1.200) compared with controls (SCM: 1.110 ± 0.265; CDIM: 2.810 ± 1.375) (SCM: $P = 0.0002$; CDIM: $P = 0.0198$). Col II expression maintained moderate elevation in Pro-Hyp treated SSEA3-positive cells, although less pronounced than on day 2.

The functional significance of these molecular changes was confirmed by Alcian blue staining of the acid mucopolysaccharides (Fig. 2C and D). After 2 weeks in CDIM, SSEA3-positive cells treated with Pro-Hyp showed enhanced matrix production (57.640 ± 7.550) compared with the control conditions (36.900 ± 3.512) ($P = 0.0360$) (Fig. 2C). Similar enhancement was observed after 2 weeks in SCM (Pro-Hyp: 50.333 ± 8.000; Control: 33.333 ± 6.000) ($P = 0.0405$) (Fig. 2D). SSEA3-negative cells consistently showed lower responses to peptide treatment across all parameters. These findings demonstrate that Pro-Hyp selectively promotes chondrogenic differentiation in SSEA3-positive hDPSCs through the time-dependent regulation of chondrogenic markers and enhanced matrix production.

3.3. Hyp-gly promotes stage-specific osteogenic differentiation in SSEA3-positive hDPSCs

The temporal progression of osteogenic differentiation in response to hydroxyproline (Hyp)-containing peptides was investigated through gene expression analysis and matrix mineralization assessment in SSEA3-positive and SSEA3-negative hDPSCs (Fig. 3). On day 2, Hyp-Gly treatment predominantly affected early osteogenic regulators in SSEA3-positive cells (Fig. 3A), with significant increases in RUNX2 (SCM: 2.101 ± 0.346; ODIM: 5.867 ± 1.882) (SCM: $P = 0.0198$; ODIM: $P = 0.0315$) and RANKL (SCM: 2.105 ± 0.361; ODIM: 4.067 ± 1.026) (SCM: $P = 0.0145$; ODIM: $P = 0.0060$) expression compared with controls. Other osteogenic markers, including ALPL, BSP, Col I, and OCN, showed moderate increases although these changes were less pronounced at this early stage.

By day 4, the expression pattern shifted toward matrix-associated genes (Fig. 3B), with SSEA3-positive cells showing significant upregulation of ALPL (3.667 ± 1.464) and Col I (4.067 ± 1.102) under ODIM conditions compared with controls (ALPL: 1.767 ± 0.351; Col I: 2.150 ± 0.700) (ALPL: $P = 0.0490$; Col I: $P = 0.0448$). This transition suggests a progression from transcription factor activation to matrix protein production, whereas the expression of the early markers RUNX2 and RANKL remained moderately elevated.

On day 8, SSEA3-positive cells exhibited robust expression of multiple matrix-associated genes under Hyp-Gly treatment (Fig. 3C). In SCM, dramatic increases were observed in ALPL (10.333 ± 2.517) ($P = 0.0001$), Col I (6.800 ± 2.621) ($P = 0.0026$) expression and OCN (2.610 ± 0.361) ($P = 0.0416$) expression. Under ODIM conditions, a significant elevation was observed in BSP (5.633 ± 1.185) and OCN (3.520 ± 0.954) expression compared with controls (BSP: $P = 0.0055$; OCN: $P = 0.0415$), indicating advanced osteogenic differentiation. The simultaneous upregulation of these matrix proteins suggests a coordinated progression toward mineralization.

The functional significance of these molecular changes was confirmed through matrix mineralization analysis after two weeks of culture (Fig. 3D and E). Under ODIM conditions, SSEA3-positive cells treated with Hyp-Gly showed significantly enhanced calcium deposition (14.330 % ± 5.033 % positive area) compared with the control conditions (1.800 % ± 0.985 %) ($P = 0.0004$) and Pro-Hyp treatment (5.333 % ± 2.309 %) ($P = 0.0078$) (Fig. 3D). However, this effect was not observed under SCM conditions, where minimal calcium deposition was detected across all treatment groups (Control: 0.633 % ± 0.577 %; Hyp-Gly: 0.967 % ± 0.681 %; Pro-Hyp: 0.967 % ± 0.862 %) (Fig. 3E), suggesting that osteogenic culture conditions are essential for Hyp-Gly-induced mineralization.

