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Original Article

The establishment of pulp polyp-derived mesenchymal stem cells with normal karyotype

Ferry Sandra [a,b*], Eko Fibryanto [c], Tien Suwartini [c], Anastasia Elsa Prahasti [c], Widya Wulandari [d], Andri Sutanto [d], Lyvia Juliana [d], Kyung Hoon Lee [e]

[a] *Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia*
[b] *Center for Molecular Biology Study, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia*
[c] *Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia*
[d] *Conservative Dentistry Specialist Program, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia*
[e] *Research Institute, Ballys Co. Ltd, Incheon, Republic of Korea*

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KEYWORDS
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Abstract *Background/Purpose:* Pulp polyp is often eliminated as dental waste. Pulp polyp cells were reported to have high proliferation activity which might be comprised of stem cells. However, little has been known on the presence of stem cells in the pulp polyp. Moreover, pulp polyp cells might contain chromosomal abnormality. The present study was conducted to investigate the presence of pulp polyp stem cells, which could later be propagated and confirmed as normal/non-pathogenic cells using karyotype analysis.
Materials and methods: Collected pulp polyps were minced, enzymatically digested, and cultured. Expression of mesenchymal stem cell (MSC) markers on pulp polyp cells were analyzed using flow cytometry. Multilineage differentiation capacity was assessed by culturing the cells in osteogenic, chondrogenic, and adipogenic differentiation media. Genomic stability of the cells was evaluated with G-banded and molecular karyotype analyses.
Results: Pulp polyp cells appeared as fibroblasts-like cells. The cells were positive for cluster of differentiation (CD)105, CD90, and CD73, and negative for CD45, CD34, CD11b, CD19, and human leukocyte antigen (HLA)-DR. The cells were capable of osteogenic, chondrogenic, and adipogenic differentiation. G-banded karyotype analysis showed that there was no abnormality in the number or structure of chromosomes in pulp polyp-derived MSCs (PP-MSCs).

* Corresponding author. Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia.
E-mail address: ferry@trisakti.ac.id (F. Sandra).

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Molecular karyotype analysis revealed that all copy number variations identified in PP-MSCs were not pathogenic.
Conclusion: PP-MSCs, which fulfill the minimal criteria for MSCs and are proven to have normal karyotype, have been successfully established. PP-MSCs might be a promising and safe candidate that can be considered for pulp-dentin complex regeneration.

Introduction

One of the advances in the field of endodontics is pulp-dentin complex regeneration, which often involves the administration of stem cells. The therapeutic effects of stem cells are recently the main focus of research in regenerative dentistry. Stem cells have a self-renewal ability and differentiation into specialized cells. Multipotent stem cells, especially mesenchymal stem cells (MSCs), are currently the subject of extensive research in pulp-dentin complex regeneration.[1,2] MSCs were initially isolated and characterized from the bone marrow.[3] More recently, MSCs can also be isolated from other tissues, including peripheral blood,[4] adipose tissue,[5–7] umbilical cord blood,[8–10] amniotic fluid,[11] and dental pulp.[12,13]

Dental pulp, which is derived from ectomesenchyme,[14] is a rich source of dental pulp stem cells (DPSCs).[15] DPSCs have similar characteristics with bone marrow-derived MSCs (BM-MSCs), but with a greater proliferation potential.[15] Although DPSCs are relatively easy to obtain with a minimally invasive procedure,[16] the dental pulp can be severely affected by caries or trauma, leading to irreversible chronic inflammation.[17] The chronic inflammation may result in the formation of enlarged granulation tissue that protrudes from the pulp chamber into the respective dentinal defect. This condition is known as pulp polyp.[17] The prevalence of pulp polyp varies from 4 to 7% in several countries.[18,19] Although the incidence of this condition is low, pulp polyps often occur in children and adolescents, who still have a good blood supply and immune response.[20,21]

Pulp polyp tissue is often eliminated as dental waste generated from endodontic treatment. Pulp polyp cells were reported to have high proliferation activity[17] which might be comprised of stem cells.[22] Isolating cells from the pulp polyp tissue is a feasible process since this source provides a relatively easy and non-invasive means of obtaining tissue.[23] Therefore, pulp polyp could be a potential source of stem cells, not a dental waste anymore. However, little has been known on the presence of stem cells in the pulp polyp. Moreover, pulp polyp cells might contain chromosomal abnormality, as reported in hyperplasia occurred in other body parts.[24–26] Therefore, the present study was conducted to investigate the presence of pulp polyp stem cells, which could later be propagated and confirmed as normal/non-pathogenic cells using karyotype analysis. In addition, the cultured pulp polyp cells were also examined for their characteristics based on the International Society for Cellular Therapy (ISCT) criteria for defining MSCs.

