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Original Article

A pilot study on biological characteristics of human CD24(+) stem cells from the apical papilla

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KEYWORDS
Stem cells from apical papilla (SCAPs);
CD24;
Cell biological characteristics;
Cell differentiation

Abstract *Background/purpose:* CD24 is a specific cell surface marker for undifferentiated dental stem cells from apical papilla (SCAPs) seen only during root development, before the tooth emerges through gum. But the comprehensive role of CD24 in the SCAPs is unclear. This study aims to clarify the exact roles of CD24 in SCAPs.
Materials and methods: SCAPs were divided into CD24 (+)-SCAPs (high percentage CD24) and CD24 (-)-SCAPs (low percentage CD24) via flow cytometry. The proliferation, migration and osteogenic/adipogenic differentiation of the two groups were detected, RT-PCR was performed to detect the expression of osteogenic/adipogenic related genes and thegene expression were analyzed.
Results: The proliferative and migratory ability of CD24 (-)-SCAPs were significantly stronger than that of CD24 (+)-SCAPs. Although, the mineralization process and the osteogenic genes expression were not significantly difference in the two groups. Both CD24 (+)-SCAPs and CD24 (-)-SCAPs differentiated into adipocytes. The adipogenic differentiation in CD24 (+)-SCAPs was better than that in CD24 (-)-SCAPs, after 3 weeks of adipogenic induction. However, the expression of adipogenic related gene, PPAR γ2 mRNA in CD24 (+)-SCAPs was lower than that in CD24 (-)-SCAPs after 1 week of adipogenic induction. But the trend changed for the opposite after 3 weeks.
Conclusion: The study proposes that CD24 has a regulatory effect on the adipogenic differentiation of SCAPs, and this may be attained by targeting the PPAR γ2 mRNA. Concurrently, it was found that CD24 plays an inhibitory role in the proliferation and migration of SCAPs, which may minimize the manifestation of diseases caused by an abnormal cell growth.

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Introduction

Stem cells from apical papilla (SCAPs) are a type of human mesenchymal stem cells (MSCs) from apical papilla of immature permanent teeth. They possess the characteristic of high proliferation, migration as well as dentin differentiation like other dental pulp stem cells (DPSC),[1–3] which help their to be a promising cell source for regeneration of bio-roots for future clinical applications.[4] Recently, SCAPs have been recogined as having therapeutic potential, which can promote the continuous root development, maturation, and eventual transformation to pulp tissues.[5,6] Since 2006, Sonoyama et al.[4] first isolated SCAPs from the root apical papilla of human teeth, and established that CD24 is a unique cell surface marker for undifferentiated SCAPs.

CD24 is a vastly glycosylated cell protein and expressed on various cell types; mainly on immune cells and cells of the central nervous system, such as immature lymphocytes,[7] developing neuronal cells,[8] dendritic cells,[9] macrophages,[10] keratinocytes,[11] and hematopoietic cells.[12] Studies have shown that CD24 binds to Siglec-10 (sialic-acid-binding Ig-like lectin10) on innate immune cells, to inhibit the destructive inflammatory response in infection, sepsis, liver disorder and chronic graft-versus-host disease.[10,13,14] Moreover, CD24 is also expressed on a variety of solid tumors,[15–20] such as breast cancer, ovarian cancer, pancreatic and small intestinal neuroendocrine tumors. Studies have shown that the expression of CD24 may be relate to the inhibitory receptor Siglec-10 expressed on tumor-associated macrophages to promote the immune escape of tumor cells.[10]

Studies have showed that CD24, as a signaling molecule, is involved in regulating the cell's homeostasis, proliferation and differentiation.[21,22] Moreover, CD24 is a biomarker on normal and malignant stem cells, and the expression of CD24 is related to the activities of these stem cells, such as self-renewal, proliferation and differentiation.[16,23–27] CD24 is expressed in 3–20% of all cells.[28–30] So, if we are to utilize SCAPs in regenerative applications, it is important to understand the function of CD24 on SCAPs. CD24 in SCAPs was speculated to be involved in osteogenic differentiation,[31] However, the exact function of CD24 in SCAPs is largely unknown.

In order to clarify the role of CD24 in SCAPs, we sorted the SCAPs into high percentage CD24 group (CD24 (+)-SCAPs) and low percentage CD24 group (CD24 (−)-SCAPs). We identified the surface stem cell markers responsible for self-renewal, cell proliferation and multi-lineage differentiation. Our results revealed an important function of CD24 expressed on SCAPs, suggesting that homeostatic proliferation and adipogenic differentiation of SCAPs are regulated by CD24.

Materials and methods

Cell isolation and culture

SCAPs were obtained from human permanent immature third molar teeth (n = 10) from 16 to 22 year-old patients (5 male, 5 female, 18.50 ± 1.78 years), approved by the Ethic Committee of the Hospital of Stomatology, Zunyi Medical University, following the principles of the Declaration of Helsinki and Written Informed Consent of the patients. The method of separation of apical papilla tissue was based on earlier studies.[3,4] Briefly, the apical papilla tissue was gently separated and cut into small pieces, and then transferred into a 3 mg/ml type I collagenase (Gibco, Grand Island, NY, USA) with a solution of 4 mg/ml trypsin (HyClone, Logan, UT, USA) for a 40 min digestion at 37 °C. The liberated single cells were harvested by gently sifting (70 μm) them, and suspended in a 35 mm cultural dish at 1×10^4 cells. The cells were then cultured in α-MEM (HyClone) containing 15% serum (Gibco), 100 U/mL L-ascorbic acid, 2 mmol/L glutamic acid, 100 U/mL penicillin, 100 μg/mL streptomycin (Solarbio, Beijing, China) at 37 °C in 5% CO_2. Then using 0.25% trypsin (HyClone) to digest and collect the cells when they have grown to about 80%, we purified the cloned SCAPs by limiting dilution method. The cultured cells were amplified for cell identification and magnetic bead sorting.

Immunofluorescence for identifying SCAPs

The fourth generation of SCAPs were prepared with good growth condition, and the cell slides were prepared at a cell density of 1×10^4/cm.[2] The cells were fixed with 4% paraformaldehyde for 30 min and ruptured with 0.5% Trion X-100 for 15 min. After blocking with goat serum for 30 min to block nonspecific protein binding, cells were treated with primary antibody (mouse anti-STRO-1 monoclonal antibody, mouse anti-human CD24 polyclonal antibody, rabbit anti-human Keratin monoclonal antibody, R&D Systems, Emeryville, CA, USA) overnight at 4 °C. Cells were washed 3 times, and then incubated for 1 h with Secondary Antibody (PE-labeled goat anti-mouse IgM, FITC-labeled goat anti-mouse IgG, FITC-labeled goat anti-rabbit IgG, Biosynthesis, Beijing, China) in the dark at 37 °C. In addition, the DAPI (1:100) nuclei were stained at room temperature for 5 min. Then the fluorescence quencher was added, and the cells were observed under an Olympus confocal microscope, equipped with a CCD camera (Olympus, Takachie, Japan). The negative control group was contrasted to the experimental group, except that the Primary Antibody was replaced with PBS.