Throughout all time points and conditions, SSEA3-negative cells showed minimal responses to peptide treatment, highlighting the selective nature of Hyp-Gly's effects on SSEA3-positive cells. These findings demonstrate that Hyp-Gly specifically promotes osteogenic differentiation in SSEA3-positive hDPSCs through the temporally coordinated regulation of gene expression, progressing from early transcription factors to matrix proteins, ultimately resulting in enhanced mineralization under appropriate culture conditions.

4. Discussion

In this study, we demonstrated three significant findings regarding the effects of hydroxyproline (Hyp)-containing peptides on SSEA3-positive human dental pulp stem cells (hDPSCs). First, we found that Hyp-containing peptides selectively enhanced SSEA3-positive hDPSCs proliferation compared with SSEA3-negative cells, suggesting a cell population-specific response mechanism. Second, Pro-Hyp specifically promoted chondrogenic differentiation in SSEA3-positive hDPSCs through the time-dependent regulation of chondrogenic markers and enhanced matrix production. Third, we demonstrated that Hyp-Gly distinctly enhanced osteogenic differentiation in SSEA3-positive hDPSCs through the temporally coordinated regulation of gene expression, progressing from early transcription factors to matrix proteins, ultimately resulting in enhanced mineralization under appropriate culture conditions. These findings provide new insights into the selective effects of specific collagen-derived peptides on SSEA3-positive hDPSC fate determination and their potential applications in tissue-specific regenerative strategies.