Materials and methods

Pulp polyp collection and single-cell suspension preparation

Pulp polyp subjects were diagnosed by a certified endodontist by intra-oral and radiographic examinations. Related medical and family histories were recorded and documented. Subjects who had not received any prior dental treatment, did not experience spontaneous pain, did not have apical periodontitis or other inflammation based on radiographs, and was free from systemic diseases or family history of genetic disorders based on anamnesis results, were included in the study. Pulp polyps with the volume of 4 mm^3 and positive response to electric pulp testing were extracted with a curette prior to the endodontic treatment. Prior to the pulp polyp extraction, subject was anesthetized locally. The collected pulp polyp sample was rinsed with phosphate-buffered saline (PBS)-ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA), placed in 500 μL of PBS-EDTA containing 100 units penicillin, 100 μg streptomycin, and 0.25 mg Amphotericin B (Sigma-Aldrich) at 4 °C, then transported to the laboratory. The tissue was cut into small pieces in sterile condition, and incubated in 3 mg/mL collagenase type I (Sigma-Aldrich) and 4 mg/mL dispase type II (Sigma-Aldrich) for 1 h at 37 °C with periodic mixing. The cell suspension was filtered using Corning 70-μm cell strainer (Sigma-Aldrich) and centrifuged at 1000×*g* for 10 min. Pulp polyp cells were isolated and cultured. This study was reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Universitas Trisakti with the approval number: 345/S2-Sp/KEPK/FKG/4/2020. All participants/patients provided written informed consent prior to the enrollment of the study.

Pulp polyp cell culture

Pulp polyp cells were cultured in MesenCult MSC Basal Medium (Human) (StemCell Technologies, Vancouver, Canada) supplemented with Mesenchymal Stem Cell Stimulatory Supplements (Human) (StemCell Technologies). The cells were maintained at 37 °C in a humidified incubator

(90% humidity, 5% CO_2). The medium was changed twice a week. Upon reaching 80% confluency, pulp polyp cells were detached using 0.05% trypsin-EDTA solution (StemCell Technologies) at 37 °C.

Flow cytometric analysis for MSC markers

Flow cytometric analysis was performed using BD Stemflow hMSC Analysis Kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, 1×10^7 cells/mL pulp polyp cells were labeled with/without antibodies for MSC-positive markers (cluster of differentiation (CD)105 peridinin-chlorophyll-protein-cyanin 5.5 (PerCP-Cy5.5), CD90 fluorescein isothiocyanate (FITC), and CD73 allophycocyanin (APC)), an MSC-negative markers cocktail (CD45/CD34/CD11b/CD19/human leukocyte antigen (HLA)-DR phycoerythrin (PE)) and corresponding isotypes antibodies in the dark at room temperature for 30 min. After that, the cells were washed twice with PBS (Gibco, Grand Island, NY, USA), resuspended in 500 μL PBS, and loaded into FACSCanto II flow cytometer (BD Biosciences). Fluorescence intensity of individual nuclei was acquired at 100,000 events. Flow cytometric data analysis was conducted using FACSDiva software (BD Biosciences). The size and granularity of pulp polyp cells were indicated by forward scatter (FSC) and side scatter (SSC), respectively. High expression of the respective marker was indicated by the curve on the histogram of expression being located on the right side of the gate line. In contrast, low expression was indicated by the curve on the histogram of expression being located on the left side of the gate line.

