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Characteristics of Mesenchymal Stem Cells Derived From the Apical Papilla of a Supernumerary Tooth Compared to Stem Cells Derived From the Dental Pulp

ORIGINAL ARTICLE

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ABSTRACT

Objective: There are several comparative studies that were conducted to explain the specific properties of oral tissue derived mesenchymal stem cells (MSC). However, apical papilla stem cells derived from supernumerary teeth (ST-APSCs) have not been characterized for their MSC properties yet.

Materials and Methods: In the present study, ST-APSCs were isolated and characterized from a nonsyndromic male patient to evaluate the MSC characteristics. Dental pulp stem cells (DPSCs) and ST-APSCs were isolated and characterized for mesenchymal surface markers. Cells were differentiated into osteo-, chondro-, and adipogenic cell types.

Results: Both cell types expressed the MSC surface markers. When DPSCs and ST-APSCs were cultured in differentiation media promoting transformation to osteo-, chondro-, and adipo-genic lineages, both showed calcium mineralization, chondrogenic mass formation, and lipid accumulation. However, DPSCs derived from a wisdom tooth demonstrated more differentiation potential to osteo- and chondro-genic cell types compared to ST-APSCs.

Conclusion: Overall, ST-APSCs were characterized by their MSC properties and were able to differentiate into three cell lineages. However, they were less potent for osteo- and chondro-genic cell lineage specification compared to DPSCs derived from a wisdom tooth.

Keywords: Supernumerary tooth, stem cell, dental pulp, apical papilla

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INTRODUCTION

The stem cell technology has been established as an important field in the regenerative medicine based on new findings related to the stem cell therapy and tissue engineering and replacement. Various mesenchymal stem cells (MSCs) have been isolated, characterized, and reported from different tissues in the past decades (1). Of those, MSCs from dental tissues have shown significant mesenchymal characteristics as alternative stem cell sources (1).

Different MSC populations from human oral tissues have been successfully isolated, such as gingival stem cells (2-5), deciduous teeth stem cells (SHED) (3), periodontal ligament derived stem cells, apical papilla stem cells (SCAP), dental follicle stem cells, and dental pulp stem cells (DPSCs) from supernumerary teeth (1), wisdom teeth (4), and immature and mature permanent teeth (5). Elucidating the advantages and limitations of each stem cell type obtained from different dental tissues might allow to regulate stem cell pluripotency, differentiation, and growth *in vitro* and *in vivo* for creating alternative techniques in regenerative medicine. DPSCs, first isolated by Gronthos and his colleagues in 2000 (2), have been obtained from deciduous and wisdom teeth (2, 6, 7), and supernumerary (1) and human wisdom teeth germs of young adults (8). DPSCs are referred to as a multipotent stromal cells which can differentiate odontoblasts, osteoblasts, and adipocytes after the comparison with bone marrow (9, 10). Among the stem cells from oral tissues, wisdom tooth DPSCs have been isolated, characterized, and reported for their MSC properties in previous studies (8, 11). As a young tissue remaining quiescent until the age of 6, high proliferative and multipotent characteristics of wisdom teeth have attracted interest in recent years. DPSCs derived from tooth germs have been proven for their significant proliferation and differentiation capacity *in vitro* (8) and dentin-like matrix formation ability *in vivo* (12). Supernumerary teeth, a condition known as hyperdontia, are additional teeth, apart from the normal dental formula (13). They can be found in almost any region of the dental cavity. These types of teeth can be observed in both syndromic and nonsyndromic patients (14, 15). DPSCs from supernumerary teeth have been reported for their MSC properties (1), differentiation capacity to osteogenic and adipogenic cell types (16, 17), and immunomodulatory activities (18). However, apical papilla stem cells from supernumerary teeth, to the best of our knowledge, have not been characterized and investigated for their MSC properties yet. The aim of the present study is to isolate the apical papilla mesenchymal stem cells derived from supernumerary teeth and compare MSC surface marker expressions and differentiation capacity with dental pulp mesenchymal stem cells that were obtained from the third molar.