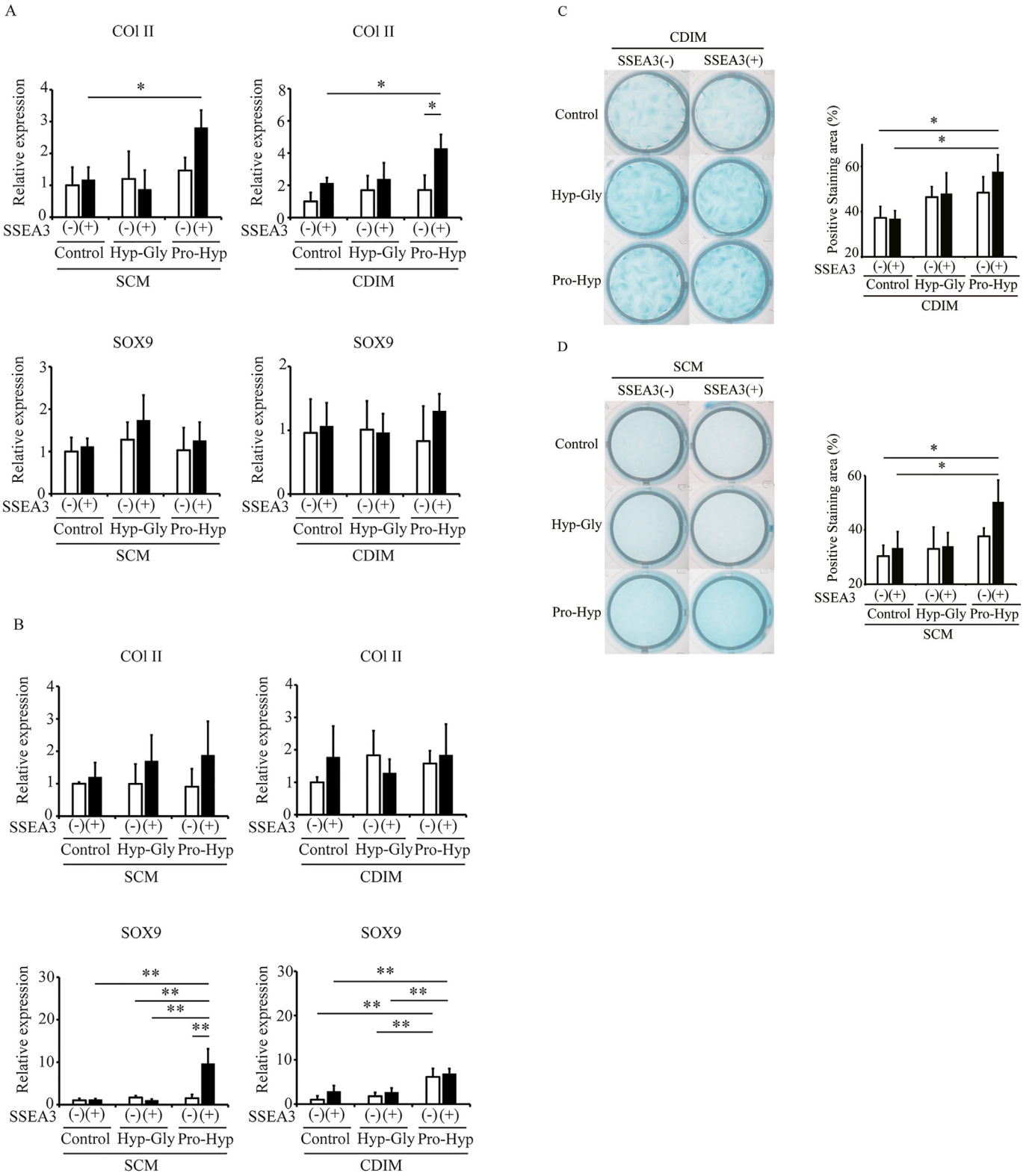

Fig. 2. Effects of Hyp-containing peptides on the chondrogenic differentiation of SSEA3-positive and SSEA3-negative hDPSCs
(A) Expression of chondrogenic marker genes (Col II and SOX9) after 2 days of culture. SSEA3-positive (+) and SSEA3-negative (−) cells were cultured in either stem cell medium (SCM) or chondrocyte differentiation-inducing medium (CDIM) with Pro-Hyp (200 μg/ml) or Hyp-Gly (200 μg/ml). Gene expression levels were normalized to ACTB and are presented as fold changes relative to SSEA3-negative control cells. Error bars represent standard deviation (n = 3). *$p < 0.05$, significant difference compared with the SSEA3-positive control group without peptides (one-way ANOVA followed by Tukey's post hoc test). (B) Expression of chondrogenic marker genes after 4 days of culture under the same conditions as described in (A). *$p < 0.05$, **$p < 0.01$, significant differences as indicated by bars (one-way ANOVA followed by Tukey's post hoc test). (C) Representative images of Alcian blue staining showing acid mucopolysaccharide production after 2 weeks of culture in CDIM. Quantification of the positive staining area is shown in the right panel. Error bars represent standard deviation (n = 3). **$p < 0.01$, significant differences as indicated by bars (one-way ANOVA followed by Tukey's post hoc test). (D) Representative images of Alcian blue staining showing acid mucopolysaccharide production after 2 weeks of culture in SCM. Quantification of the positive staining area is shown in the right panel. Error bars represent standard deviation (n = 3). White bars represent SSEA3-negative cells; black bars represent SSEA3-positive cells throughout all graphs.