Osteogenic, chondrogenic, and adipogenic differentiations

Differentiation of pulp polyp cells into osteoblasts, chondroblasts, and adipocytes was performed according to the manufacturer's instructions, using MesenCult Osteogenic Differentiation Kit (Human) (StemCell Technologies), MesenCult-ACF Chondrogenic Differentiation Kit (StemCell Technologies), and MesenCult Adipogenic Differentiation Kit (Human) (StemCell Technologies), respectively. For chondrogenic differentiation, micromass culture was performed. Pulp polyp cells were cultured in the differentiation media for 21 days. After that, the cells were fixed in 4% paraformaldehyde. To evaluate osteogenic, chondrogenic, and adipogenic differentiations, the cells were stained using 2% alizarin red (Sigma-Aldrich), 1% alcian blue (Sigma-Aldrich), and 0.18% oil red O (Sigma-Aldrich), respectively. Stained results were observed and documented under an Axiovert inverted light microscope (Zeiss, Jena, Germany).

G-banded karyotype analysis

Pulp polyp cells were seeded at a density of 15×10^3 cells/cm^2. After overnight culture, the cells were incubated with 10 μL/mL colcemid (Roche, Vienna, Austria) for 1 h, washed and treated with 0.05% trypsin (Sigma-Aldrich). The cells were then incubated with a hypotonic solution of 0.56% KCl for 1 h at room temperature, washed and fixed thrice with fresh Carnoy's fixative containing 3:1 ratio of methanol:-acetic acid. Cell suspension was placed on a wet, cold microscope slide, and the slide was air-dried. After the slides were digested with 0.25% trypsin (Sigma-Aldrich), chromosomes were stained with Giemsa (Sigma-Aldrich). Metaphase plates with scattered chromosomes were examined by an experienced cytogeneticist under a light microscope (Olympus, Tokyo, Japan), using a 100 × objective and a 20 × ocular. For karyogram generation and analysis, HiBand 9-slide Scanning & Capture System (Applied Spectral Imaging, Carlsbad, CA, USA) was employed. Chromosomes were identified according to the International System for Human Cytogenomic Nomenclature (ISCN) 2020.[27]

Molecular karyotype analysis

DNA was extracted from the pulp polyp cell using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), amplified, precipitated, resuspended, and hybridized on beadchip version 3.0 of the Infinium Global Screening Array with Cytogenetics (GSACyto) (Illumina, San Diego, CA, USA) with SNP-FASST2 segmentation algorithm, which comprised of ~700,000 markers of single nucleotide polymorphism (SNP) with 4885 disease-associated-genes based on National Human Genome Research Institute (NHGRI); American College of Medical Genetics and Genomics (ACMG); ClinVar; Absorption, Distribution, Metabolism, Excretion (ADME); Developmental Disorders Genotype-to-Phenotype database (DDG2P); Online Mendelian Inheritance in Man (OMIM); and Exome Aggregation Consortium (ExAC) probability of loss of function intolerance (pLI). The resolution of the beadchip was ~10 kb. After that, the hybridized beadchips were scanned with the iScan microarray scanning system (Illumina). Copy number variant (CNV) analysis was performed with NxClinical analysis software version 6.1 (BioDiscovery, El Segundo, CA, USA). Threshold was set at >200 kb for copy number (CN) loss and homozygous copy loss, >400 kb for CN gain and >10 Mb for absence of heterozygosity (AOH). CNVs below the thresholds were excluded to avoid variants misinterpretation. CNVs were classified and interpretated based on the guidelines of the ACMG[28] using Genome Reference Consortium Human (GRCh) Build 38 into five categories: pathogenic, likely pathogenic, variants of uncertain significance (VUS), benign, and likely benign. ISCN 2020[27] was used to determine chromosome region which was affected by CNV. CNVs were further evaluated using Database of Genomic Variation and Phenotype in Humans using Ensembl Resources (DECIPHER) (EMBL-EBI, Wellcome Genome Campus, Hinxton, UK) and The Human ncRNA Gene Database (GeneCaRNA) (LifeMap Sciences, Covina, CA, USA).