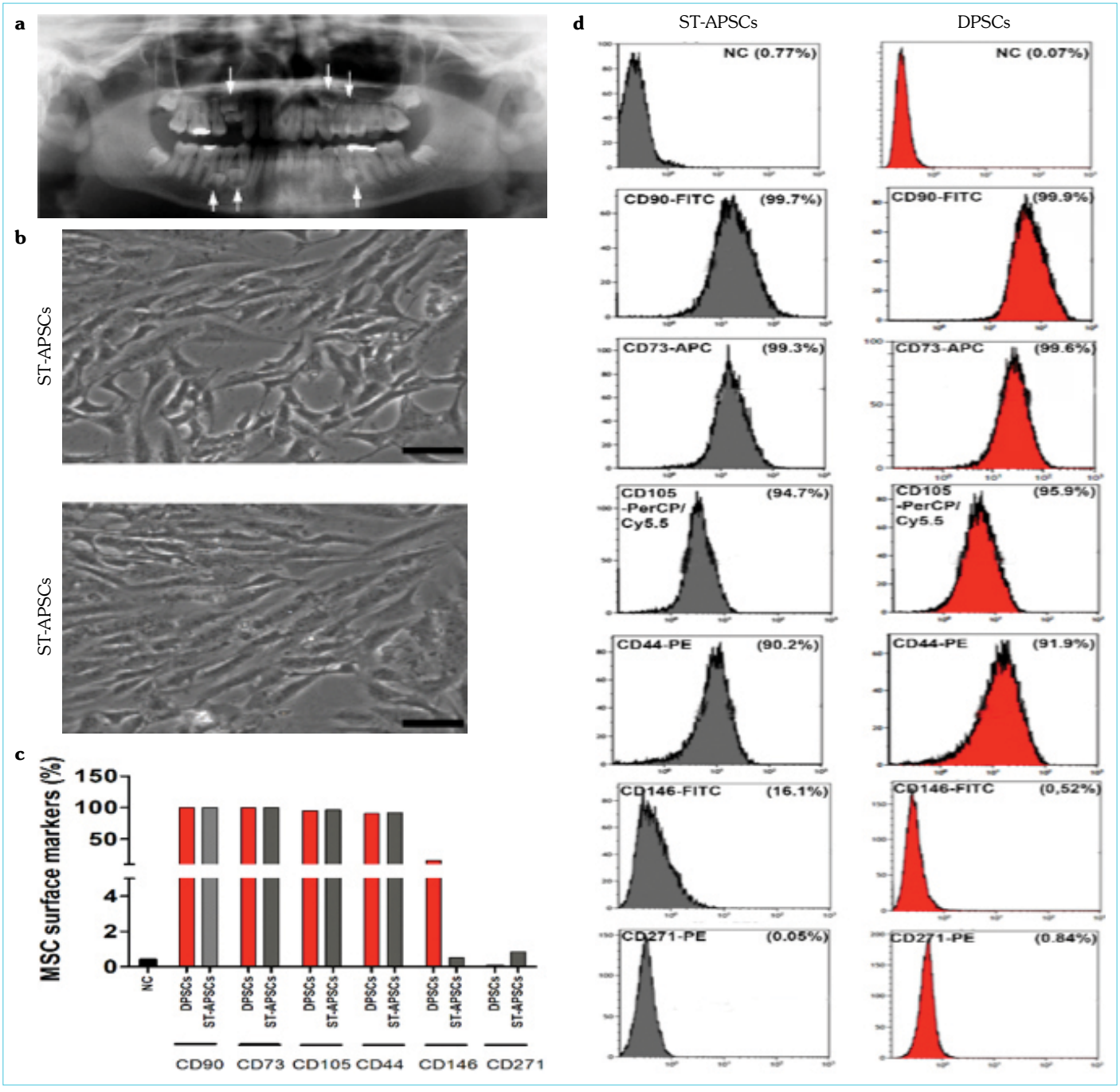

Figure 1. a–d. Isolation and surface marker analysis of DPSCs and ST-APSCs. (a) Panoramic radiography of the 16-year-old male patient. White arrows show the supernumerary teeth on panoramic radiography. (b) Fibroblastic cell morphology of DPSCs and ST-APSCs. (c) Percentage of MSC surface marker expression. (d) Immunophenotypic characteristics of DPSCs and ST-APSCs. Surface marker expressions were represented in percentage expression levels. Scale bar, 100μm

MATERIALS AND METHODS

Source of Supernumerary Teeth

Multiple supernumerary teeth were identified on panoramic radiography in a healthy 16-year-old male patient (Fig. 1a). The patient had no significant medical or family history. The supernumerary teeth in the right maxillary region were extracted under local anesthesia and delivered to the laboratory for the ST-APSCs isolation. Tooth germs were obtained from three young adults aged 18–20 years and used for DPSCs isolation. Written consent of the patients and their parents was obtained for the use of extracted teeth according to the approval of the local ethical committee (2013/508) and the guidelines of the Helsinki Declaration.