Identify the differentiation ability of SCAPs

Identify the osteogenic differentiation of SCAPs

The fourth generation SCAPs were prepared with good growth condition. After digested with 0.25% trypsin, the cells were suspend to 2.5×10^5/mL cell. Two drops of cell suspension per well were cultured in 6-well plate at 37 °C in 5% CO_2. After cell growth and fusion up to 80%—90%, add 2 mL osteogenic induction solution per well (containing 10% FBS, 10 mM β-phosphoglycerol, 50 ug/mL vitamin C, 10 nM dexamethasone α-MEM; HyClone) at 37 °C in 5% CO_2. After cultured for 3 weeks, the cells were fixed with 4% paraformaldehyde at room temperature for 20 min, and 1 mL of alizarin red dye solution was added to each well, stained for 15 min at 37 °C in 5% CO_2. Moreover, rinsed with sterile distilled H_2O until the liquid was clear and colorless, and observed under an inverted phase contrast microscope (Leica, Weztlar, Germany).

Identify the adipogenic ability of SCAPs

The fourth generation SCAPs were prepared with good growth condition. After digested with 0.25% trypsin, the cell suspension was prepared with 2.5×10^5/mL cell. Two drops of cell suspension per well were cultured in 6-well plate at 37 °C in 5% CO_2. Until the cell growth is fused to about 80% of the bottom area of the well, and 2 mL of adipogenic inducted solution (containing 10% FBS, 0.5 mM IBMX, 10 ug/mL insulin, 1uM dexamethasone, 0.5 nM indomethacin α-MEM; HyClone) was added to each well, and continuous cultured for 3 weeks at 37 °C in 5% CO_2. In addition, the cells were fixed with 4% paraformaldehyde for 30 min, and then aspirated the dye solution, rinsed with sterile deionized water until no floating color, and the reaction was terminated by adding 1.5 mL phosphate buffer saline (PBS), and observed under an inverted phase contrast microscope (Leica).

Immuno-magnetic beads sorting of CD24(+)-SCAPs and CD24(−)-SCAPs

To determine the expression of STRO-1 and CD24 on SCAPs surface by flow cytometry

The fourth generation SCAPs were prepared with good growth condition. After digested with 0.25% trypsin, the cells were centrifugated (1000 rpm) for 5 min, and suspended to 10^7 cells/mL by adding PBS buffer (PBS containing 0.5% BSA, 2 mol EDTA, pH 7.2). Each 100 μL SCAPs suspension was added with 5 μL of CD24-PE or STRO-1-APC mouse anti-human monoclonal antibody. The cells were incubated at 4 °C for overnight in the dark. After centrifugation at 1000 rpm for 5 min at 4 °C, Subsequently, the SCAPs were re-suspended in 300 μL of PBS buffer. STRO-1 and CD24 dependent fluorescence intensity were measured via flow cytometry. Isotype monoclonal STRO-1 and CD24 antibodies (Biolegend, San Diego, CA, USA) were used to determine background markers. The positive expression rate of cell surface antigens, STRO-1 and CD24, were calculated by using the dedicated software for flow cytometry (Becton, Dickinson and Company (BD), Franklin Lake, NJ, USA), which was expressed by %.

Immune-magnetic beads sorting CD24(+)-SCAPs and CD24(−)-SCAPs

For magnetic activated cell sorting, cells were first immuno-labeled with CD24-Biotin (Miltenyi Biotec, Bergisch-Gladbach, Germany). Magnetic labeling was performed, strictly, following the manufacturer's instructions. In short, the cell pellets were re-suspended in 40 μL precooled sorting buffer, for up to 10^7 total cells, after centrifugation. Then 10 μL of CD24-Biotin was added and the cell pellets were incubated at 4 °C for 15 min, in the dark. Next, the cells were resuspended in 80 μL of sorting buffer, and then incubated with 20 μL anti-Biotin magnetic beads (Miltenyi Biotec) for 15 min at 4 °C. The cells were wash three times and resuspended by adding 500 μL buffer to them.

The resuspended solution was carefully added to the magnetic beads sorting column, and the column was washed 3 times with 3 mL PBS buffer. Simultaneously, the discharging liquid, CD24 (−)-SCAPs suspension was collected from the sorting column. Afterwards, the sorting column was remove, and placed on the top of a 15 ml sterile centrifuge tube. 5 mL sorting buffer was added to the column, and a plunger was used, that matched with the sorting column to pressurize the liquid in the column to separate quickly and push out the cells that were adsorped in the magnetic beads sorting. The latest liquid collected was CD24 (+)-SCAPs suspension.

Determine the purity of cells after sorting

Centrifuged the cells suspension separately for obtaining the CD24 (+)-SCAPs and CD24 (−)-SCAPs. After removed the supernatant, the cells were suspended in culture medium at a concentration of 10^7 cells/mL at 37 °C in 5% CO_2. The positive expression rates of STRO-1 and CD24 on the surface of the two groups after sorting were determined by flow cytometry (according to 4.1), and expressed as %.

The effect of CD24 on the proliferation ability of SCAPs

Using 5-ethynyl-2′-deoxyuridine (EdU) method to detect the proliferative ability of CD24(+)-SCAPs and CD24(−)-SCAPs

The fifth generation CD24 (+)-SCAPs and CD24 (−)-SCAPs were prepared with good growth condition. After digested with 0.25% trypsin, 100 ul cells (4×10^4/mL) were incubated in a 96-well palatefor 4 h at 37 °C in 5% CO_2. Each group of cells were reconstituted in 3 wells. After discarded the culture medium, 150 μL of EdU medium (containing 25 μmol EdU, 10% FBS α-MEM, Hyclone) was added to each well of the cells. After incubating for 48 h at 37 °C in 5% CO_2, the cells were washed twice with PBS for 5 min, and 50 μL of 4% paraformaldehyde was added to each well and the cells were fixed for 30 min at room temperature. Then, added 50 μL 2 mg/ml glycine to the cells and shaken for 5 min, and, added 100 μL of penetrant (0.5% Triton X-100) to each well and shaken for 10 min. After washed the cells with PBS for 5 min, 100 μL Apollo staining reaction solution (Ribobio, Guangzhou, China) were added to each

well and shaken for 30 min in the dark. After washed 3 times with formaldehyde solution, 100 μL Hoechst 33,342 reaction solution (Ribobio) was added to each well and incubated for 30 min at room temperature. Then washed the cells 5 times, and added 100 μL PBS to each well for observing under an inverted phase-contrast microscope (Olympus).

Using tetrazolium salt colorimetric test (MTT) to detect the proliferative ability of CD24(+)-SCAPs and CD24(−)-SCAPs

CD24 (+)-SCAPs and CD24 (−)-SCAPs (5th generation cells) were prepared with good growth conditions. And digested with 0.25% trypsin and inoculated into 96-well plates at 10^5/mL cells, 100 μL per well. The number of cells was 10^4/well, and each group of cells was duplicated in 5 wells, and cultured under normal conditions, and the status of cell growth were observed under a microscope. Cell proliferative capability was measured at 1, 3, 5 and 7 days after cell inoculation. Added 20 μL of 5 mg/mL MTT solution (Solarbio) per well and incubated at 37 °C in 5% CO_2 for 4 h, then added 150 μL of DMSO to each well, and shaken for 10 min in the dark. After the reaction was completed, the optical density values (OD = 490 nm) of each well were measured by an enzyme-labeled instrument (Bio-Tek, Burlington, VT, USA), and the proliferative capability of the two groups were detected.