Results

Morphology of pulp polyp cells

Ten pulp polyp samples were obtained in the present study, which were then processed further to obtain a single-cell suspension for culturing. Pulp polyp cells were successfully cultured. Morphology of pulp polyp cells in passage 4 was shown in Fig. 1. The isolated pulp polyp cell population

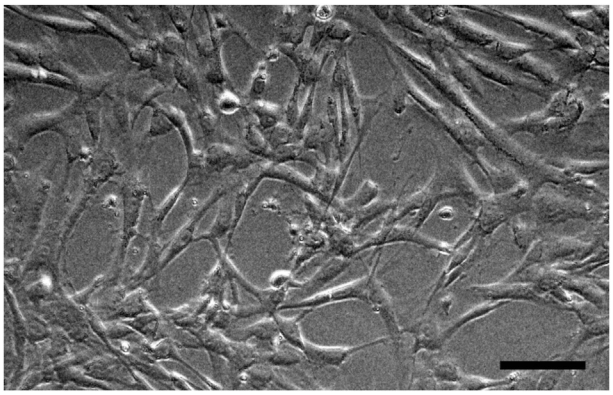

Figure 1 Morphology of pulp polyp cells. Pulp polyp was minced, and enzymatically digested to obtain single-cell suspension. Pulp polyp cell suspension was cultured as described in Materials and methods. Pulp polyp cells in passage 4 were documented under an inverted light microscope. Scale bar: 200 μm.

appeared as thin, elongated, spindle-shaped fibroblasts-like cells.

Pulp polyp cells expressed MSC surface markers

The size and granularity of pulp polyp cells were shown in a dot plot in Fig. 2 1a–c. MSC-positive markers CD105, CD90, and CD73 were highly expressed on antibody-labeled pulp polyp cells (Fig. 2 2a, 3a, 4a). On the contrary, CD105 (Fig. 2 2b, 2c), CD90 (Fig. 2 3b, 3c), and CD73 (Fig. 2 4b, 4c) expressions were not detected in isotype-labeled and unlabeled cells. Furthermore, lacked expression of MSC-negative markers (CD45, CD34, CD11b, CD19, and HLA-DR) was found in all groups (antibodies-labeled, isotype-labeled, and unlabeled cells) (Fig. 2 5a–c). The mean percentages of CD105, CD90 and CD73 expression in the pulp polyp cells were 98.5%, 99.8%, and 99.7%, respectively (Table 1). Meanwhile, the mean percentage of negative marker expression was 1.2%. Since the expression of CD90, CD105, and CD73 was >95%, and the expression of negative markers was <2%, thus the isolated pulp polyp cells could be considered to have MSC properties.

Pulp polyp cells had trilineage differentiation capacity

Pulp polyp cells were positive for alizarin red staining after 21 days of culture in osteogenic differentiation medium, as indicated by the presence of calcified nodules produced by differentiated osteoblasts, which stained red in the culture. Chondrogenic differentiation was also apparent after 21 days of culture with chondrogenic induction medium, as shown by the formation of Alcian blue-stained chondrocyte-associated extracellular proteoglycans. Similarly, after 21 days of adipogenic induction, oil red O-positive red-colored lipid droplets were observed in and between the differentiated cells (Fig. 3). These results suggest that the isolated pulp polyp cells had capacity to differentiate into osteoblasts, chondroblasts, and adipocytes, strengthening the notion that these cells might possess MSCs characteristics.

Pulp polyp cells exhibited no chromosomal abnormality

A total of 57 pulp polyp cells were randomly selected for chromosome counting. All pulp polyp cells had a normal chromosome number of 46. A total of 18 cells in mitosis metaphase were then analyzed for G-bands. The results of G-banded karyotype analysis revealed that there was no structural chromosomal abnormality occurred in all tested cells. Fig. 4 presented a karyogram showing the 23rd pair of chromosomes was XX, suggesting that the pulp polyp cell was obtained from a female subject.

Furthermore, based on the molecular karyotyping analysis, several CNVs were detected in pulp polyp cells, and some of them were classified as VUS, likely benign, and benign. No variants were classified as pathogenic or likely pathogenic. Two CNVs were categorized as CN loss. CN loss in 7p21.3 affected *Thrombospondin Type-1 Domain-Containing Protein 7A* (*THSD7A*) gene and was considered as VUS (Table 2). Further investigation using the DECIPHER databases indicated that variant in the *THSD7A* gene might not be associated with any malignancies or other diseases. Meanwhile, CN loss in 1p21.1 affected *Long Intergenic Nonprotein Coding RNA 1676* (*LINC01676*) gene and was considered as a benign variant (Table 2). The other CNVs were categorized as CN gain and homozygous copy loss, both considered likely benign variants. CN gain in 10q11.22 affected *Anthrax Toxin Receptor* (*ANTXR*)*-like Pseudogene 1* (*ANTXRLP1*), *Locus of Control* (*LOC)105378577*, and *ANTXR-like* (*ANTXRL*) genes, while homozygous copy loss in 19p12 affected *Zinc Finger Protein 826, Pseudogene* (*ZNF826P*) (Table 2). However, the *LINC01676*, *ANTXRLP1*, *ANTXRL* and *ZNF826P* genes were not recorded as being associated with any malignancies or diseases in the DECIPHER database. Meanwhile, based on GeneCaRNA database, *LOC105378577* was listed as uncharacterized gene. Therefore, these results might indicate chromosomal variations instead of abnormalities.