Isolation and Characterization of the Cells

DPSCs and ST-APSCs were isolated and characterized as described previously by our group (8, 19, 20). As cells reached the 80% confluence at the second passage, they were removed via

the trypsin-EDTA solution (Invitrogen) and incubated with primer antibodies for 1 hour. Primary antibodies against CD271, CD90, CD146, CD44, CD73, and CD105 obtained from BD were analyzed using Navios (Beckman Coulter, USA).

MSCs Differentiation

To show the MCS characteristics, the cells were induced to differentiate into three mesenchymal cell lineages (8, 19). Both types of cells were seeded onto 12-well plates at a concentration of $3x10^4$ cells/well, and pre-made differentiation mediums were added. Differentiation medium contents are presented in Table 1. Cells were incubated in a 5% CO_2 humidified incubator at 37°C for 7–10 days. The differentiation media were replaced the day after.

RT-PCR Analysis

Primers synthesized by Invitrogen for collagen type I (Col1A1), osteonectin (ON), collagen type II (Col2A1), aggrecan (ACAN), and fatty-acid-binding protein-4 (FABP4) were used in this study (Table 2). Total RNAs from differentiated MSCs from both sources were isolated, and mRNA levels were determined using the SYBR Green staining method. The Maxima SYBR Green qPCR Master Mix was mixed with cDNAs in a final volume (20 µL). β-actin was used as a housekeeping gene for data normalization. iCycler RT-PCR system (Bio-Rad, Hercules, CA) was used for the experiments.

Alkaline Phosphatase Enzyme Activity

Osteogenic differentiation was confirmed by the alkaline phosphatase (ALP) enzyme activity assay. Cells were collected, and lysis buffer containing triton-X 100 (0.2%) in phosphate-buffered saline (PBS) were used for protein extraction. Subsequently, 25 µL of protein lysate was mixed with 75 µL of ALP ligand (Randox ALP detection kit; Randox, Antrim, UK) in a 96-well plate and incubated for 15 minutes. The enzyme activity was detected by measuring the absorbance at 405 nm using an enzyme-linked immunosorbent assay plate reader (Biotek).

von Kossa Staining

von Kossa staining was performed to show the mineralization and calcium deposition of differentiated cells as a marker of osteogenic transformation. Formaldehyde-fixed cells were rinsed with distilled water and stained with a von Kossa kit (Polysciences Inc, Warrington, PA) according to manufacturer's instructions.

Alcian Blue Staining

Alcian blue staining was performed to examine the chondrogenic differentiation levels of differentiated cells. Briefly, formaldehyde-fixed cells were stained with a solution prepared with Alcian blue in 3% acetic acid. After the incubation, it was rinsed three times with PBS. Observation was performed under the light microscope (Primo Vert, Zeiss, Germany).

Oil Red Staining

Oil red staining was conducted to show lipid vesicles to confirm the adipogenic differentiation. Formaldehyde-fixed cells were washed with PBS three times and stained with oil red diluted (6:4) in PBS for 1 hour. After rinsing with PBS, observation was performed under the light microscope (Primo Vert, Zeiss, Germany).

Immunocytochemical Analysis

We used 4% paraformaldehyde as a fixation agent for the cells.