The effect of CD24 on the migratory ability of SCAPs

Using transwell migration chamber to detect the migratory ability of CD24(+)-SCAPs and CD24(−)-SCAPs

CD24 (+)-SCAPs and CD24 (−)-SCAPs (5th generation cells) with good growth condition were cultured for 12 h in serum-free culture. After digested with 0.25% trypsin, α-MEM medium containing 2% FBS was added to the cells. Then, adjusted the number of cells to 5×10^5/1.5 mL, and inoculated the two groups of cells into the upper of transwell chamber (Corning, Corning, NY, USA). Each chamber was inoculated with 150 μL cells suspension, and 500 μL of α-MEM medium containing 10% FBS was added to the chamber. After incubated for 12 h at 37 °C in 5% CO_2, the upper and lower chamber fluids were aspirated, then washed the cells with PBS twice. 600 μL of methanol was added to the

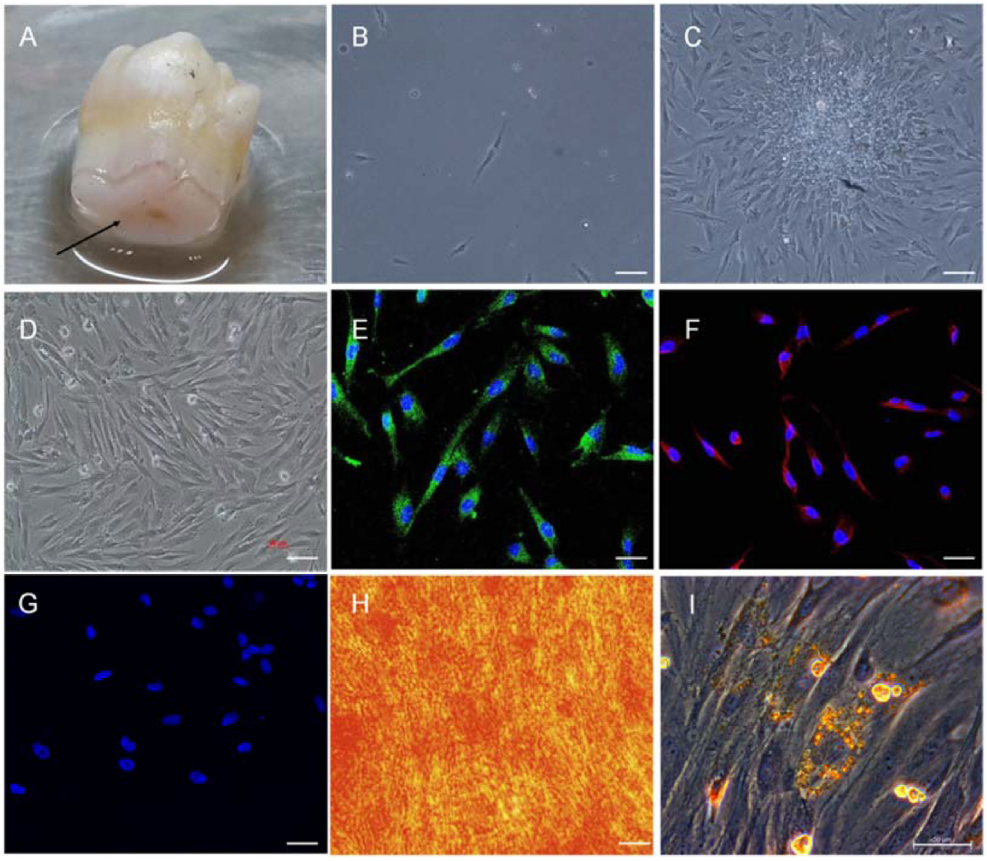

Figure 1 Identification of the origin and differentiative ability of SCAPs. (A) Apical papilla from an extracted human third molar with developing root tissues (arrow). (B) Single adherent SCAP after 3 days of cell culture. (C) SCAPs grown in colonies. (D) Purified cultured cells grown and fused up to 80%–90%. (E) 4th generation cell CD24 immunofluorescence staining (green, nuclear DAPI staining is blue). (F) The STRO immunofluorescence staining (red, nuclear DAPI staining is blue) of 4th generation cells. (G) The Keratin immunofluorescence staining (nuclear DAPI staining was blue). (H) Alizarin red S staining for mineralized nodules after SCAPs were cultured in osteogenic- inducing medium for 3 weeks. (I) Positive staining for lipid droplets with oil red O after adipogenic induction for 3 weeks.

lower chamber for fixing the cells for 20 min. The upper chamber was stained for 20 min in the lower chamber, which was added 0.1% crystal violet (Solarbio). Then washed the upper chamber with PBS. Used a small cotton swab to wipe off the cells gently and the excessive floating color on the surface of the upper chamber, then recorded and counted the cells under an inverted phase-contrast microscope (Olympus).

Determine the effect of CD24 on SCAPs migratory ability by cell wound-healing test

Prepared the two group cells with good growth (5th generation cells), adjusted the cell number to 5×10^5/mL after 0.25% trypsin digestion. Added 1 mL of cell suspension to the labeled 6-well plate, and repeated 3 wells in each group. Gently shaken the 6-well plate to spread the cell suspension evenly in at 37 °C in 5% CO_2. After 1 h, 1 mL of α-MEM medium containing 10% FBS was added to each well, and cultured overnight in an incubator. Removed the 6-well plate and observed the cells under a microscope. And then, used 200 μL pipette tip perpendicular to the bottom mark line to quickly and evenly scratched the scratch line (the intersection of each hole and the mark line were not marked). Washed each hole with 5 ml PBS twice, added 2 mL α-MEM medium containing 1% FBS to each well, and photographed the spots under an inverted microscope. The samples were placed in a 37 °C, 5% CO_2 cell incubator for 12 h and then photographed under the same multiple inverted phase-contrast microscope (Olympus).

Detection of CD24(+)-SCAPs differentiation ability

Osteogenesis

To promote osteogenic differentiation, the cells were cultured in an osteogenic induction medium (Osteogenic basal medium containing 10% FBS, 10 mM β-phosphoglycerol, 50ug/mL vitamin C, 10 nM dexamethasone α-MEM). The medium was replaced twice a week for 3 weeks. After differentiation, the cells were fixed with 4% formaldehyde (Sigma—Aldrich, San Francisco, CA, USA) for 30 min and stained with 1% Alizarin Red (Sigma—Aldrich) for 5 min at 37 °C.

The mineralized nodules were analyzed by cetylpyridinium quantitative detective method, as described elsewhere.[32] Briefly, 600 μL of 10% cetylpyridinium solution was added to each well, and shaken the plate for 30 min at room temperature to avoid the complete dissolution of the mineralized nodules. Each group of samples was added to a 96-well plate, and each sample was refilled with 5 wells. The optical density value (OD = 570 nm) of each well was measured by an enzyme-labeled instrument (Bio-Tek).