Discussion

In the present study, pulp polyp cells exhibited high expression (>95%) of MSC-positive markers, namely CD105, CD90, and CD73, and low expression (<2%) of MSC-negative markers, namely CD45, CD34, CD11b, CD19, and HLA-DR. Moreover, these cells possessed the capacity to differentiate *in vitro* into the mesodermal lineages of osteoblasts, adipocytes, and chondroblasts. Therefore, cells derived from pulp polyp tissue in the present study have fulfilled the minimal criteria for MSCs proposed by the ISCT.[29] These findings showed that the pulp polyp-derived MSCs (PP-MSCs) isolated and cultured in the present study had concordant surface markers and differentiation capacities.

In the present study, PP-MSCs were differentiated into osteoblasts, chondroblasts, and adipocytes after 21 days of culture with the respective induction media. These results are similar with osteogenic[12] and adipogenic[30] differentiation of DPSCs, and chondrogenic differentiation of BM-MSCs,[31] which also took 21 days to undergo the differentiation.

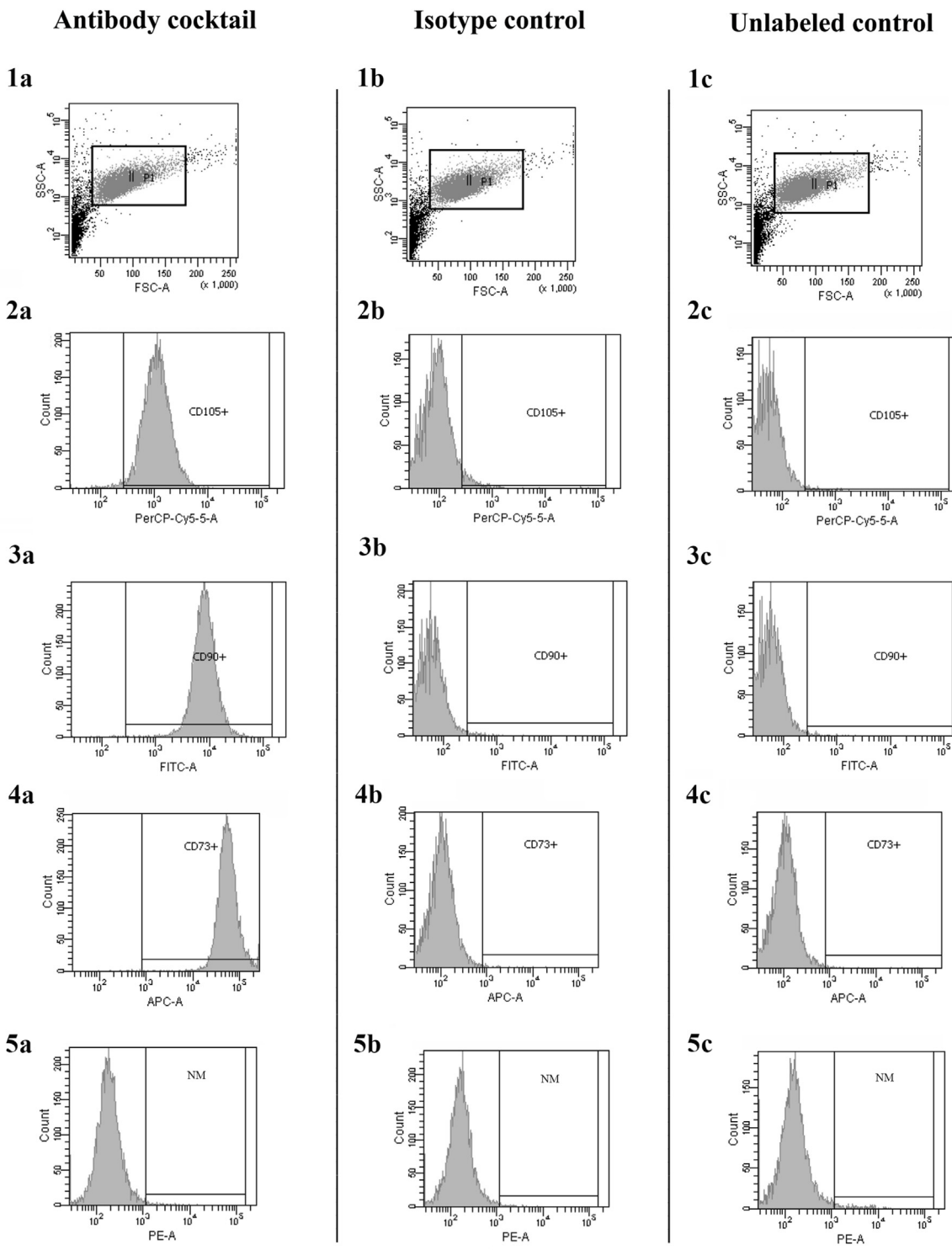

Figure 2 Flow cytometric analysis of pulp polyp cells. Pulp polyp cells in passage 4 were harvested and labeled with/without antibodies for MSC-positive and negative markers with the corresponding isotype controls as described in Materials and methods. The expression of MSC surface marker was analyzed using a flow cytometer. The experiment was carried out in triplicate. 1a—c: Dot plots of FSC and SSC; 2a—c: Histograms of CD105 expression; 3a—c: Histograms of CD90 expression; 4a—c: Histograms of CD73 expression; 5a—c: Histograms of negative markers (NM) (CD45, CD34, CD11b, CD19 and HLA-DR) expression.

