Proliferation and osteo/odontoblastic differentiation of stem cells from dental apical papilla in mineralization-inducing medium containing additional KH_2PO_4

L. Wang^{*,†,2}, M. Yan^{*,2}, Y. Wang^{*}, G. Lei^{*}, Y. Yu^{*}, C. Zhao[†], Z. Tang^{*}, G. Zhang^{*}, C. Tang^{*}, J. Yu^{*,‡} and H. Liao[†]

^{*}Institute of Stomatology, Nanjing Medical University, Nanjing 210029, Jiangsu, China, [†]College of Stomatology, Guangxi Medical University, Nanning 530021, Guangxi, China, and [‡]Endodontic Department, School of Stomatology, Nanjing Medical University, Nanjing 210029, Jiangsu, China

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Abstract

Objectives: Stem cells from the dental apical papilla (SCAPs) can be induced to differentiate along both osteoblast and odontoblast lineages. However, little knowledge is available concerning their differentiation efficiency in osteogenic media containing additional KH_2PO_4 .

Materials and methods: Stem cells from the dental apical papilla were isolated from apical papillae of immature third molars and treated with two kinds of mineralization-inducing media, MM1 and MM2, differing in KH_2PO_4 concentration. Proliferation and osteo/odontogenic differentiation capacity of MM1/MM2-treated SCAPs were investigated and compared both *in vitro* and *in vivo*.

Results: Cell counting and flow cytometry demonstrated that MM2 containing 1.8 mM additional KH_2PO_4 significantly enhanced proliferative potential of SCAPs, compared to MM1. Osteo/odontogenic capacity of SCAPs was much better in MM2 medium than in MM1, as indicated by elevated alkaline phosphatase activity, increased calcium deposition and upregulated expression of osteo/odontoblast-specific genes/proteins (for example, runt-related transcription factor 2, osterix, osteocalcin, dentin sialoprotein, and dentin sialophosphoprotein).

In vivo transplantation findings proved that SCAPs in MM2 group generated more mineralized tissues, and presented higher expression of osteo/odontoblast-specific proteins (osteocalcin and dentin sialoprotein) than those in the MM1 group.

Conclusion: Mineralization-inducing media supplemented with 1.8 mM additional KH_2PO_4 significantly enhanced cell proliferation and improved differentiation capacity of SCAPs along osteo/odontogenic cell lineages, compared to counterparts lacking additional KH_2PO_4 .

Introduction

Stem cells from the dental apical papilla (SCAPs) are a population of stem/progenitor cells isolated from soft tissues at apices of developing permanent teeth (1–4). They are important for continued root development, maturation and apexogenesis of immature permanent teeth (5,6). These stem cells can undergo dentinogenic, osteogenic, adipogenic, and neurogenic differentiation under different inductive conditions *in vitro* (2,3,7). Compared to dental pulp stem cells (DPSCs), SCAPs have greater proliferation potential and higher efficiency for *in vivo* dentin regeneration (2,8). When combined with periodontal ligament stem cells and hydroxyapatite/tricalcium phosphate (HA/TCP), SCAPs can bring about formation of a biological root-periodontal complex *in vivo*, strong enough to support a porcelain crown (1,2).

Several studies have revealed that biological features of SCAPs can be affected by various factors, including insulin-like growth factor, resinous monomers, acellular amniotic membrane matrix, mineral trioxide aggregates and mineralization-inducing media (3,4,6,8–10). Of these, mineralization-inducing media are commonly used to

Correspondence: J. Yu, Institute of Stomatology, Nanjing Medical University, 136 Hanzhong Road, Nanjing 210029, Jiangsu, China. Tel.: +86 25 86862843; Fax: +86 25 85031861; E-mail: yuziyi_yjh@hotmail.com and H. Liao, Prosthetic Department, College of Stomatology, Guangxi Medical University, 10 Shuangyong Road, Nanning 530021, Guangxi, China. Tel.: +86 771 5358349; E-mail: dr_liaohb2005@126.com
2These authors contributed equally to this work and should be considered as co-first authors.