Table 1. Differentiation medium contents used to differentiate stem cells to osteo-, chondro-, and adipogenic cell lineages

Differentiation	Content
Osteogenic medium	100 nM dexamethasone,
	10 mM β-glycerophosphate,
	0.2 mM ascorbic acid
Chondrogenic medium	1x insulin–transferrin–selenium,
	100 nM dexamethasone,
	100 ng/ml TGF-β,
	14 µg/ml ascorbic acid,
	1 mg/ml BSA
Adipogenic medium	100 nM dexamethasone,
	5 µg/ml insulin,
	0.5 mM 3-isobutyl-1-methylxanthine,
	60 µM indomethacin

Table 2. The list of primers used in RT-PCR

Gene	Sequences
COL1A1	5′-CCACGCATGAGCGGACGCTAA-3′
	5′-ATTGGTGGGATGTC TTCGTCTTGG-3′
COL2A1	5′-GTGTGGAAGCCGGAGCCCTG-3′
	5′-GGTCCTGGTTGCCCACTGGC-3′
ON	ATGAGGGCCTGGATCTTCTT
	CTGCTTCTCAGTCAGAAGGT
ACAN	ACTGCTGCAGACCAGGAGGT
	TCCTCGGGGGTGACGATGCT
FABP4	5′-GGGTCACAGCACCCTCCTGA-3′
	5′-TGGTGGCAAAGCCCACTCCTAC-3′
β-actin	5′-GACAGGATGCAGAAGGAGATTACT-3′
	5′-TGATCCACATCTGCTGGAAGGT-3′

Permeabilization was obtained with 0.1% Triton-X 100/PBS solution, and 1% bovine serum albumin (BSA) was used as a blocking reagent. Cells were incubated with anti-COL1A1 (Santa Cruz Biotechnology#59772), anti-OCN (Santa Cruz Biotechnology # 30044), anti-COL2A1 (Santa Cruz Biotechnology #28887), and anti-FABP4 (Santa Cruz Biotechnology #271) overnight. Then, AlexaFluor 488 conjugated secondary antibodies were used for 1 h at room temperature. DAPI staining was performed, and samples were visualized under a fluorescence microscope (Zeiss Primo Vert, Göttingen, Germany).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California, the USA). A histogram, q-q plots, and Shapiro–Wilk's test were applied to assess the data normality. The Levene test was used to test variance homogeneity. The data were statistically analyzed using Student's t-test. A p-value <0.05 was considered as statistically significant.

