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Chondrogenic potential of stem cells from human exfoliated deciduous teeth *in vitro* and *in vivo*

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Abstract

Objective. The aim of this study was to investigate the chondrogenic potential of stem cells from human exfoliated teeth (SHED). ***Materials and methods.*** SHED cultures were isolated from human exfoliated deciduous teeth. Colony-forming capacity, odonto/osteogenic and adipogenic potential were measured. SHED were cultured for 2 weeks in chondrogenic differentiation medium containing dexamethasone, insulin, ascorbate phosphate, TGF-β3 and bFGF. Toluidine blue staining and safranin O staining were used for chondrogenesis analysis. The related markers, type II collagen and aggrecan, were also investigated using immunohistochemistry. SHED were seeded onto the β-TCP scaffolds and transplanted into the subcutaneous space on the back of nude mice. The transplants were recovered at 2, 4 and 8 weeks post-transplantation for analysis. ***Results.*** SHED showed colony-forming capacity, odonto/osteogenic and adipogenic differentiation capacity. Chondrogenic differentiation was confirmed by toluidine blue staining, safranin O staining, type II collagen and aggrecan immunostaining. After *in vivo* transplantation, SHED recombined with β-TCP scaffolds were able to generate new cartilage-like tissues. ***Conclusions.*** The findings demonstrate the chondrogenic differentiation capacity of SHED both *in vitro* and *in vivo* models, suggesting the potential of SHED in cartilage tissue engineering.

Key Words: *stem cells, exfoliated deciduous teeth, chondrogenic, tissue engineering*

Introduction

Cartilage has little capacity for repair [1]. However, treatment for lesions of articular surfaces has historically included clinical procedures ranging from conservation therapy to invasive surgery [2]. In recent years, several 'tissue engineering' approaches have been developed to improve cartilage repair by transplanting cells or engineered constructs into an injured site [3,4]. As this tissue engineering technology moves closer to clinical feasibility, it is becoming clear that the selection of suitable seed cells must become a priority.

Stem cells can be divided broadly into two categories: embryonic and adult [5]. Embryonic stem cells are pluripotent and are capable of differentiating into any mature cell type [6]. Due to ethical issues associated with the use of embryonic stem cells, however, recent attention has focused on stem cells derived from adult tissues. Post-natal stem cells have been isolated from a variety of tissues such as bone marrow [7], skin [8] and dental pulp [9]. They are characterized by their high proliferative capacity, while maintaining their ability to differentiate into multiple lineages. Several types of stem cells or progenitor cells from dental tissues have been isolated and characterized. They include post-natal dental pulp stem cells (DPSCs) [9], stem cells from exfoliated deciduous teeth (SHED) [10], periodontal ligament stem cells (PLDSCs) [11], stem cells from apical papilla (SCAP) [12] and dental follicle progenitor cells [13]. SHED have been isolated from naturally exfoliated deciduous teeth with the capacity to differentiate into osteogenic cells, adipocytes and neural-like cells [10]. Additionally, SHED were able to form a dentin-like structure when transplanted into immunocompromised mice [10] and offer obvious bone regeneration for repairing mandibular defects in a swine model

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[14]. However, few reports have shown that SHED could differentiate into chondrocytes. Therefore, the aim of our study was to investigate the chondrogenic potential of SHED both *in vitro* and in an *in vivo* model.

Materials and methods

Sample collection and cell culture

Normal exfoliated human deciduous incisors ($n = 13$) were collected from 6–8-year-old children after approval of the Medical Ethics Committee of Guangzhou Women and Children's Medical Centre. The pulp was separated from the remnant crowns (Figure 1A) and then digested in a solution of 3 mg/ml collagenase type I (Sigma-Aldrich Co., St Louis, MO) and 4 mg/ml dispase (Sigma-Aldrich) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer. The suspensions were seeded at 1×10^4 into a T25 flask (Costar, Cambridge, MA) and were then cultured with alpha-Modification of Eagle's Medium (α-MEM) (Hyclone, Utah, USA) supplemented with 10% FBS (Gibco, Life Technologies, Grand Island, NY), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. Cell culture was continued with a medium change every 3 days until the cells were sub-confluent. After the cells reached sub-confluency, they were passaged at a 1:3 ratio.

Colony-forming assay

To assess colony-forming efficiency, a total of 200 cells at passage 1 were seeded into 35 mm dishes (Costar). After 10 days of culture, the cultures were fixed with 4% formalin and then stained with 0.5% crystal violet (Sigma-Aldrich). Aggregates of ≥50 cells were scored as colonies. Experiments were performed in triplicate and were repeated three times.

Flow cytometric analysis

Cells at passage 2 were harvested and cell-surface antigen expression was analysed by flow cytometric analysis. Approximately 1×10^6 cells were washed with PBS and incubated with mouse anti-human STRO-1, CD90 and CD44 IgG or IgM (Santa Cruz, CA) for 30 min at 4°C. Cells were then washed twice with cold PBS containing 2% FBS and incubated with 1 mg of fluorescein isothiocyanate-conjugated goat anti-mouse IgG or IgM (Santa Cruz) for 30 min at 4°C. Mouse isotype antibody served as a control. Labelled cells were analysed using a BD FACSCanto™ II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Experiments were performed in triplicate and were repeated three times.