induce differentiation of SCAPs into osteo/odontoblastic lineages; these media usually contain dexamethasone and ascorbic acid (or an ascorbic acid analogue) and β -glycerophosphate (4,8,11–13). Dexamethasone has been thought to be the leading osteoinductive factor during mineralization, and is also used as an indicator of *in vivo* osteogenic potency of mesenchymal cells (14). It can increase proportions of cells expressing STRO-1, upregulate alkaline phosphatase activity, enhance proportion of ALP-positive colony-forming unit (CFU) critical to the mineral deposition, and induce expression of *DSPP* (14–16). Ascorbic acid can promote osteogenic differentiation, increase cell viability and stimulate synthesis of collagen matrix in osteo/odontogenic cells (14,17,18). Moreover, ascorbic acid can significantly enhance generation of induced pluripotent stem cells (iPSC) derived from fibroblasts or by somatic cell nuclear transfer (SCNT) from embryos (19). β -glycerophosphate can provide organic phosphate ions that facilitate biomineralization, induce dental stem cells to express odontoblastic phenotype and stimulate cell polarization/extension (8,20). Furthermore, some scholars have proposed that KH_2PO_4 can be used as an osteogenic medium supplement, providing inorganic phosphate to promote mineralization (8,21,22).

To date, many studies have proven that SCAPs can be driven into osteo/odontoblast lineages in a range of mineralization-inducing media (3,4,8,10). However, little knowledge is available concerning their differentiation capacity in mineralization-inducing media containing KH_2PO_4 . For this study, we have hypothesized that SCAPs in osteogenic media supplemented with additional KH_2PO_4 have some distinctive biological features. For this purpose, SCAPs were isolated from immature tooth apices and co-cultured with osteogenic media containing 1.8 mM additional KH_2PO_4 . Proliferation and differentiation of SCAPs in inductive media were investigated both *in vitro* and *in vivo*. Our findings showed that mineralization-inducing medium containing additional KH_2PO_4 exerted potent influence on proliferation and osteo/odontogenic differentiation of SCAPs in contrast to conditions with lack of additional KH_2PO_4 . This indicates that KH_2PO_4 can enhance differentiation efficiency of dental stem cells along osteo/odontoblastic lineages and serve as cost-effective supplement to mineralization media.

Materials and methods

Cell isolation and purification

All experiments were performed with the approval of the Ethics Committee of the Stomatological School of Nanjing Medical University. Human impacted third

molars with immature roots were collected from 7 healthy patients (16–24 years old) at the Department of Oral and Maxillofacial Surgery of Jiangsu Stomatological Hospital. After informed consent was signed by the patient, molars were removed and preserved in Hanks solution. Then, root apical papillae were gently separated from surfaces of roots, minced and digested using 3 mg/ml type I collagenase (Worthington Biochem., Freehold, NJ, USA), for 1 h at 37 °C. Single-cell suspension was obtained by passing digestate through a 70- μm sieve and seeding at 1×10^4 cells/ml in 10-cm culture dishes (Costar, Cambridge, MA, USA). Multicolony-derived SCAPs were isolated as previously described (2), cultured in alpha minimum essential medium (α -MEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco), then incubated at 37 °C in 5% CO_2 . Cells were purified using rabbit anti-STRO-1 antibody (Santa Cruz, Delaware, CA, USA) and sheep anti-rabbit IgG Dynabeads (Dyna Biotech, Oslo, Norway) according to protocols for magnetic activated cell sorting (MACS). Cells at 3–5 passages were used in subsequent experiments.

SCAPs were treated with three types of culture media: (i) Control group, α -MEM; (ii) MM1 group, mineralization-inducing medium (α -MEM supplemented with 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 10 nM dexamethasone and 10 mM β -glycerophosphate); (iii) MM2 group, mineralization-inducing medium supplemented with 1.8 mM additional KH_2PO_4 (Sigma, St. Louis, MO, USA) (8). 1.8 mM difference of KH_2PO_4 between MM1 and MM2 was confirmed by detecting final potassium/phosphorous ion concentrations in different groups (potassium concentrations: 6.05 ± 0.09 mM control group, 6.35 ± 0.17 mM in MM1 and 8.13 ± 0.16 mM in MM2; phosphorous concentrations: 1.27 ± 0.07 mM in the control group, 9.52 ± 0.1 mM in MM1, 11.53 ± 0.13 mM in MM2,) using Inductively Coupled Plasma Optical Emission Spectrometer (Optima 7000DV; PerkinElmer Inc., Waltham, MA, USA).