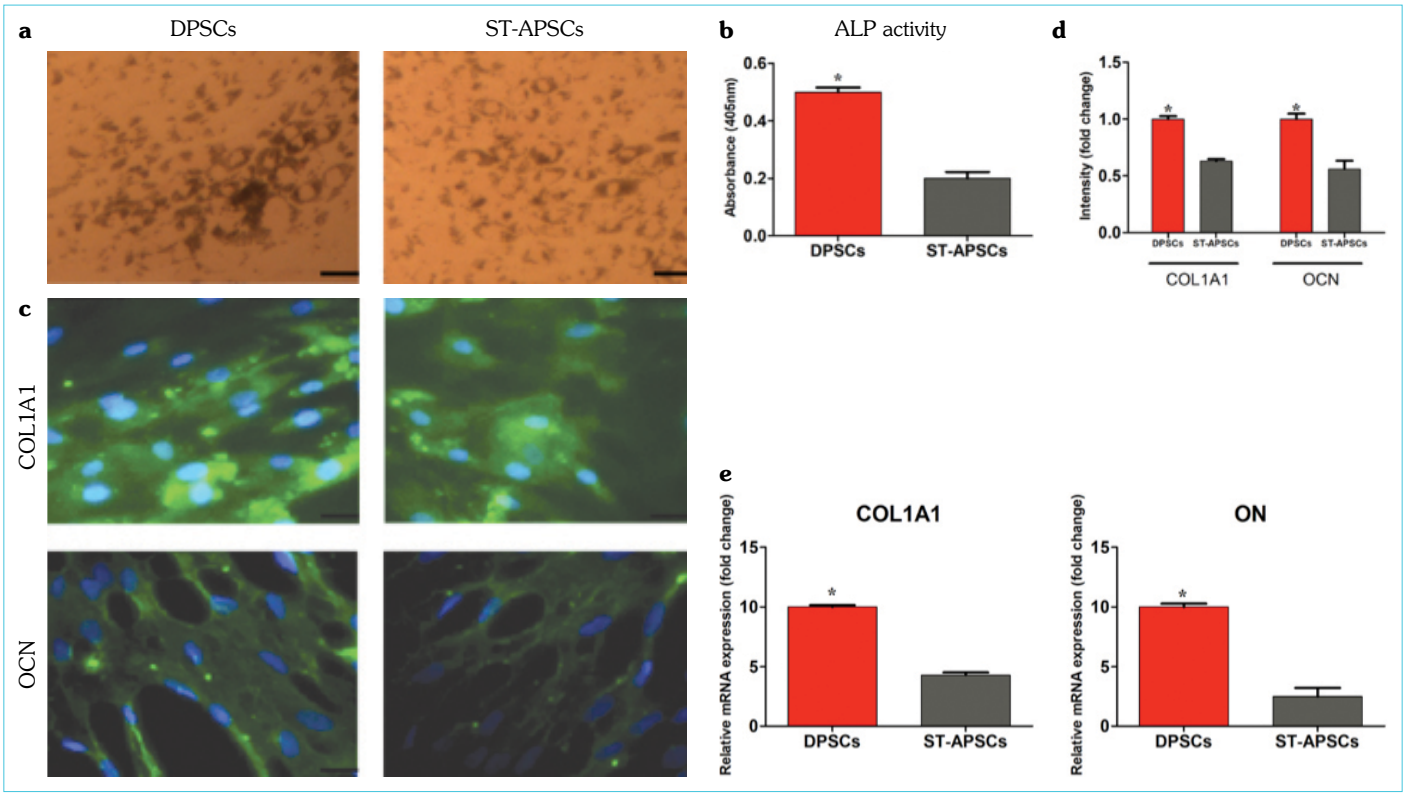

Figure 2. a–e. Osteogenic differentiation of DPSCs and ST-APSCs. (a) von Kossa staining results. Scale bar, 100μm. (b) ALP activity measurements. (c) COL1A1 and OCN protein expression. Scale bar, 50μm. (d) Intensity measurements of COL2A1 and OCN immunocytochemistry. (e) COL1A1 and ON gene expression analysis. *p<0.05

RESULTS

Isolation and Characterization

DPSCs and ST-APSCs were successfully isolated and expanded, and they demonstrated fibroblast-like cell morphology (Fig. 1b). Freshly isolated DPSCs and ST-APSCs were exerted MSC characteristics according to the surface protein expression profile. Both showed high CD73, CD90, CD105, and CD44 expression, and low CD146 and CD271 (Fig. 1c, d) expression. There was no significant difference in all markers except CD146. ST-APSCs expressed 16% of CD146, while DPSCs are negative for this marker.

Stainings

DPSCs and ST-APSCs were differentiated into the three cell lineages to prove and compare MSC characteristics. von Kossa staining indicated the osteogenic differentiation by calcium deposition. The results revealed that DPSCs exerted relatively increased calcium mineralization with respect to ST-APSCs, indicating the osteogenic transformation capacity of both cells (Fig. 2a). The chondrogenic differentiation was confirmed by staining the mucopolysaccharydes and glycosaminoglycans with Alcian blue. DPSCs exhibited more intense staining for chondrogenic transformation, indicating a remarkable cartilage differentiation capacity (Fig. 3a). Lipid vesicles were visualized with oil red staining as the result of adipogenic differentiation. No significant difference in the adipogenic transformation capacity was detected. Both cells were successfully differentiated into adipocyte-like cells, and lipid vesicles were stained with oil red in both experimental groups (Fig. 4a).

ALP Activity in Differentiating Stem Cells

As an osteogenic marker, ALP is found in the bone and teeth at high concentrations and is involved in mineralization. The ALP level was significantly increased in DPSCs compared to ST-APSCs at the end of the osteogenic differentiation (Fig. 2b).

Immunostaining Analysis

To confirm the differentiation of cells, both cell types were labeled with specific antibodies against markers of osteo¬genic (COL1A1, OCN), chondrogenic (COL2A1), and adipogenic (FABP4) cell types. The results indicate that DPSCs expressed higher levels of COL1A1 and OCN proteins (Fig. 2c, d). COL2A1, a marker of chondrogenesis, was increased two times in DPSCs compared to ST-APSCs (Fig. 3b, c). On the other hand, FABP4 was expressed in similar amounts in the adipogenic transformation (Fig. 4b, c).

RT-PCR Analysis

The mRNA levels for COl1A1 and ON were upregulated in DPSCs compared to ST-APSCs after the osteogenic differentiation (Fig. 2e). The COl1A1 and ACAN gene levels were high in differentiated DPSCs compared to ST-APSCs (Fig. 2d). Adipogenic differentiation results revealed that DPSCs and ST-APSCs expressed the FABP4 gene at equal amounts (Fig. 3d).