Adipogenesis

For adipogenic differentiation, CD24 (+)-SCAPs and CD24 (−)-SCAPs were treated for 3 days with adipogenic induction medium consisting of 10% FBS, 0.5 mM IBMX, 10μg/mL insulin, 1μM dexamethasone, 0.5 nM indomethacin α-MEM. Adipogenic differentiation was confirmed by the formation of neutral lipid vacuoles stainable with Oil Red O (Sigma—Aldrich) after 3 weeks of induction.

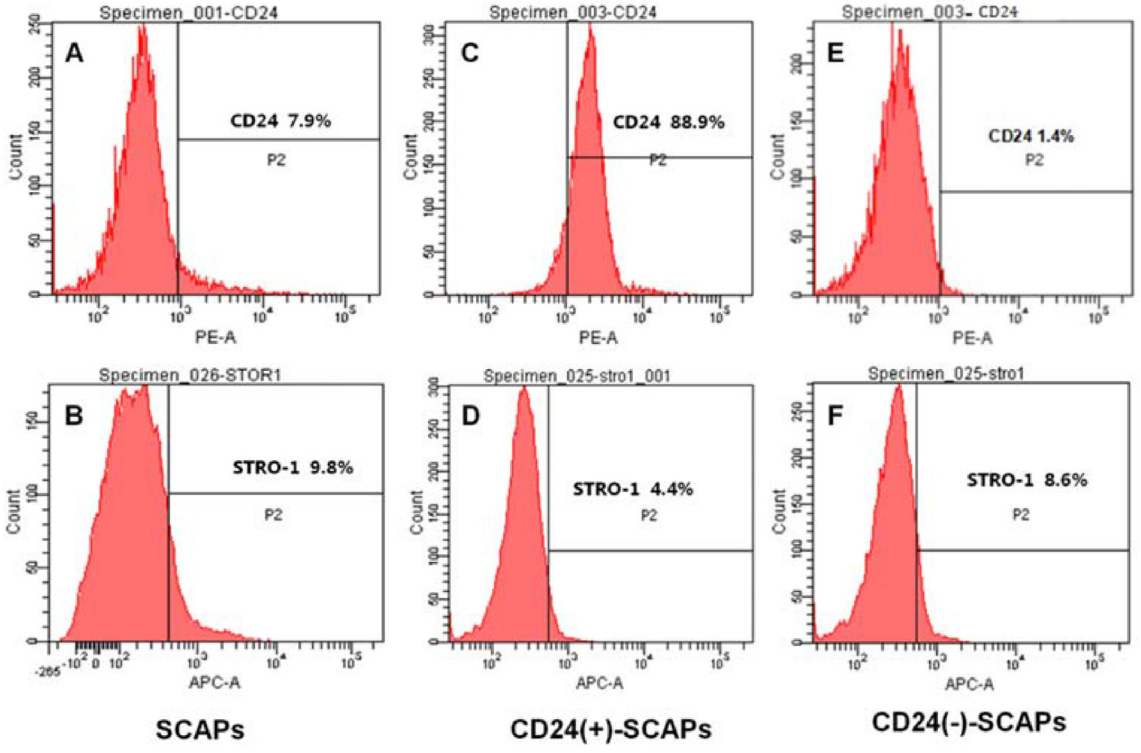

Figure 2 The positive expression rate of CD24 before sorting was 7.9%. (B) The positive expression rate of STRO-1 before sorting was 9.8%. (C, D) The positive expression rate of STRO-1 and CD24 of CD24 (+)-SCAPs group after sorting were 88.9% and 4.4%, respectively. (E, F) The positive expression rates of CD24 and STRO-1 in CD24(−)-SCAPs group after sorting were 1.4% and 8.6% respectively.

RT-PCR detection of mRNA expression of osteogenic and adipogenic genes

The expression of osteogenic differentiation markers DSPP, BSP, OCN, ALP, RUNX2 and adipogenic differentiation marker PPAR-γ2 were examined to reflect the ability of experimental cells to differentiate into bone and lipid. Total RNA was extracted by RNA lysate (Takara, Kyoto, Japan), and transcribed into cDNA by RNA reverse transcription kit (Takara).

Statistical analysis

Statistical analysis was performed with the Statistics Package for Social Sciences for Windows software package version 18.0 (SPSS, Chicago, IL, USA). Data from the MTT experiment and the cell scratch experiment were analyzed by *t*-test, and the data from the EdU and Transwell experiments were analyzed by non-parametric tests. Data were presented as the mean ± standard deviation (SD) unless described otherwise. Differences were considered to be significant and highly significant at $p < 0\ 0.05$ and $p < 0\ 0.01$ respectively.

Results

Isolation and culture of SCAPs

The third molar apex (Fig. 1A) was ascertained to be a healthy pink colour. The surface morphology was similar to the root of the molar, the apical nipple was medium in hardness and easy to separate from the apical foramen. Enzyme digestion of human primary apical papilla cells were observed for 3 days, and adherent cells were allowed