Cell counting assay

SCAPs were cultured in 96-well plates (Costar) at initial density of 3×10^3 cells/well in α -MEM containing 10% FBS, up to 60% confluence, serum-starved for 24 h, then respectively cultured in MM1 and MM2 media. For nine consecutive days, cells were harvested and counted every day using a Coulter counter (Beckman Coulter, Fullerton, CA, USA). Trypan blue was added to cell suspensions to identify and exclude non-viable cells. Cell number results are expressed as

means $\pm$ SD, and graphs were plotted based on average cell numbers. Population doubling time (PDT) was calculated according to the Patterson formulation (23). This procedure was repeated three times.

Flow cytometry

Cells were plated into 6-cm culture dishes (Costar), cultured in α -MEM supplemented with 10% FBS up to 60% confluence, serum-starved for 24 h, then respectively co-cultured in MM1 and MM2 media. After 3 days incubation, cells were harvested and fixed in 75% ice-cold ethanol at 4 °C, for 30 min in the dark. DNA content was measured by FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), and cell cycle fractions (G_0G_1 , S and G_2M phases) were also determined by flow cytometry (FCM). The procedure was repeated in triplicate.

Alkaline phosphatase assay

Cells were cultured in 96-well plates at initial density of 3×10^3 cells/well, in α -MEM containing 10% FBS up to 60% confluence, serum-starved for 24 h and then cultured in MM1 or MM2. After 3 days incubation, ALP assay was performed using an ALP kit (Sigma-Aldrich). First, 50 μ l alkaline buffer solution and 50 μ l stock substrate solution were added to each well. Second, 10 μ l sample was added to each well, mixed and incubated at 37 °C for 15 min, then, 110 μ l 0.5M NaOH was added to stop the reaction. Optical density at 405 nm was obtained. Finally, ALP activity was calculated according to our standard curve and normalized on the basis of equivalent protein concentrations. ALP assays were repeated three times.

Alizarin red staining

Cells were cultured in 12-well plates at initial density of 5×10^4 cells/well, in α -MEM containing 10% FBS up to 60% confluence, serum-starved for 24 h, then induced by MM1 or MM2. After 14 days incubation, alizarin red staining was performed to detect calcium deposition. Alizarin red was then destained with 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate, for 60 min at room temperature. Calcium concentrations were determined by absorbance measurement on a multiplate reader at 562 nm using a standard calcium curve. Protein content was quantitatively determined using Bio-Rad protein assay solution (Bio-Rad Laboratories Inc., Hercules, CA, USA) after removal of CPC solution from the samples. Final calcium concentrations were normalized with total protein content obtained from duplicate plates. The procedure was repeated in triplicate.

Real-time reverse transcription-polymerase chain reaction

Total cell RNA was isolated by adding TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to SCAP samples after 3 days and 7 days of incubation in MM1 or MM2 media and total RNA was extracted according to the manufacturer's protocol. Concentration and purity of RNA samples were determined by absorbance of RNA at 230, 260 and 280 nm, respectively. First-strand cDNA was synthesized using a first-strand cDNA synthesis kit (Takara, Bio, Otsu, Japan). Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR) was performed using SYBR Premix Ex Taq™ kit (TaKaRa, Bio) in a quantitative PCR System (ABI 7300, CA); all primers were synthesized by the same manufacturer (Sangon Biotech, Shanghai, China). The following primer sets were used: *ALP*, 5'-GACCTCCTCGGAAGACACTC-3' (forward) and 5'-TGAAGGGCTTCTTGTCTGTG-3' (reverse); dentin sialophosphoprotein (*DSPP*), 5'-ATATTGAGGGCTGG-AATGGGGA-3' (forward) and 5'-TTTGTGGCTCCAG-CATTGTCA-3' (reverse); osteocalcin (*OCN*), 5'-AGCA AAGGTGCAGCCTTTGT-3' (forward) and 5'-GCGCCT GGGTCTCTTCACT-3' (reverse); osterix (*OSX*), 5'-CCTCCTCAGCTCACCTTCTC-3' (forward) and 5'-GT TGGGAGCCCAAATAGAAA-3' (reverse); runt-related transcription factor 2 (*RUNX2*), 5'-TCTTAGAA-CAAATTCTGCCCTTT-3' (forward) and 5'-TGCTTT GGTCTTGAAATCACA-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GAAGGT GAAGGTCGGAGTC-3' (forward) and 5'-GAGA-TGGTGATGGGATTTC-3' (reverse). Real-time RT-PCR reaction conditions were: 95 °C for 30 s; followed by 40 cycles of 95 °C for 5 s, 60 °C for 31 s. Relative gene expression values were evaluated by the $2^{-\Delta\Delta C_t}$ method (4). Data were expressed as means $\pm$ standard deviation of three independent experiments.