Pulp polyp is a kind of inflammatory hyperplasia in the dental pulp which occurs due to exposure to caries or trauma.[17] In some cases, cells in hyperplastic tissues may experience chromosomal abnormalities,[24—26] which might be associated with an increased risk of pathogenicity.[32] In the present PP-MSCs study, G-banded karyotype analysis was performed and the results showed that there was no abnormality in the number or structure of chromosomes. In

Table 1 Percentage of CD105, CD90, CD73, and negative markers (CD45, CD34, CD11b, CD19 and HLA-DR) expression. Results of the flow cytometric analysis of pulp polyp cells in triplicate (Fig. 2) were documented and listed.

Group	Replicate	Biomarkers			
		CD105	CD90	CD73	Negative markers
Antibody cocktail	1	98.50	99.80	99.80	1.10
	2	98.50	99.70	99.60	1.20
	3	98.50	99.80	99.80	1.20
Isotype control	1	1.60	0.80	0.30	0.60
	2	1.50	0.90	0.50	0.60
	3	1.90	0.80	1.50	2.00
Unlabeled control	1	1.10	1.00	0.20	0.10
	2	0.90	0.80	0.10	0.00
	3	0.70	0.70	0.00	0.00

addition, molecular karyotype analysis was also performed using the Infinium Global Screening Array. The results of this analysis showed that most CNVs detected in PP-MSCs were considered likely benign and benign variants. Genes affected by these CNVs, such as *LINC01676*, *ANTXRLP1*, *ANTXRL* and *ZNF826P*, were confirmed to be not associated with any malignancies or other diseases in the DECIPHER database. This is consistent with the results of searches in other databases, such as the OMIM database, which show that alterations in these genes have not been recorded as being associated with any specific diseases. In addition, there was one CNV in PP-MSCs that was classified as VUS, which was the CNV affecting the *THSD7A* gene caused by CN loss. As confirmed by the DECIPHER database, this VUS was also not associated with any malignancies or other diseases. This is in accordance with the information in the UniProt database, which states that the *THSD7A* gene encodes a protein that plays a crucial role in actin cytoskeleton