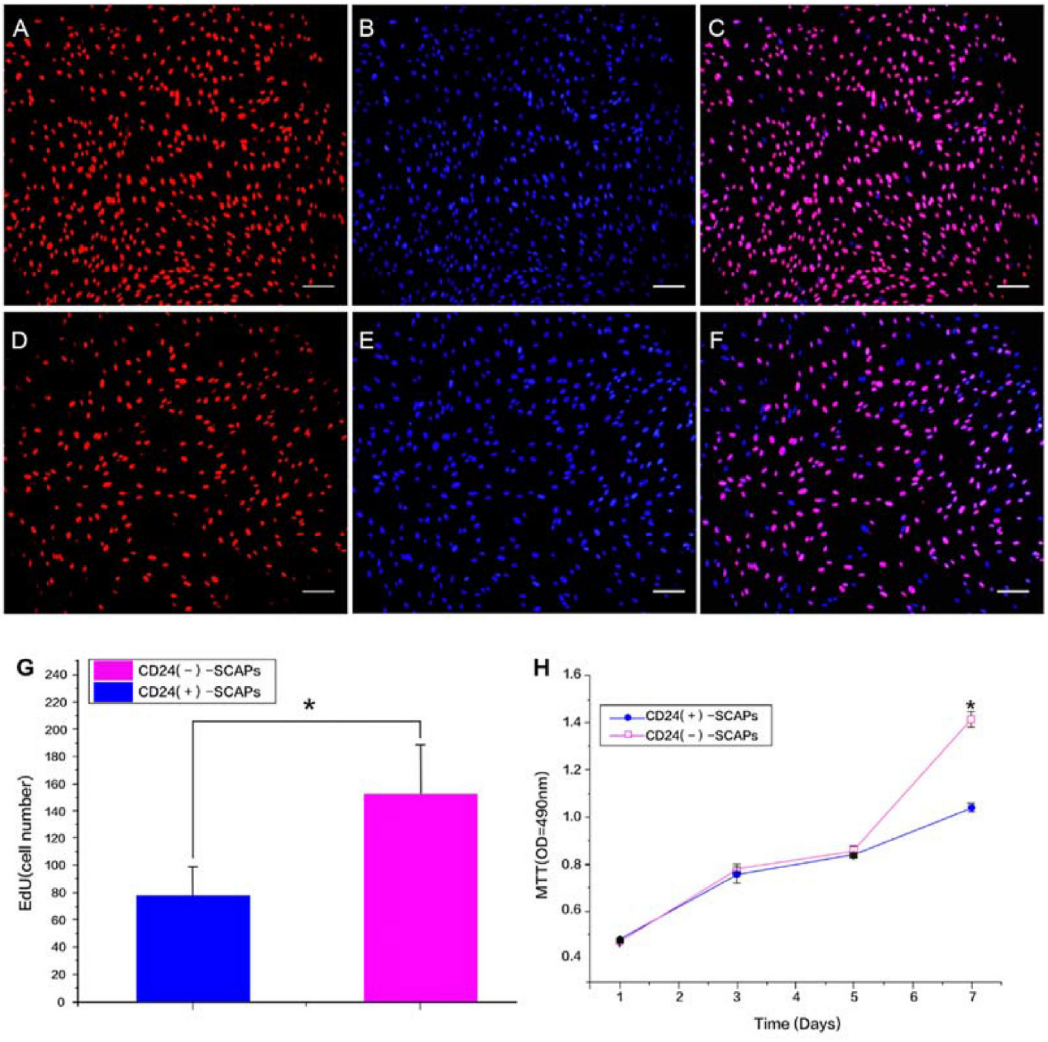

Figure 3 Detection of proliferative capacity of CD24(+)-SCAP. (A–C) The EdU immunofluorescence stainning of CD24(−)-SCAP group (A. red, EdU staining; B. nuclear Hoechst staining; C. the emerge of EdU and Hoechst staining). (D–F) The EdU immunofluorescence stainning of CD24(+)-SCAP group (D. red, EdU staining; E. nuclear Hoechst staining; F. the emerge of EdU and Hoechst staining). (G) The expression of EdU in CD24(+)-SCAPs and CD24(−)-SCAPs. (H) The proliferative capability of CD24(+)-SCAP on CD24(−)-SCAP 1, 3, 5, and 7 days. Data are presented as the mean ± SD, n = 10. Difference from corresponding control group at the same hypoxia time:* $p < 0.05$.

to grow and transform into fibroblast-like cells (Fig. 1B). A small number of cells were seen in square, elliptical and polygonal shapes, and the cells grew in nests or colonies (Fig. 1C). These cells began to be confluent in 7—10 days, and the purified apical papillary stem cells were cloned and isolated by limiting dilution. After reaching a ratio of 1:3, the cell growth fusion reached 80%—90% in about 3—5 days (Fig. 1D). The expression of CD24 and STRO-1 were positive (Fig. 1E and F). The expression of keratin was negative (Fig. 1G); The 4th-generation cultured cells developed mineralized granules after being cultured for 3 weeks in the osteogenic induction solution (Fig. 1H). The 4th generation cells were cultured for 3 weeks in the adipogenic induction solution, as well, and some of them can formed vacuolar round lipid droplets, and red lipid droplets, which were observed after oil red O staining (Fig. 1I).

Magnetic beads sorting CD24(+)-SCAPs and CD24(−)-SCAPs

To obtain a population of SCAPs with high expression of CD24 (CD24 (+)-SCAPs), we sorted CD24 (+)-SCAPs and CD24 (−)-SCAPs using flow cytometry. The positive expression rates of CD24 and STRO-1 on the surface of apical papilla stem cells before magnetic bead sorting were 7.9% (Fig. 2A), and 9.8% (Fig. 2B), respectively. The positive expression rates of CD24 and STRO-1 in CD24 (+)-SCAPs group were 88.9% (Fig.2C), 4.4% (Fig. 2D), and in the CD24 (−)-SCAPs group were CD24, respectively. The positive expression rates of STRO-1 were 1.4% (Fig. 2E) and 8.6% (Fig. 2F), respectively.

Detection of the proliferative capacity of CD24(+)-SCAPs

EdU staining method helped detect the proliferative capacity of CD24 (+)-SCAPs. The positive rate of EdU staining in CD24 (−)-SCAPs cells (Fig. 3A—C) was higher than that in CD24 (+)-SCAPs cells (Fig. 3D—F). The cell proliferative ability of CD24 (+)-SCAPs group was lower than CD24 (−)-SCAPs (Fig. 3G, $p < 0.05$). The cell proliferation of the two groups were detected by MTT after culture, on days 1, 3, 5, and 7 (Fig. 3H). The proliferation of the two groups showed an upward trend, and there was no significant difference in cell proliferative capacity between the two groups from day 1 to day 5 ($p > 0.05$). From day 5 to day 7, the cell proliferative capacity of the two groups was