Western blot analysis

Cells were collected after 3 and 7 days incubation respectively of MM1 and MM2 groups, washed twice in cold PBS and lysed in RIPA lysis buffer (Beyotime, Beijing, China) containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Cell debris was eliminated by centrifugation at 10800 g for 10 min. Protein concentrations were determined using a Bio-Rad protein assay kit (Pierce, Rockford, IL, USA). 40 μ g protein per lane was loaded on 10% SDS-polyacrylamide gel for electrophoresis, then transferred to PVDF membranes (Millipore Co. Bedford, MA, USA) at 300 mA for 1 h, in blotting apparatus (Bio-Rad). Membranes were blocked at room temperature for 2 h with blocking solution (5% w/v

skimmed milk, 0.01 mol/l PBS, 0.1% Tween-20), and subsequently incubated overnight at 4 °C with primary polyclonal antibodies to (DSP, 1:1000, Santa Cruz, sc-33587; RUNX2, 1:1000, Abcam, ab76956; OSX, 1:1000, Abcam, ab22552; OCN, 1:1000, Abcam, ab13418; β -actin, 1:1000, Bioworld, AP0060). Then, membranes were rinsed in PBST (0.1% Tween-20 in 0.01 mol/l PBS), and incubated with appropriate horseradish peroxidase conjugated secondary antibodies at 1:10 000 (Boster) at room temperature for an additional 1 h, visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo, Rockford, IL, USA), and exposed to Kodak X-ray films. β -actin served as internal control. This procedure was performed in triplicate.

Preparation of root segments

Freshly extracted rat incisors were collected and horizontally sectioned to obtain root segments of 5 mm length using an IsoMet low speed saw (Buehler, Lake Bluff, IL, USA). Soft tissues inside or outside the root segments were carefully removed. Empty root canals were exposed to 17% ethylenediamine tetraacetic acid (EDTA, Sigma) for 10 min and 19% citric acid (Sigma) for 1 min to remove smear layers and washed 3 times in PBS. Then, all segments were exposed to 75% ethanol and soaked in sterile PBS with 500 units/ml penicillin (Gibco, Life Technologies) and 500 μ g/ml streptomycin (Gibco, Life Technologies) for 1 h, washed 3 times in sterile PBS for 5 min and finally stored in α -MEM containing 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C for 3–7 days to remove potential microbial contamination (6).

Cell transplantation

After co-culture with MM1 and MM2 culture medium for 3 days, 1×10^6 SCAPs (isolated from the immature rat third molars) at the 3rd passage were collected as a pellet and carefully transferred into canal spaces of root segments. Then, root segments containing SCAP pellets were embedded into absorbable gelatin sponges (AGS; Nanjing Pharmaceuticals Inc., Nanjing, China), pre-immersed in corresponding inductive media (MM1 or MM2) and transplanted into renal capsules of adult rats. Twenty-four renal capsules from 12 adult Sprague-Dawley rats were used for allogenic transplantation. All retrieved tissues at 2 weeks post-transplantation were fixed in 4% polyoxymethylene for 48 h, decalcified in 10% formic acid for 1 month, paraffin wax embedded, serially sectioned and stained with haematoxylin and eosin (H&E) for histological analysis.