DISCUSSION

Identification of MSCs in the adult body has led to the development of treatment regimens involving stem cell therapy applications. BMSCs, as the primary source of adult stem cells, are well

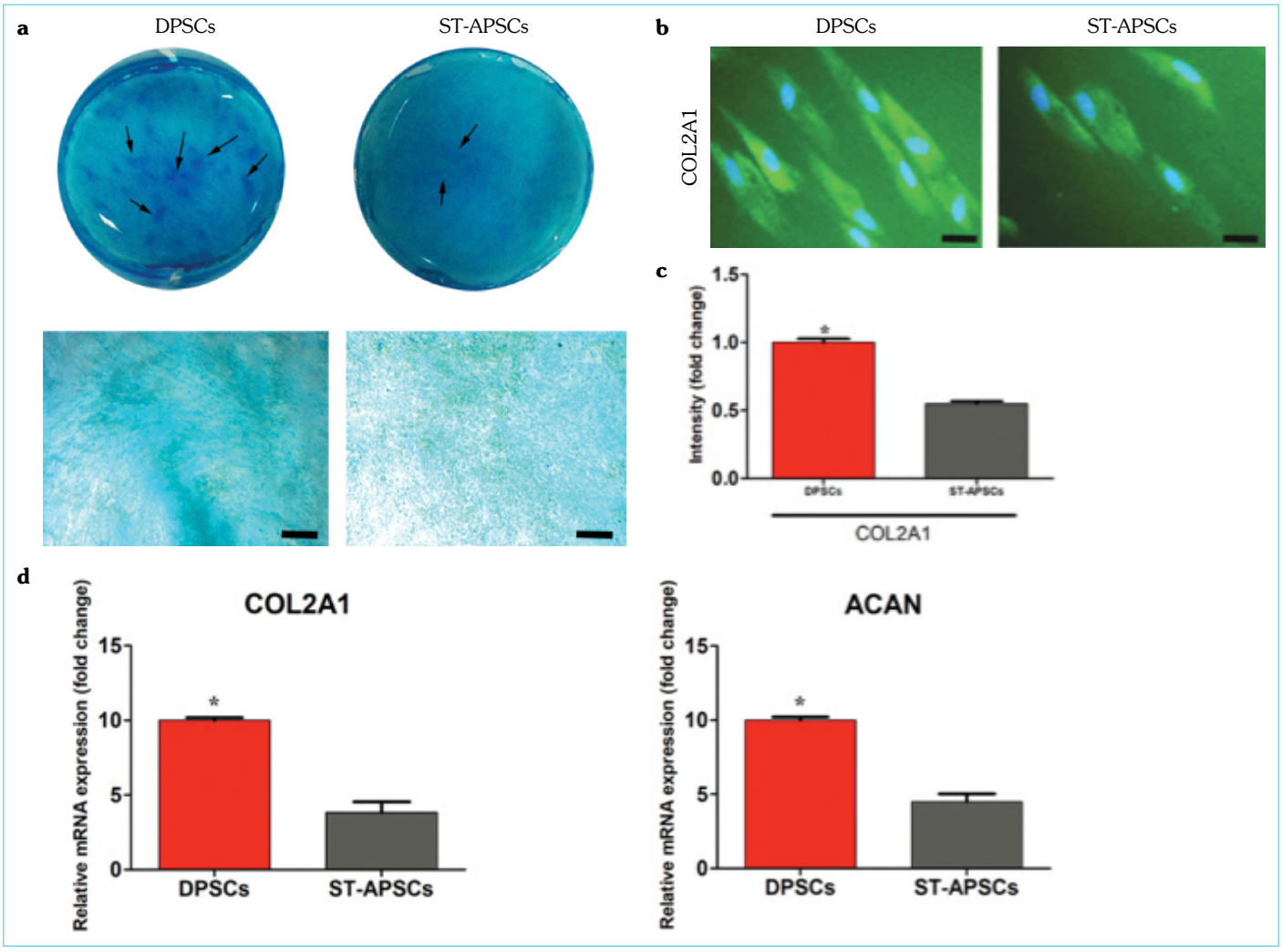

Figure 3. a–d. Chonrogenic differentiation of DPSCs and ST-APSCs. (a) Alcian blue staining of chondrogenic masses. Scale bar, 100μm. (b) COL2A1 protein expression. Scale bar, 50μm. (c) Intensity measurements of COL2A1. (d) COL2A1 and ACAN gene expression analysis. *p<0.05

characterized and have been proven to display a multipotent differentiation capacity. However, painful surgical procedures during resection, contamination risk, and an inadequate cell number yield restrict clinical applications. Therefore, the isolation of adult MSCs from alternative sources that are enabling the generation of a high number of proliferative cells has been of great interest in recent years.