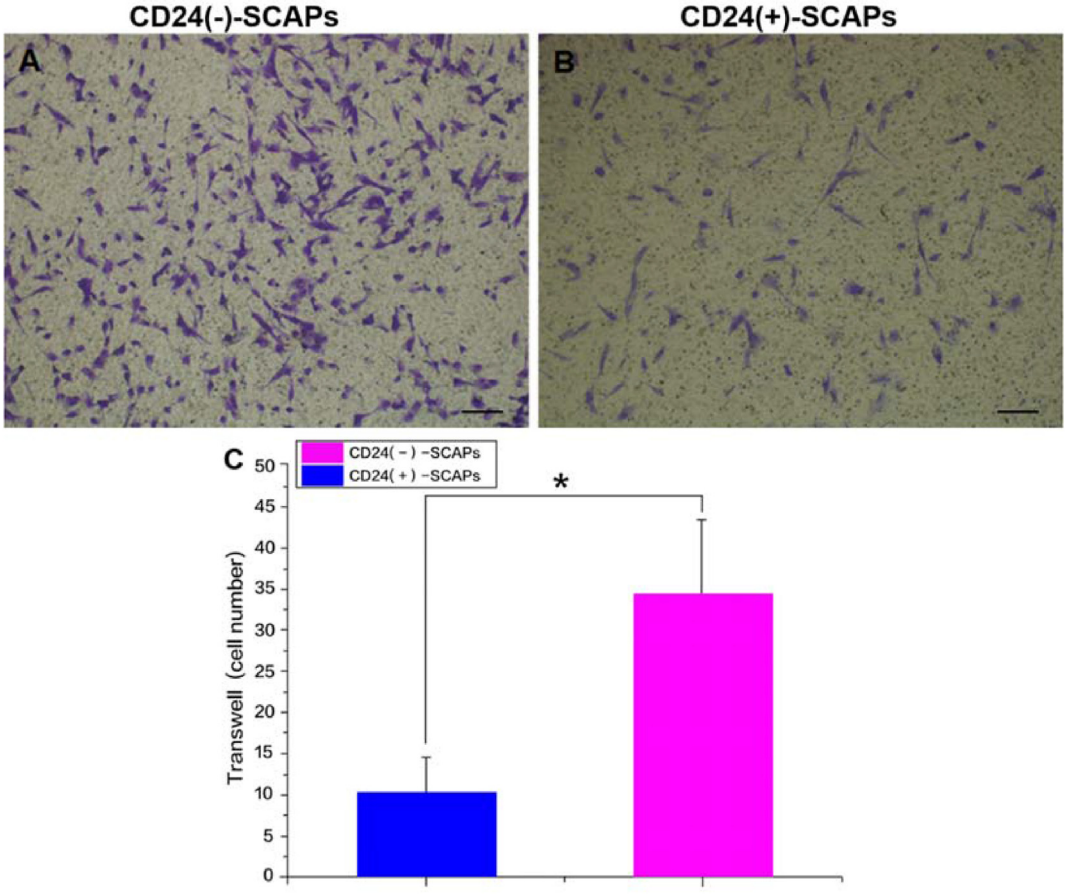

Figure 4 Detection of the migration ability of CD24(+)-SCAPs and CD24(−)-SCAPs by transwell migration chamber method. (A) Crystal violet staining of CD24(−)-SCAPs after migration 12hours. (B) Crystal violet staining of CD24(+)-SCAPs after migration 12 hours. (C) The migration ability analysis of CD24(+)-SCAPs and CD24(−)-SCAPs. Data are presented as the mean ± SD, n = 10. Difference from corresponding control group at the same hypoxia time:* $p < 0.05$.

enhanced significantly, and the peak within the observation points were reached on day 7 of the MTT test. The cell proliferation of the two groups was weaker than that of the CD24 (+)-SCAPs group ($p < 0.05$).

Detection of the migratory ability in CD24(+)-SCAPs

Transwell migration chamber method

The transwell migration chamber was used for detecting the migratory ability of the two group SCAPs. From Fig. 4, it can be shown that CD24 (−)-SCAPs had more crystal violet stains formation (Fig. 4A) than CD24 (+)-SCAPs after 12 h. It was observed that the migratory ability of CD24 (+)-SCAPs was weaker than that of CD24 (−)-SCAPs (Fig. 4C, $p < 0.05$).

Cell wound-healing test

In order to further display the differences in the migratory ability between CD24 (+)-SCAPs and CD24 (−)-SCAPs, we tested the cell migratory ability through the cell wound-healing test. The two groups of cells were cultured at regular spots and scratched spots (Fig. 5A–C). After 12 h, the migration of cells at the 2 different sites were observed (Fig. 5B–D). After statistical analysis, the results further indicated that the

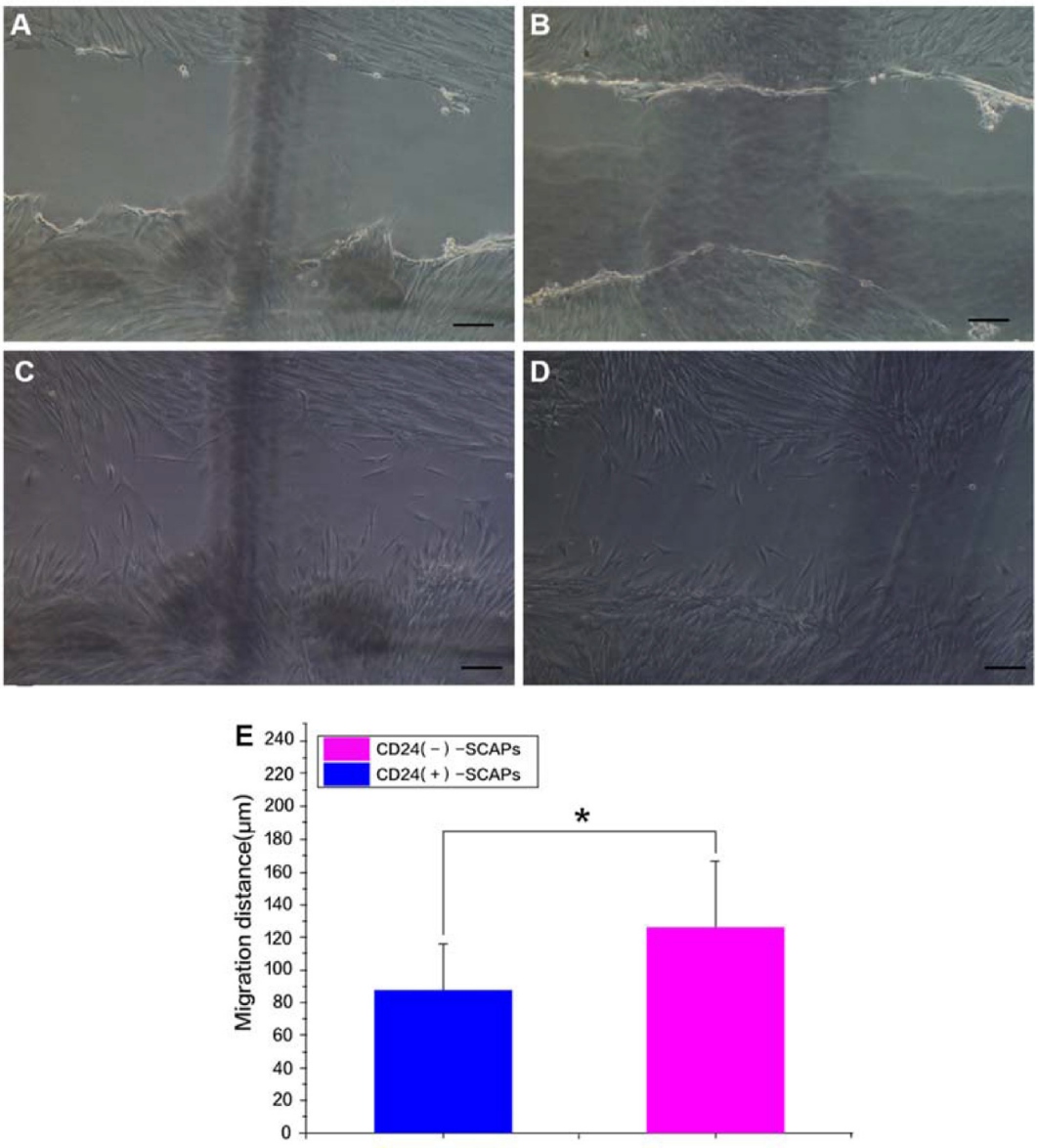

Figure 5 Detection of the migratory ability of CD24(+)-SCAPs and CD24(−)-SCAPs by cell scratch test. CD24(−)-SCAPs scratched immediately (A) and after 12 hours (B). CD24(+)-SCAPs scratched immediately (C) and after 12 hours (D). (E) The migratory ability analysis of CD24(+)-SCAPs and CD24(−)-SCAPs. Data are presented as the mean ± SD, n = 10. Difference from corresponding control group at the same hypoxia time:* $p < 0.05$.