Immunohistochemistry

Immunohistochemical analyses of retrieved implants were performed using the streptavidin-biotin complex (SABC) method according to the manufacturer's recommended protocol. Briefly, tissue sections (5 μ m) from representative paraffin wax blocks were deparaffinized in xylene and rehydrated through a gradient of ethanol solutions. For antigen-epitope retrieval, sections were processed by conventional microwave heating in 0.01 M citrate buffer (0.01 M sodium citrate and 0.01 M citric acid, pH 6.0) for 5 min. Sections were treated with 100 μ l 3% H_2O_2 to suppress endogenous peroxidase activity, for 10 min at room temperature. Then, sections were blocked in 5% normal goat serum for 1 h and incubated with primary antibodies anti- (DSP, 1:200 Santa Cruz, sc-33587; OCN, 1:100 Abcam, ab13418) overnight at 4 °C. Incubation in PBS alone instead of primary antibodies served as negative controls. Sections were rinsed in PBST and incubated in biotinylated secondary antibodies for 45 min, at room temperature. Finally, sections were washed three times in PBST, incubated in SABC for 30 min and stained with 100 μ l DAB solution. When brown colouration was detected, slides were rinsed then counterstained with haematoxylin for 1 min and observed under a light microscope.

Statistical analysis

Quantitative results were expressed as means $\pm$ SD. Chi-square testing, one-way analysis of variance and Tukey multiple comparison tests were performed using SPSS-Windows v.12.0 software (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Proliferation properties of SCAPs in mineralization-inducing media containing additional KH_2PO_4

All isolated SCAPs were immunopositive for mesenchymal stem-cell marker STRO-1 (Fig. 1a). Then, SCAPs were incubated in different mineralization media for 9 consecutive days, and cell counting assay was performed to investigate their proliferation features. Cells in MM2 containing additional KH_2PO_4 had higher growth rates (Fig. 1b, PDT = 38.22 h) than those in MM1 lack of additional KH_2PO_4 (PDT = 39.47 h, $P < 0.05$) or control group (PDT = 40.54 h, $P < 0.05$). Flow cytometry results (Fig. 1c–d) further revealed that proliferation index (percentage of cells in S and G_2M phases) in

Figure 1. Proliferation characteristics of SCAPs in different mineralization-inducing media. (a) Isolated SCAPs appeared microscopically as spindle-shaped cells and immunopositive for STRO-1. (b) Growth curves of SCAPs in the control medium, MM1 and MM2, respectively. * $P < 0.05$, ** $P < 0.01$, one-way analysis of variance and Tukey multiple comparison test. The asterisks indicate a statistical significance between the MM1/MM2 and control groups. (c–e) Flow cytometry analyses for SCAPs in the control group (c), MM1 group (d), and MM2 group (e), respectively. (f) There was a statistically significant difference in S + G₂M phases between the MM2 group (16.23% in average) and other two groups (11.59% in the control group and 12.85% in the MM1 group). Values are the means $\pm$ SD, $n = 3$, ** $P < 0.01$, chi-squared test.

MM2 (16.23%) was higher than that in MM1 (12.85%) or in the control group (11.59%, $P < 0.01$).

Differentiation capacity of SCAPs in mineralization-inducing media containing additional KH_2PO_4

Both MM1 and MM2 groups had higher ALP activity than the control group, in which ALP levels at day 3 in MM2 group were significantly higher than those in the MM1 group (Fig. 2a, $P < 0.01$). At day 14, SCAPs in MM2 generated more calcium nodules (Fig. 2b) and showed higher calcium concentrations (Fig. 2c, $P < 0.01$) than those of MM1, indicated by alizarin red staining and cetylpyridinium chloride assay, respectively. Real-time RT-PCR revealed that *ALP*, *DSPP*, *OSX*, and *OCN* mRNA were significantly upregulated in MM2 group at day 3 and day 7, respectively, as compared to the MM1 group. *RUNX2* mRNA was significantly upregulated in the MM2 group at day 3, but downregulated in

MM2 group by day 7 (Fig. 3a and 3b; $P < 0.01$). Western blotting results showed that mineralization-related proteins (*RUNX2*, *DSP*, *OSX*, and *OCN*) in the MM2 group at day 3 were significantly enhanced, in comparison with the MM1 group (Fig. 3c). Protein levels of *DSP*, *OSX* and *OCN* at day 7 were also elevated in the MM2 group compared to the MM1 group (Fig. 3c).