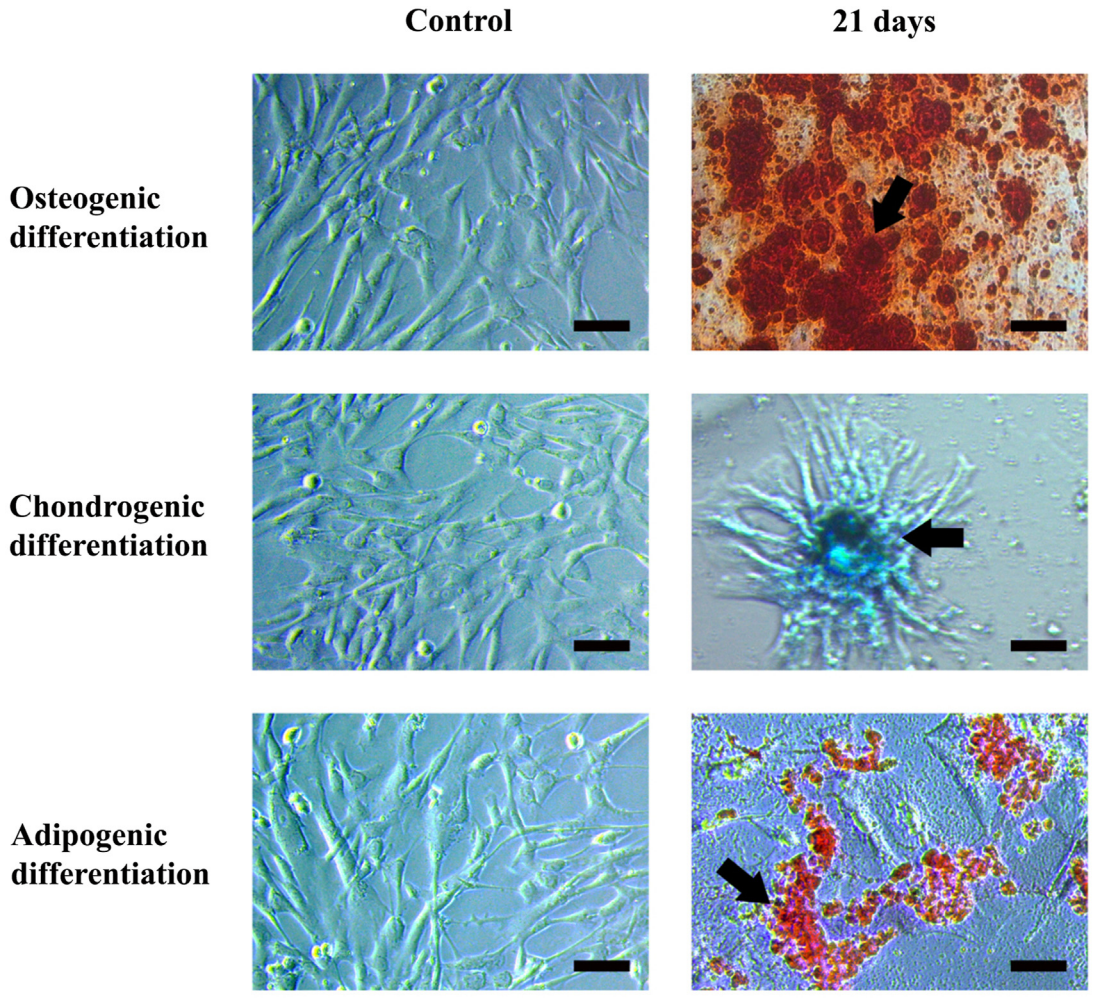

Figure 3 **Differentiation of pulp polyp cells.** Pulp polyp cells in passage 4 were cultured in osteogenic, chondrogenic, and adipogenic differentiation media for 21 days. Osteogenic, chondrogenic, and adipogenic differentiation were assessed using alizarin red, alcian blue, and oil red O staining, respectively. The differentiated cells were documented under an inverted light microscope as described in Materials and methods. The experiment was carried out in triplicate. Arrows indicated calcified nodules for osteogenic differentiation; chondroblasts aggregation for chondrogenic differentiation; and lipid droplet for adipogenic differentiation. White bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