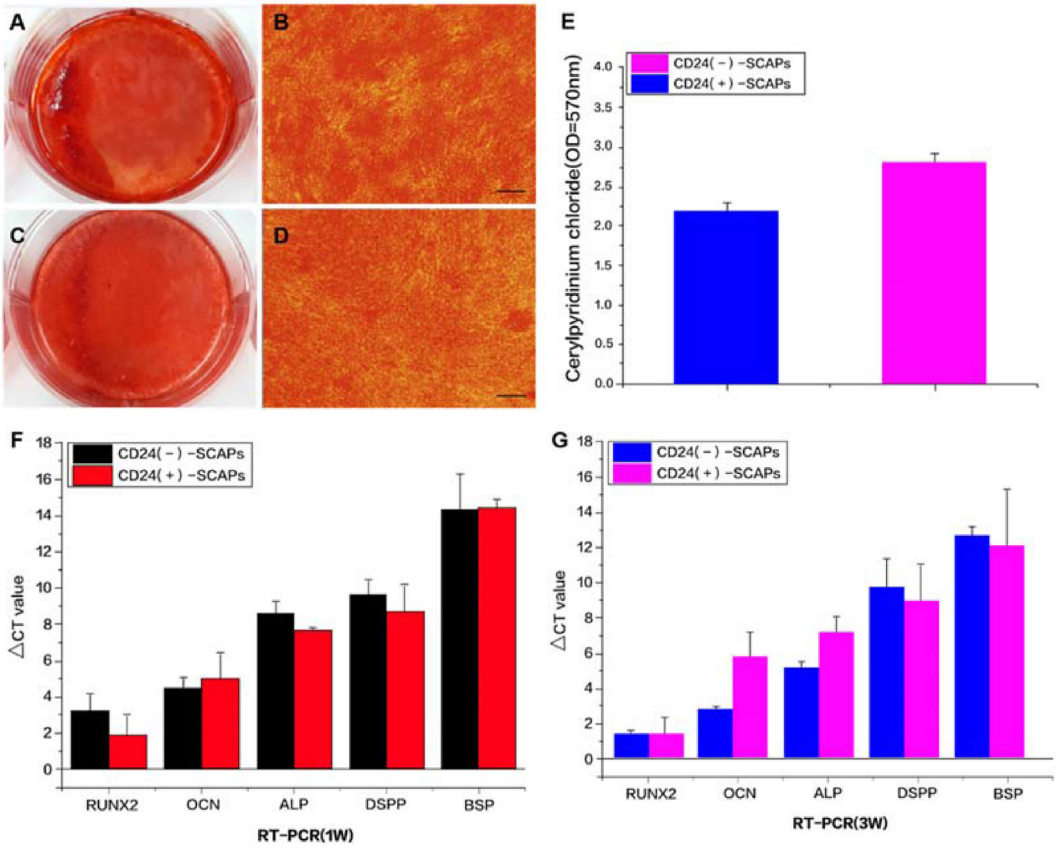

Figure 6 The osteogenic differentiation ability and the expression of osteogenic related genes (RUNX2, OCN, ALP, DSPP and BSP mRNA) of CD24(+)-SCAPs and CD24(−)-SCAPs. (A) Alizarin Red staining of CD24(−)-SCAPs. (B) Alizarin Red staining of CD24(−)-SCAPs (×40). (C) Alizarin Red staining of CD24(+)-SCAPs. (D) Alizarin Red staining of CD24(+)-SCAPs (×40). (E) The quantitative analysis of osteogenic ability between CD24(+)-SCAPs and CD24(−)-SCAPs. (F) The expression of osteogenic related genes after osteogenic induction 1 week. (G) The expression of osteogenic related genes after osteogenic induction 3 weeks. Data are presented as the mean ± SD, n = 10. Difference from corresponding control group at the same hypoxia time:* $p < 0.05$.

migratory ability of CD24 (−)-SCAPs was significantly stronger than that of CD24 (+)-SCAPs (Fig. 5E, $p < 0.05$).

The differentiation ability of CD24(+)-SCAPs and CD24(−)-SCAPs

The osteogenic differentiation ability

The cells of the two groups were cultured in the osteogenic induction solution for 3 weeks. The results of alizarin red staining are shown in Fig. 6 (A–D) and quantitatively analyzed by cetylpyridinium chloride (Fig. 6E). From the results, it was discovered that there was no statistically significant difference between the two groups ($p > 0.05$). It is indicated that CD24 may not have a significant effect on the osteogenic differentiation ability of SCAPs.

To further inspect the effects of CD24 on the osteogenic differentiation ability of SCAPs, we examined the expression of osteogenic related genes in CD24 (+)-SCAPs and CD24 (−)-SCAPs. From the results, we can found there are no significant differences in the expression of osteogenic related genes (RNUX2、OCN、ALP、DSPP and BSP mRNA) between the two groups after osteogenic induction 1 week (Fig. 6F) to 3 weeks (Fig. 6G). These data also suggest that CD24 may not be involved in the regulation of the osteogenic differentiation of SCAPs.

The adipogenic differentiation ability

To determine the adipogenic differentiation ability of CD24 (+)-SCAPs and CD24 (−)-SCAPs, the cells of the two groups were cultured in the adipogenic induction solution for 3 weeks. It was observed that the two groups both formed oil droplets after the oil red O staining. The oil red O staining was verified by looking under an Inverted Phase-Contrast microscope (Fig. 7A and B). It showed that the cells' volume increased and had oil droplets forming in the cytoplasm in both of the two groups.

Furthermore, to ascertain the effect of CD24 on the adipogenic differentiation ability of SCAPs, we observed the expression of adipogenesis-related genes PPAR-γ2 after adipogenic induction for 1 week and 3 weeks. The results (Fig. 7C) show that the expression is significantly higher in CD24 (+)-SCAPs than in CD24 (−)-SCAPs ($p < 0.05$). It demonstrated that CD 24 is a regulator of the adipogenic differentiation of SCAPs.