Mineralization capability of induced SCAPs in vivo

SCAP pellets in the different groups were seeded into root canals and transplanted into renal capsules of adult rat hosts for 2 weeks. HE staining showed that pellets in all three groups generated dental pulp-like tissues inside the canal space of root segments. There were no mineralized tissues formed near the canal orifice in the control group (Fig. 4a and 4b). However, SCAPs in MM1 and MM2 groups had formation of new mineralized tissues inside the root canal space or covering the canal orifice

Figure 2. ALP assay and alizarin red staining of SCAPs in different mineralization-inducing media. (a) ALP activities of SCAPs in the control medium, MM1 and MM2, respectively. The MM2 group presented a higher ALP level than the other two groups after 3 days of osteo/odontogenic induction. $**P < 0.01$. (b) Alizarin red staining demonstrated that the MM2 group generated more calcified nodules than the MM1 and control groups after a 14-day induction. (c) After the 14-day induction, calcium contents in the MM2 group were significantly elevated as compared with those in the other two groups. Values are the means $\pm$ SD, $n = 3$. $**P < 0.01$, one-way analysis of variance and Tukey multiple comparison test.

Figure 3. Real-time reverse transcription-polymerase chain reaction and western blot analyses of SCAPs in different mineralization-inducing media. (a and b) Gene expression for *ALP*, *DSPP*, *RUNX2*, *OSX*, and *OCN* in SCAPs after 3 days (a) or 7 days (b) of osteo/odontogenic induction. *GAPDH* was used as an internal control for each group. Values are the means $\pm$ SD, $n = 3$, $**2^{-\Delta\Delta Ct} \geq 2$, $P < 0.01$; $*1 < 2^{-\Delta\Delta Ct} < 2$, $P < 0.01$, one-way analysis of variance and Tukey multiple comparison test. (c) Protein expression for *RUNX2*, *DSP*, *OSX* and *OCN* in SCAPs after 3 days or 7 days of osteo/odontogenic induction. β -actin was used as a control.

(Fig. 4c–f). The MM2 group (Fig. 4e and 4f) produced larger amounts of mineralized tissues near the canal orifice than MM1 group (Fig. 4c and 4d).

To identify newly formed calcified tissues in retrieved implants, expression of mineralization-related proteins (OCN and DSP) were illustrated using immunohistochemical techniques. No mineralized tissue was generated near canal orifices of root segments in the control group (Fig. 5a and 5b). Immunopositive staining against OCN and DSP was detected respectively in newly formed hard tissues of both MM1 and MM2 groups (Fig. 5c–f). The MM2 group presented stronger positive staining for OCN

(Fig. 5e) and DSP (Fig. 5f) than MM1 groups for newly formed mineralized tissues (Fig. 5c and 5d).

Collectively, SCAPs in MM2 containing 1.8 mM additional KH_2PO_4 had higher proliferative activity, stronger osteo/odontogenic differentiation potential *in vitro* and greater mineralized matrix-forming capacity *in vivo*, than those in MM1.

Discussion

Knowledge concerning differentiation of mesenchymal stem cells is growing. Current evidence indicates that at

Figure 4. *In vivo* histological evaluation of SCAPs in different groups. (a) Retrieved implants in the control group. Dental pulp-like tissues were generated inside the dentin wall of root segments, and no obvious mineralization appeared inside the canal space seeded with untreated SCAPs pellets. (b) A higher magnification of Fig. 4a. (c) Retrieved implants in the MM1 group. SCAPs pellets grew well in the root segments, and MM1-treated SCAPs pellets produced a small amount of mineralized tissues (MT) in the root canal. (d) A higher magnification of Fig. 4c. (e) Retrieved implants in the MM2 group. MM2-cultured SCAPs pellets produced many mineralized tissues near the root canal orifice. (f) A higher magnification of Fig. 4e. Scale bars = 200 µm. D, dentin; DP, dental pulp-like tissue; MT, mineralized tissue.

least five different types of dental stem/progenitor cell have been isolated and characterized, including dental pulp stem cells, stem cells from exfoliated deciduous teeth, stem cells from apical papilla, periodontal ligament stem cells, and dental follicle progenitor cells (1,24). So far, mineralization-inducing media have been commonly used to induce osteo/odontogenic differentiation of these dental stem cells (4,8,25–30). Each kind of mineralization-inducing medium contains different ingredients or has different proportions of ascorbic acid, dexamethasone and β -glycerophosphate. In this study, our findings have revealed that osteogenic media containing additional KH_2PO_4 clearly exerted the required distinctive effects on proliferation and differentiation capacity of SCAPs.