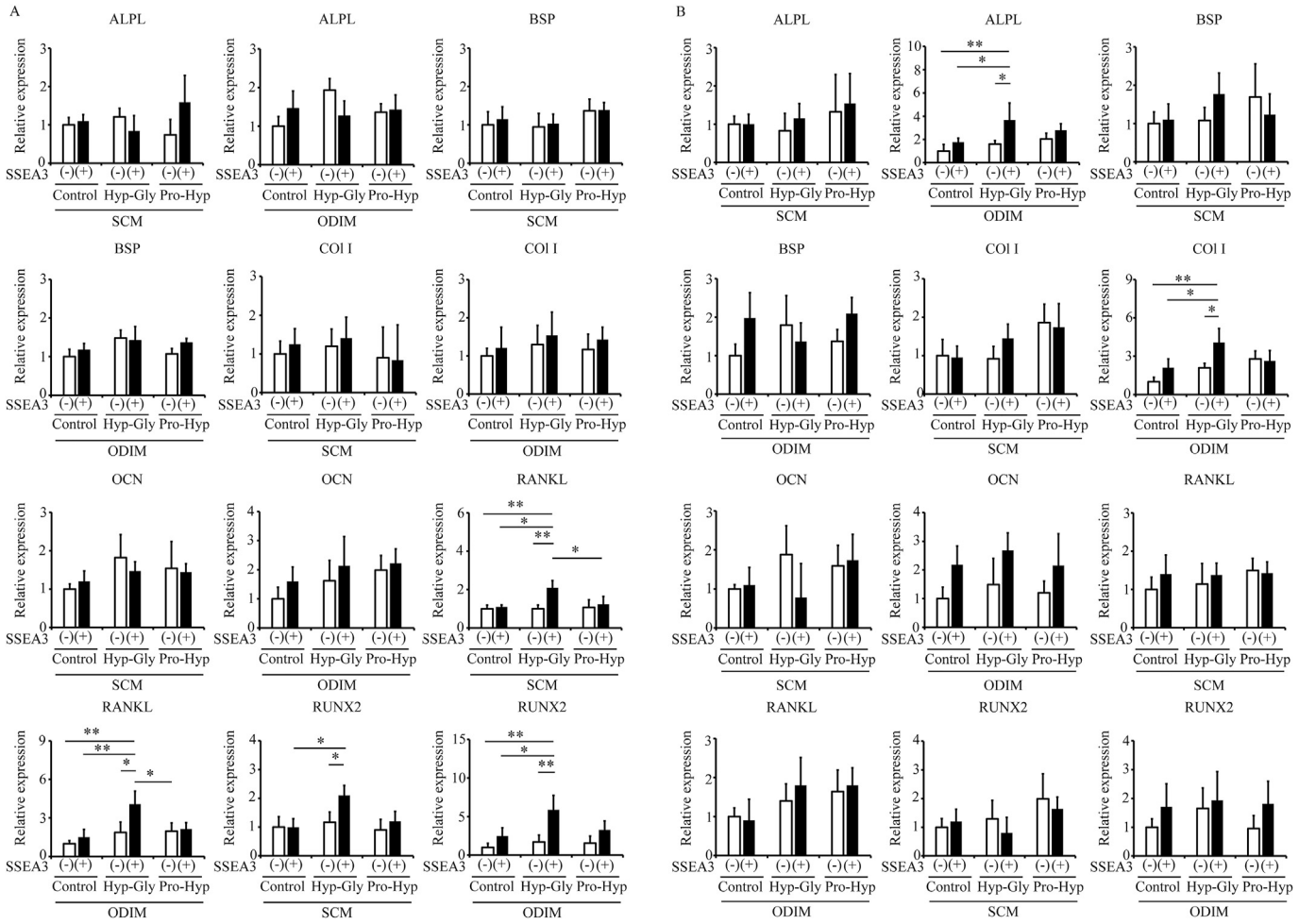

Fig. 3. Effects of Hyp-containing peptides on the osteogenic differentiation of SSEA3-positive and SSEA3-negative hDPSCs
(A) Expression of the osteogenic marker genes (RUNX2, RANKL, Col I, ALPL, OCN, and BSP) after 2 days of culture. SSEA3-positive (+) and SSEA3-negative (−) cells were cultured in either stem cell medium (SCM) or osteoblast differentiation-inducing medium (ODIM) with Pro-Hyp (200 μg/ml) or Hyp-Gly (200 μg/ml). Gene expression levels were normalized to ACTB and are presented as fold changes relative to SSEA3-negative control cells. Error bars represent standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$, significant differences as indicated by bars (one-way ANOVA followed by Tukey's post hoc test). (B) Expression of osteogenic marker genes after 4 days of culture under the same conditions as described in (A). *$p < 0.05$, **$p < 0.01$, significant differences as indicated by bars (one-way ANOVA followed by Tukey's post hoc test). (C) Expression of osteogenic marker genes after 8 days of culture under the same conditions as described in (A). *$p < 0.05$, **$p < 0.01$, significant differences as indicated by bars (one-way ANOVA followed by Tukey's post hoc test). (D) Representative images of Alizarin red S staining showing calcium deposition after 2 weeks of culture in ODIM. Quantification of the positive staining area is shown in the right panel. Error bars represent standard deviation (n = 3). **$p < 0.01$, significant differences as indicated by bars (one-way ANOVA followed by Tukey's post hoc test). (E) Representative images of Alizarin red S staining showing calcium deposition after 2 weeks of culture in SCM. Quantification of the positive staining area is shown in the right panel. Error bars represent standard deviation (n = 3).
White bars represent SSEA3-negative cells; black bars represent SSEA3-positive cells throughout all graphs.