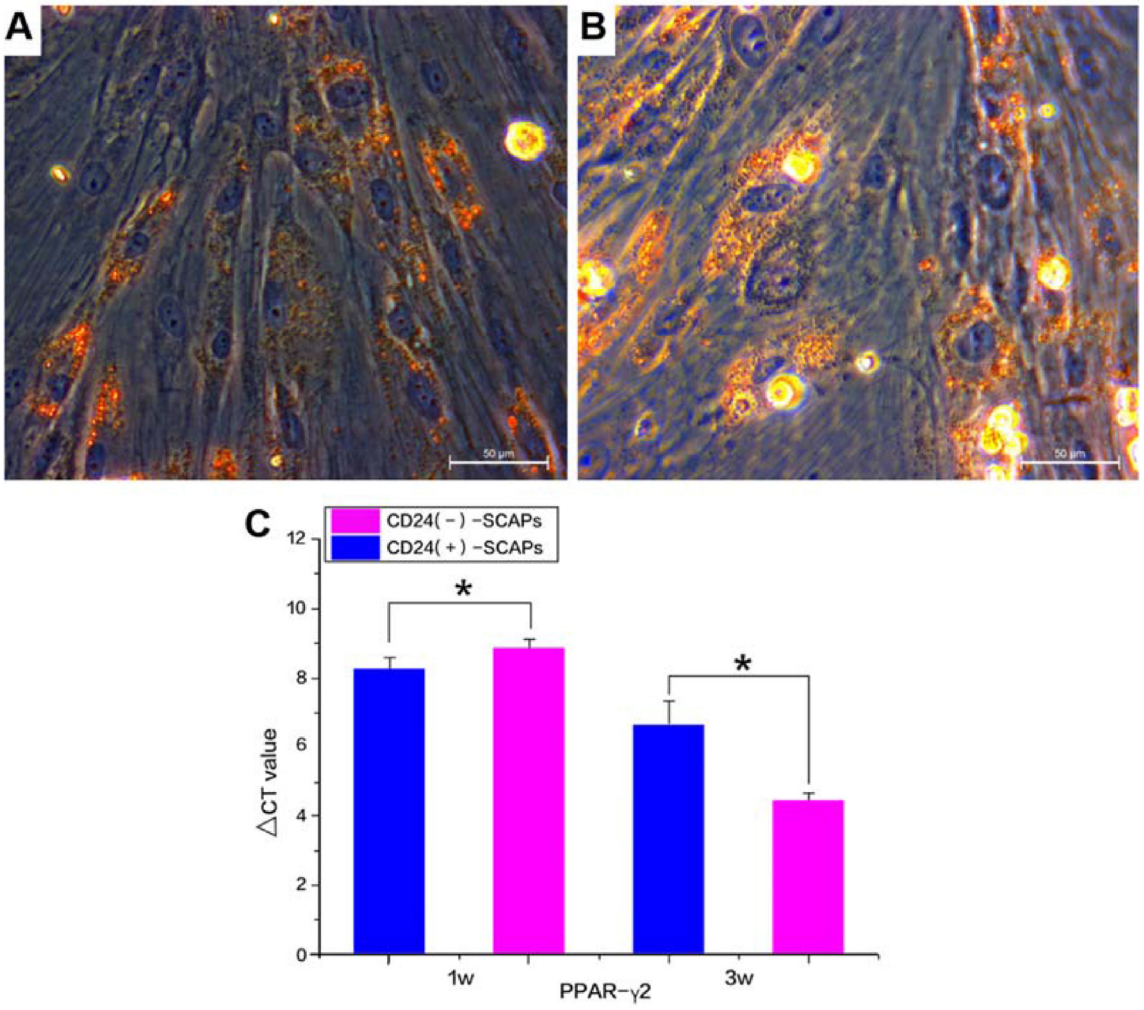

Figure 7 The adipogenic differentiation ability of CD24(+)-SCAPs and CD24(−)-SCAPs. (A) The oil red O stainning of CD24(−)-SCAPs after adipogenic induction 3 weeks (×400). (B) The oil red O stainning of CD24(+)-SCAPs after adipogenic induction 3 weeks (×400). (C) The expression of adipogenic differentiation related gene PPAR-γ2mRNA in CD24(+)-SCAPs and CD24(−)-SCAPs after adipogenic induction for 1 week and 3 weeks. Data are presented as the mean ± SD, n = 10. Difference from corresponding control group at the same hypoxia time:* $p < 0.05$.

Discussion

The major findings of this study include: (i) previously confirmed report of CD24 acting as a regulatory molecule in the differentiation of SCAPs.[33] (ii) Providing new results that demonstrate that CD24 is required for the optimal adipogenic differentiation of SCAPs, but revealing that it plays a negligible role in the osteogenic differentiation of SCAPs, and (iii) CD24 may promote the adipogenic differentiation of SCAPs by regulating the expression of PPAR-γ2mRNA.

SCAPs are a unique mass of postnatal stem cells, and are a promising cell source to regenerate bio-roots for future clinical applications. Studies have found that the multi-lineage differentiation potential of SCAPs were regulated by various factors, such as transforming growth factor beta 1 (TGF-β1),[34] granulocyte colony stimulating factor (G-CSF), fibroblast growth factor 2 (FGF-2),[35] insulin-like growth factor I (IGF-1),[36] CD146 and stromal cell antigen1 (STRO1),[6] and silent information regulator 1 (SIRT1).[37] It is an established fact that CD24 is involved in stem cell activities such as self-renewal, proliferation and differentiation, and researchers have found that CD24 is a unique marker for undifferentiated SCAPs.[4] For revealing the exact role of CD24 expression in SCAPs, Panuroot and coauthors[33] have compared the stem cell behaviors between indigenous high and low-CD24 percentage expressing cells of SCAPs, they have discovered the quantity of CD24 expressing cells influenced SCAPs self-renewal and multi-lineage differentiation, but did not influence on cell proliferation.

In our study, we detected the osteogenic differentiation capability of CD24 (+)-SCAPs and CD24 (−)-SCAPs and analyzed the expression of marker genes for osteogenic differentiation of these stem cells. Surprisingly, the results indicated that, although both groups of cells showed osteogenic differentiation, CD24 does not seem to be involved in regulating the osteogenic differentiation of SCAPs. Moreover, the adipogenic differentiation of SCAPs were

determined after adipogenic induction of 1—3 weeks. As shown in Fig. 7, the expression of PPAR-γ2 gene on CD24 (−)-SCAP was higher than CD24 (+)-SCAP after adipogenic induction 1 week, but in the third week of adipogenesis induction, the opposite result was shown, that is, the gene expression in CD24 (+)-SCAP was higher than in CD24 (−)-SCAPs. It reiterates for us the fact that as time passes, CD24 can begin to promote the adipogenic differentiation potential of SCAPs. Furthermore, to determine if CD24 was required for the survival of SCAPs, we compared the proliferation and migration of CD24 (+)-SCAPs and CD24 (−)-SCAPs. As shown in Figs. 3—5, the proliferative and migratory ability of CD24 (+)-SCAPs is lower than that of CD24 (−)-SCAPs. Thus, the data presented in this section demonstrates that CD24 expression inhibits the proliferation and migration of SCAPs, which is not conducive to the survival of SCAPs.

It has been reported that in pathological conditions, such as tumors,[17,38,39] inflammations[40,41] and autoimmune diseases, CD24 often plays a role in promoting tissue proliferation, cell differentiation, and damage repair. However, under physiological conditions, especially during the development of immature tissues, the expression of CD24 can limit the continued proliferation and differentiation of normal tissues and effectively prevents the occurrence of diseases. Cremers et al.[42] have found that the expression of CD24 has a certain inhibitory effect on the normal growth and development of mammary glands and the formation of collateral ducts. Belvindrah et al.[43] found that CD24 inhibits the proliferation of neural precursor cells under physiological conditions. In addition, the number of neural precursor cells in the S phase is significantly increased, the proliferation is more obvious, and the cell differentiation cascade was amplified after knocking out the CD24 gene,[14] thus confirming that the expression of CD24 can negatively regulate the proliferation and differentiation of neural precursor cells under physiological conditions.[44] Previous studies have shown that CD24 is downregulated following odontogenic differentiation.[4] Our research also shows that CD24 does not play a positive role in the differentiation of odontogenesis/osteogenesis in SCAPs, but instead promotes the differentiation of adipogenesis. Moreover, its regulation of SCAPs' proliferative and migratory ability is also consistent with its regulation of other cells, that is, it inhibits cell proliferation and migration under physiological conditions.

The study reveals that CD24 has a regulatory effect on the adipogenic differentiation of apical dental papilla stem cells, and this may be achieved by regulating the transcription factor PPARγ2. Meantime, we also found that CD24 plays an inhibitory role in the proliferation and migration of SCAPs, which may reduce the occurrence of related diseases caused by abnormal cell proliferation. However, the results in this study were obtained from cellular experiments, and whether CD24 has the same effect in vivo needs further verification.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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