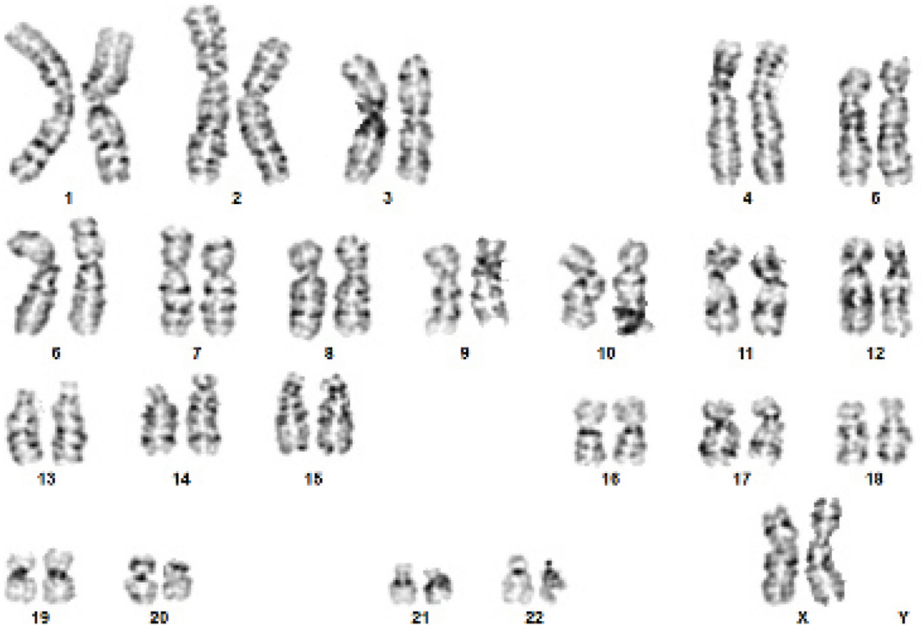

Figure 4 G-banded karyogram of pulp polyp cells. Cultured pulp polyp cells were collected and processed for G-banded karyotype analysis as described in Materials and methods. The karyogram represented 18 cells analyzed for their karyotypes.

Table 2 Results of molecular karyotyping analysis of pulp polyp cells.

Category	Chromosome region	Cytoband	Length (bp)	Classification	ISCN nomenclature	Genes
CN loss	chr1:105,492,887—105,720,493	1p21.1	227,607	Benign	1p21.1(105492887_105720493)x1	1 gene: *LINC01676*
CN loss	chr7:11,512,747—11,540,829	7p21.3	28,083	VUS	7p21.3(11512747_11540829)x1	1 gene: *THSD7A*
CN gain	chr10:46,241,791—46,332,633	10q11.22	90,843	Likely benign	10q11.22(46241791_46332633)x3	3 genes: *ANTXRLP1, LOC105378577, ANTXRL*
Homozygous copy loss	chr19:20,406,130—20,537,969	19p12	131,840	Likely benign	19p12(20406130_20537969)x0	1 gene: *ZNF826P*

The list showed typical results of two independent molecular karyotype analyses. CN: Copy number, VUS: Variants of uncertain significance.

rearrangement, as well as promotes migration of endothelial cell and formation of filopodia during sprouting angiogenesis. Therefore, this VUS could be considered normal, suggesting that the variant in this gene might not affect its function. Thus, the results of this molecular analysis revealed that all CNVs identified in PP-MSCs were not pathogenic. These findings confirmed that PP-MSCs retained genomic stability. Although there were several studies have reported the characteristics of MSCs derived from inflamed dental tissues,[33—35] including the pulp polyp,[23,36] however, the genomic stability of the obtained MSCs were not pursued.

Although often discarded as a dental waste, pulp polyp could be a potential source of MSCs to be used in regenerative medicine, especially pulp-dentin complex regeneration. Comprehensive understanding of the activation of specific genes in PP-MSCs is essential for directing the differentiation process towards the desired cell lineage.[37] Since the inflammatory profile of PP-MSCs has not been investigated in the present study, further studies are necessary to evaluate whether administration of PP-MSC can induce inflammatory reaction in both *in vitro* and *in vivo* settings. Moreover, the results of molecular karyotype analysis (CN gain/loss and homozygous copy loss) should be further analyzed because it is crucial for acquiring convincing clues to exclude karyotypic abnormality in PP-MSCs.

In conclusion, PP-MSCs, which fulfill the minimal criteria for MSCs defined by the ISCT and are proven to have normal karyotype, have been successfully established in the present study. PP-MSCs might be a promising and safe candidate that can be considered for pulp-dentin complex regeneration.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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