The selective enhancement of SSEA3-positive hDPSC proliferation by Hyp-containing peptides represents a significant advancement in the understanding of peptide-mediated stem cell regulation. Our results showed that both Pro-Hyp and Hyp-Gly peptides comparably enhanced the proliferation of SSEA3-positive cells, while showing minimal effects on SSEA3-negative populations. This selective response differs from previous observations in other cell types, where Pro-Hyp has distinct effects on cell proliferation compared with other Hyp-containing peptides [2]. The cell population-specific response may be related to the unique characteristics of SSEA3-positive cells, which have been identified as a distinct subpopulation with enhanced regenerative potential [13]. This finding suggests that both peptides may share common mechanisms for promoting proliferation in SSEA3-positive hDPSCs, possibly through similar cellular uptake pathways or downstream signaling cascades. The selective proliferation effect observed in our study is particularly noteworthy as it indicates that these peptides could be used to specifically expand the SSEA3-positive cell population, which is crucial for regenerative medicine applications.

The temporal regulation of chondrogenic markers and enhanced matrix production by Pro-Hyp in SSEA3-positive hDPSCs represents a significant advancement in understanding peptide-mediated differentiation control. Our observation of early Col II upregulation followed by sustained SOX9 expression aligns with recent findings demonstrating that Pro-Hyp promotes chondrogenic differentiation under physiological conditions [5] and enhances extracellular matrix production [3]. The enhanced matrix production observed in our study may be explained by Pro-Hyp's reported ability to activate specific cellular signaling pathways and influence matrix synthesis [2,4]. The selective response of SSEA3-positive cells to Pro-Hyp-induced chondrogenic differentiation parallels