DPSCs obtained from wisdom teeth of young adults were characterized by our group in previous reports, and their remarkable multipotent differentiation capacity to osteo-, odonto-, chonro-, adipo-, and neuro-genic cell lineages was shown (8, 19, 20). In addition to easy isolation of highly proliferative and multipotent stem cells, using the pulp tissue as a cell source might provide the number of cells sufficient enough for a functional stem cell therapy. Such sources of dental stem cells are valuable in regenerative medicine applications and should be well characterized for improvements in stem-cell-based therapy applications.

Supernumerary teeth, residing in the oral cavity instead of normal permanent teeth, are generally recognized at radiographic examinations for routine dental applications (21-23). This type of extra teeth might cause several complications, including the prevention of permanent teeth eruption, diastemas, dentigerous cysts, rotations, crowding, and root resorption problems (14, 15). To avoid such complications, supernumerary teeth should be removed by standard dental treatment applications. The patient in the current investigation had multiple supernumerary teeth without any systemic conditions, which is uncommon. Although multiple supernumerary teeth, in most cases, are associated with different syndromes, such as cleidocranial dysplasia, familial adenomatous polyposis, chondroectodermal dysplasia, trichorhinophalangeal syndrome, and Robinow syndrome (24-27), multiple supernumerary teeth without an accompanying syndrome were reported in previous studies (14, 22). In the current study, supernumerary teeth from a nonsyndromic patient were used to isolate apical papilla stem cells, and these cells were compared with the DPSCs derived from wisdom tooth pulps for their MSC characteristics.

A colony-forming ability and differentiation capacity of stem cells derived from supernumerary tooth were shown in previ-