Here, SCAPs in MM2 containing additional KH_2PO_4 had higher proliferative potential than those in MM1 medium. Usually, cell proliferation is regulated by vari-

Figure 5. Immunohistological staining of retrieved SCAPs implants in different groups. (a and b) Control group. Weak positive staining against OCN (a) and DSP (b) was detected in the dental pulp-like structures generated by untreated SCAPs pellets inside the root canal. There were almost no newly formed hard tissues near the canal orifice (CO). (c and d) MM1 group. Weak positive staining against OCN (c) and DSP (d) was observed in both cell components of pulp like tissues and newly formed mineralized tissues near the canal orifice (CO) in the MM1 group. (e and f) MM2 group. Positive staining against OCN (e) and DSP (f) was detected in the cell components of dental pulp-like structures and newly formed hard tissues near the canal orifice (CO) in the MM2 group. (g and h) Negative control using PBS instead of primary antibodies in SCAPs implants. Scale bars = 100 µm. CO, canal orifice; D, dentin; DP, dental pulp-like tissue; MT, mineralized tissue.

ous factors including telomerase activity, osmolarity, ions and superoxide dismutase activity (2,31,32). Potassium is a major ionic component of normal cells, and K^+ channels play an important role in regulating cell proliferation and apoptosis. In detail, K^+ channels control cell anchorage to the stromal matrix and cell migration as well as secretion of paracrine growth factors (33,34). Inhibition of K^+ channels or elevated extracellular K^+ concentration can reduce efflux of intracellular

K⁺, which finally leads to inhibition of apoptosis and stimulation of cell proliferation. Thus, additional KH₂PO₄ in the MM2 exposed group increases extracellular K⁺ concentration, causes inhibition of efflux of intracellular K⁺, and subsequently results in acceleration of cell proliferation.

In addition, SCAPs in MM2 had better defined osteo/odontogenic potential than those in MM1, as indicated by elevated ALP activity, increased calcium deposition, upregulated expression of osteo/odontoblast-specific markers and enhanced mineralization capacity *in vitro* and *in vivo*. The most plausible explanation for the increased differentiation ability is associated with high levels of local phosphate ions released by KH₂PO₄ that provide the chemical microenvironment necessary for cell differentiation, biomineralization, cell maturation, polarization and morphological extension (8,20–22). Phosphorus is an essential mineral nutrient for all organisms, it is essential for synthesis of nucleotides (thus for ATP, DNA, and RNA) and regulate protein phosphorylation. Inorganic phosphate is thought to be the major form of phosphorus that can be directly absorbed by cells from their environment (35). Higher levels of KH₂PO₄ as used in this study, can provide sufficient phosphorus necessary for nucleotide and DNA synthesis, which subsequently facilitate upregulation of mineralization-related genes/proteins (such as *DSP/DSPP*, *RUNX2/RUNX2*, *OSX/OSX*, and *OCN/OCN*) (8). *RUNX2* and *OSX* are early-stage markers of osteoblast differentiation (36–38) and *RUNX2* is a transcription factor involved in differentiation of mesenchymal cells into preosteoblasts; it can upregulate *DSPP* transcription in pre-odontoblasts (39). *OSX* is a downstream gene of *RUNX2* and is also essential for osteoblast differentiation as well as biomineralization (40). *DSPP* is highly expressed in mature odontoblasts, whose over-expression can increase expression of *OSX* (39). *OCN* is an osteoblast-specific protein that is thought to be a late-stage marker of osteoblast differentiation (8). Together, increased expression of *OCN*, *RUNX2*, *DSPP* and *OSX* in the MM2 group indicates that mineralization medium containing additional KH₂PO₄ can efficiently drive SCAPs into osteo/odontoblast lineages at both early and late stages.

Summing up the findings of this study, mineralization-inducing media containing 1.8 mM additional KH₂PO₄ can significantly promote proliferation and improve differentiation efficiency of SCAPs compared to counterparts lacking additional KH₂PO₄. Thus, we recommend that KH₂PO₄ be routinely added to mineralization-inducing media to ensure sufficient proliferation and osteo/odontogenic differentiation of SCAPs. These findings may have general implications for other dental mesenchymal stem cells induced by osteogenic media.

Further studies are required to explore optimal concentration of KH₂PO₄ in mineralization-inducing media necessary for efficient proliferation and differentiation of this type of stem cell.

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