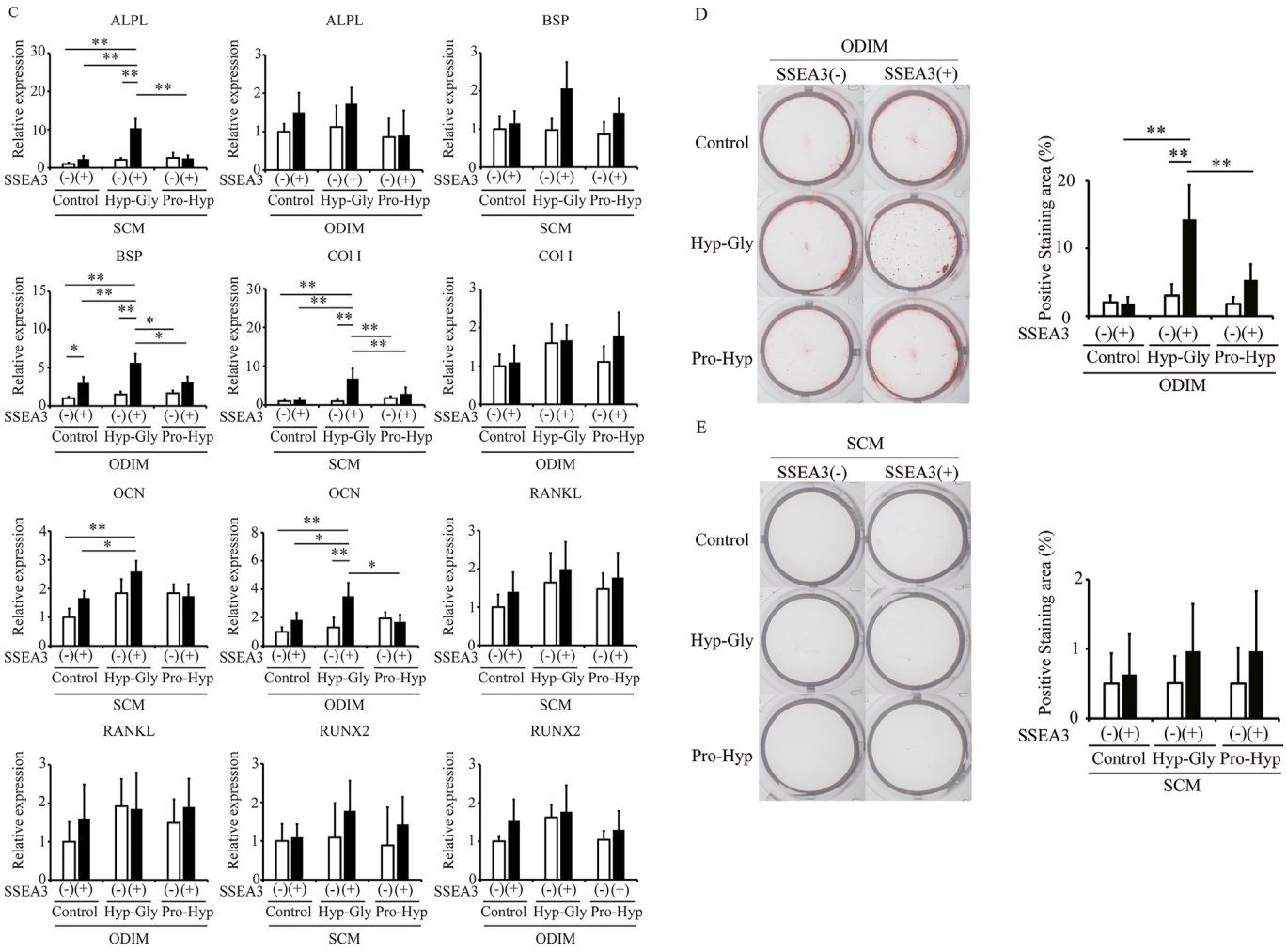

Fig. 3. (*continued*).

findings in other stem cell populations, where specific subpopulations show enhanced responsiveness to differentiation signals [13,20]. Moreover, the observed time-dependent regulation suggests a coordinated mechanism similar to that reported in ATDC5 cells, where Pro-Hyp enhanced chondrogenic differentiation through the sequential activation of key regulatory pathways [5] and influenced matrix organization [16].

The observed temporal regulation of osteogenic differentiation by Hyp-Gly in SSEA3-positive hDPSCs demonstrates a sophisticated control of cell fate determination. Our findings of sequential activation, from early transcription factors to matrix proteins, align with established osteogenic differentiation pathways but reveal a novel peptide-specific regulation. Previous studies have shown that Pro-Hyp promotes osteoblast differentiation through the Foxg1-mediated regulation of Runx2 [8], whereas our results demonstrate that Hyp-Gly exhibits a distinct pattern of temporal regulation. The requirement for appropriate culture conditions for mineralization is consistent with reports showing that the cellular response to collagen-derived peptides is context-dependent [8]. The enhanced mineralization observed specifically in SSEA3-positive cells suggests a unique interaction between Hyp-Gly and this stem cell subpopulation, possibly related to their distinct molecular signature [13]. This finding expands on previous observations of the effects of collagen peptides on osteoblast differentiation by demonstrating cell type-specific responses and defining the temporal sequence of molecular events leading to enhanced mineralization.

The selective effects of the collagen-derived peptides Pro-Hyp and Hyp-Gly on chondrogenic and osteogenic differentiation, respectively, highlight the importance of the peptide structure in regulating stem cell fate. Pro-Hyp was found to promote chondrogenic differentiation in SSEA3-positive hDPSCs, characterized by the early upregulation of Col II followed by sustained SOX9 expression and enhanced acid mucopolysaccharide matrix production. Interestingly, previous studies reported that Pro-Hyp enhances osteoblast differentiation through Foxg1-mediated activation of Runx2 [8,9], raising the question of how these findings align. One possible explanation is cell type specificity: while previous studies focused on pre-osteoblasts and bone-marrow-derived mesenchymal stem cells (BM-MSCs), our study investigated hDPSCs, which originate from ectomesenchyme and exhibit distinct differentiation potentials. Differences in experimental conditions may also contribute, as osteogenic differentiation in BM-MSCs was examined under serum-supplemented conditions, whereas our chondrogenic experiments utilized defined differentiation media. Furthermore, Pro-Hyp may regulate multiple signaling pathways depending on cellular context. In hDPSCs, the Sox9-Col II axis appears dominant, leading to chondrogenic differentiation, whereas in osteoblast-lineage cells, the Foxg1-Runx2 pathway may be more active. Further mechanistic studies are

needed to delineate these pathways and determine whether Pro-Hyp exerts dual effects depending on lineage-specific transcriptional programs.