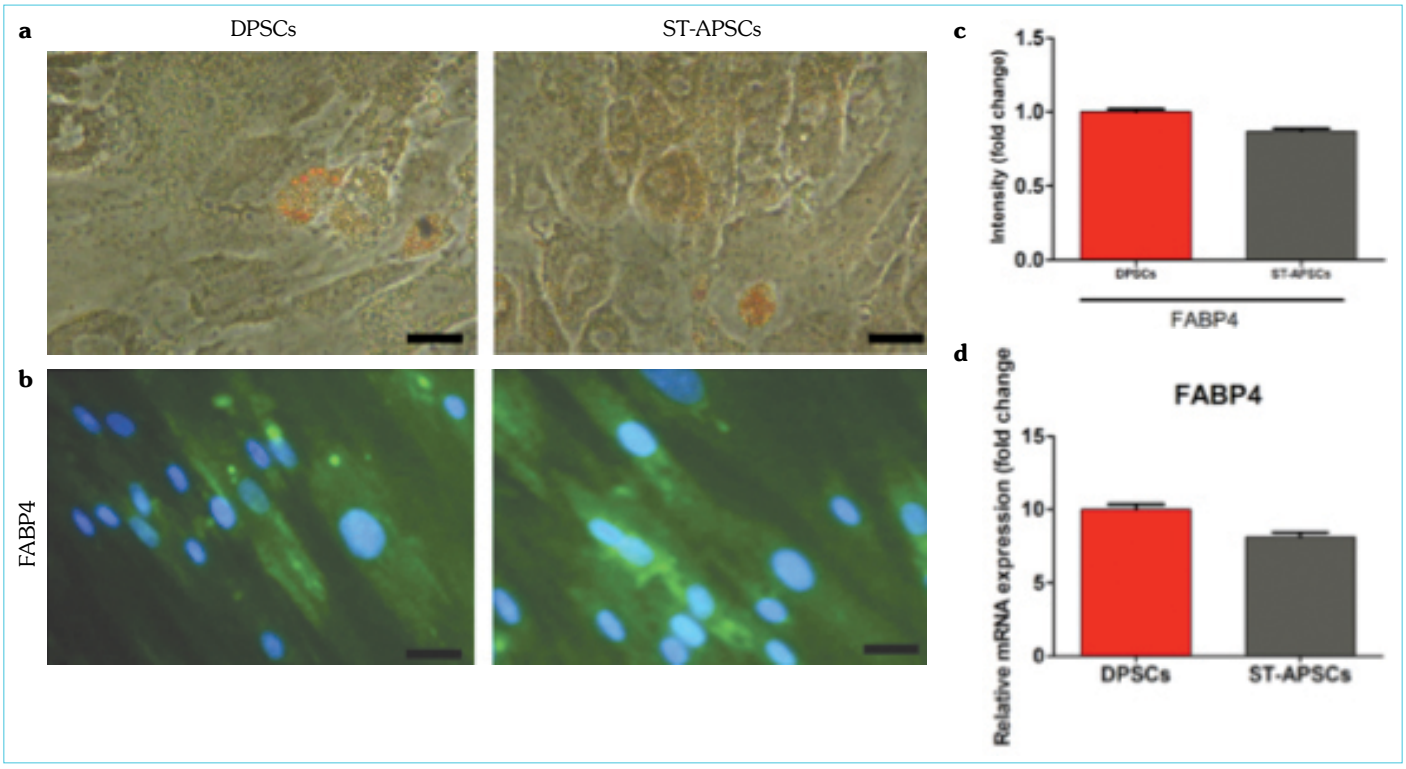

Figure 4. a–d. Adipogenic differentiation of DPSCs and ST-APSCs. (a) Oil red staining of lipid droplets after adipogenesis. Scale bar, 100μm. (b) FABP4 protein expression. Scale bar, 50μm. (c) Intensity measurements of FABP4. (d) FABP4 gene expression analysis. *p<0.05

ous reports (1, 17). Supernumerary-tooth-derived DPSCs were compared with SHED in a previous study (17). Similar results were obtained for immunophenotypic characteristics and differentiation abilities. ST-APSCs and DPSCs have a similar surface marker expression for all MSC markers. The only differentially expressed surface antigen is CD146, which was shown to be associated with vascular smooth muscle commitment (28). The CD146 level was high in ST-APSCs compared to DPSCs, which should be analyzed in further studies. The cells were also compared for their differentiation capacity by inducing the osteo-, chondro-, and adipogenic cell transformation. The current investigation proved that DPSCs obtained from tooth germs of young adults were found to be superior to ST-APSCs in terms of the osteo- and chondro-genic differentiation. High levels of calcium mineralization and osteogenic gene and protein expression were observed in DPSCs, indicating a better bone formation capacity compared to ST-APCSs. When both cell types were stained with Alcian blue, chondrogenic differentiation was similar in both cell sources. More prominent chondrogenic masses appeared in the DPSCs group. COL2A1 as a chondrogenic marker protein was expressed at low levels in ST-APSCs as well as is chondrogenic genes.

On the other hand, both cell types exerted similar characteristics for adipogenic differentiation. The present study clearly indicates that supernumerary teeth can be used for the MSC isolation with some limitations. Although ST-APSCs have the differentiation ability for three cell lineages, osteo-, chondro-, and adipogenic, originated from mesenchyme, they are less potent adult stem cells compared to DPSCs derived from wisdom teeth.

CONCLUSION

In conclusion, this report described the isolation of apical papilla stem cells from supernumerary teeth for the first time, to the best of our knowledge, and their stem cell potential was compared with DPSCs derived from wisdom teeth. As waste materials of dental applications, supernumerary teeth could be used to isolate MSCs.

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Ethics Committee Approval: Written consent of the patients and their parents was obtained for the use of extracted teeth according to the approval of the local ethical committee (2013/508) and the guidelines of the Helsinki Declaration.

Informed Consent: Written informed consent was obtained from the participants in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Conceived and designed the experiments or case: ZBG, SD, AE. Performed the experiments or case: ZBG, SD, AD, AS, AE Analyzed the data: SD, AD, AA, FŞ. Wrote the paper: ZBG, SD, AD, AE. All authors have read and approved the final manuscript.

Conflict of Interest: The authors have no conflict of interest to declare.

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