In contrast, Hyp-Gly specifically enhanced osteogenic differentiation through the temporal activation of early transcription factors (RUNX2, RANKL) and subsequent matrix-associated proteins (ALPL, Col I, BSP, OCN), culminating in increased calcium deposition under osteogenic culture conditions. These selective effects may be attributed to differences in the molecular interactions of Pro-Hyp and Hyp-Gly with cellular receptors or transporters. For instance, Pro-Hyp is known to interact with peptide/histidine transporter 1 (PHT1) and solute carrier family 15 member 4 (SLC15A4), which mediate its uptake and activate integrin β1 signaling, a pathway previously shown to regulate chondrogenic differentiation [2,8]. Hyp-Gly, on the other hand, has been implicated in stabilizing hypoxia-inducible factor-2α (HIF-2α), thereby promoting osteogenesis through enhanced RUNX2 activity and matrix protein synthesis [5,6]. The ability of these peptides to differentially influence stem cell differentiation emphasizes their potential as bioactive molecules for tissue-specific regenerative strategies. SSEA3-positive cells, which are characterized by their enhanced regenerative capacity, respond selectively to these peptides, further underlining the importance of identifying specific markers for peptide-responsive subpopulations. Future studies should aim to elucidate the precise molecular mechanisms underlying these effects, including the role of peptide structure in receptor binding and intracellular signaling, to optimize their application in regenerative medicine.

These findings have significant implications for both basic research and therapeutic applications in regenerative medicine. The selective effects of Pro-Hyp and Hyp-Gly on SSEA3-positive hDPSCs suggest the possibility of targeted manipulation of stem cell fate through specific collagen-derived peptides. The ability to selectively promote either chondrogenic or osteogenic differentiation through different peptides offers new opportunities for tissue-specific regeneration strategies [4]. This is particularly relevant given that collagen-derived peptides can be detected in the circulation after oral administration, suggesting potential therapeutic applications through dietary supplementation [6]. Furthermore, the identification of SSEA3 as a marker for peptide-responsive cells provides a new tool for selecting cells with enhanced regenerative potential [13]. The temporal regulation of the differentiation markers observed in this study also provides valuable insights for optimizing the timing of therapeutic interventions. These findings could lead to the development of more effective treatments for bone and cartilage tissue regeneration and potentially extend to other applications in regenerative medicine where precise control of stem cell differentiation is crucial.

Although our findings demonstrate the selective effects of collagen-derived peptides on SSEA3-positive hDPSCs and their potential in tissue-specific regeneration, several limitations should be considered in future research. First, although we observed clear temporal patterns in differentiation marker expression and matrix production, the precise molecular mechanisms underlying the selective response of SSEA3-positive cells to specific peptides remain to be fully elucidated. Further investigation of peptide uptake mechanisms, downstream signaling pathways, and the role of SSEA3 in peptide responsiveness would provide valuable insights. Second, although our *in vitro* results are promising, *in vivo* studies are necessary to confirm the therapeutic potential of these peptides. Additionally, the long-term stability of the induced differentiation and the potential effects of repeated peptide administration need to be evaluated. Future studies should also explore the potential synergistic effects of combining different peptides or incorporating them into existing tissue engineering protocols. Furthermore, future investigation of the effects of these peptides on other stem cell populations will help determine the broader applicability of our findings in regenerative medicine.

Availability of data and materials

The datasets used and/or analyzed for the case report are available from the corresponding author upon reasonable request.

Author contributions

R.S., K.I. and H.N.—study concept, design, acquisition of data, analysis of data, interpretation of data, writing of the original draft, critical revision of the manuscript, and supervision. S.T., T.K., and M.M.—acquisition of data, interpretation of data, and writing review. E.H.N., H.S. and Y.S.—critical revision of the manuscript. All authors have read and agreed to the submission and publication of this manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Abbreviations

ALPL	Alkaline phosphatase
BSP	Bone sialoprotein
CDIM	Chondrocyte differentiation-inducing medium
Col I	Type I collagen
Col II	Type II collagen
GABA	γ-aminobutyric acid
Gly	Glycine
hDPSCs	Human dental pulp stem cells
Hyp	Hydroxyproline
Hyp-Gly	Hydroxyproline-glycine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OCN	Osteocalcin
ODIM	Osteoblast differentiation-inducing medium
Pro-Hyp	Prolyl-hydroxyproline
RANKL	Receptor activator of nuclear factor-kappa B ligand
RUNX2	Runt-related transcription factor 2
SCM	Stem cell medium
SOX9	SRY-box transcription factor 9
SSEA3	Stage-specific embryonic antigen 3

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