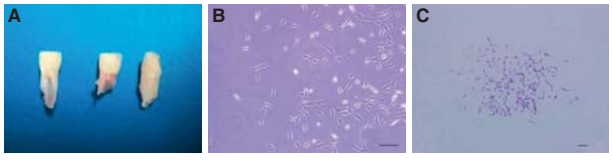

Figure 1. Morphologic appearance of SHED. (A) Human exfoliated deciduous teeth were extracted, with resorption of root and opened pulp chamber. (B) SHED presented a spindle or fibroblastic appearance at day 3. (C) Single colonies were formed after cells were plated at low density and cultured as described in the Materials and methods section. Scale bar: 100 μm.

Odonto/osteogenic differentiation

To induce mineralization, cells at passage 3 were seeded into 6-well plates, grown to sub-confluence and incubated in the differentiation medium containing 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbate phosphate (all from Sigma-Aldrich) and 10% FBS. The cultures exposed to normal medium without the additional supplements were used as the negative control (uninduced control). The cells were cultured for 3 weeks with the differentiation medium changed every 3 days. Then, both the induced and uninduced cells were evaluated for mineralization by Alizarin red staining and processed for the immunocytofluorescence analysis of ALP, BSP and OC.

Adipogenic differentiation

Cells at passage 3 were seeded into 6-well plates, grown to sub-confluence and incubated in the adipogenic medium containing 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 100 μM indomethacin, 10 μM insulin (all from Sigma-Aldrich) and 10% FBS. The cultures exposed to normal medium without the additional supplements were used as the negative control (uninduced control). The cells were cultured for 3 weeks with the differentiation medium changed every 3 days. Lipid droplets were stained with 2% (w/v) Oil Red O.

Chondrogenic differentiation

Cells at passage 3 were seeded into 6-well plates, grown to sub-confluence and incubated for 2 weeks in the chondrogenic differentiation medium containing 100 nM dexamethasone (Sigma-Aldrich), 6.25 μg/mL insulin (Sigma-Aldrich), 50 mg/L ascorbate phosphate (Sigma-Aldrich), 10 ng/mL TGF-β 3 (Boehringer Mannheim), 10 ng/mL bFGF (Boehringer Mannheim) and 10% FBS. The cultures exposed to normal medium without the additional supplements were used as the negative control (uninduced control). Then, the cultures were analysed by haematoxylin and eosin (H&E) staining, toluidine

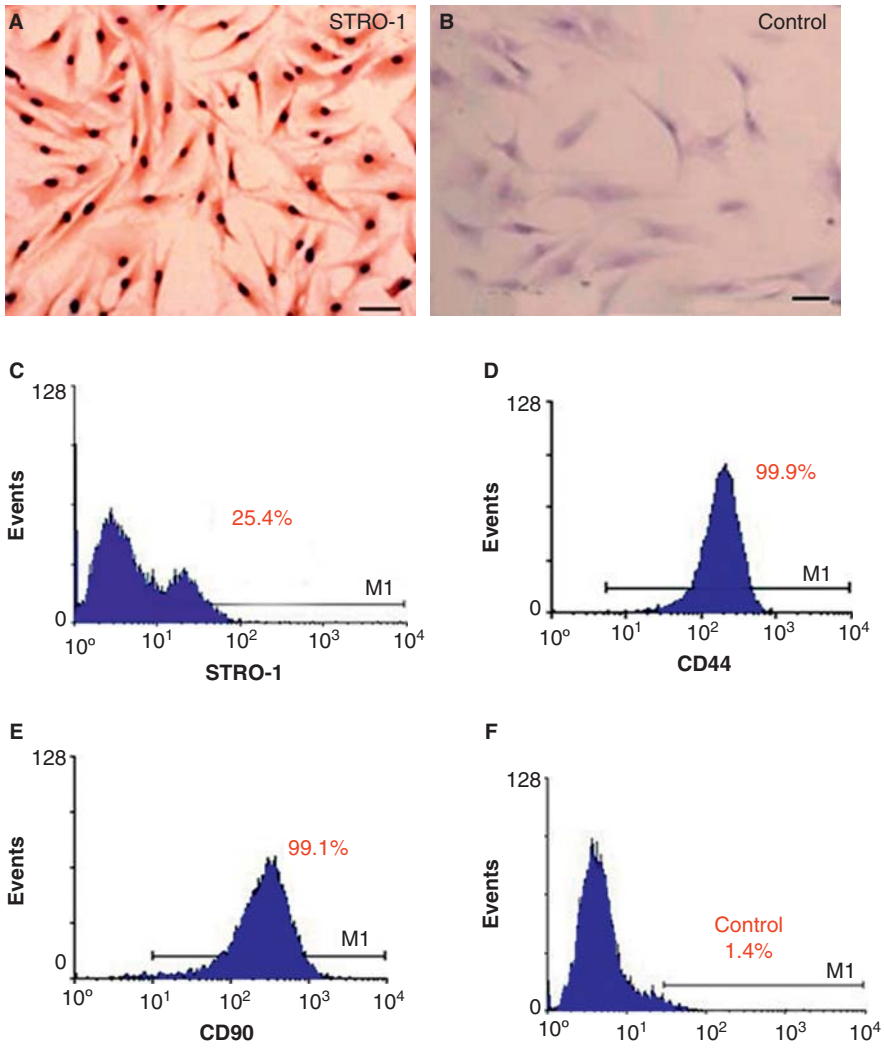

Figure 2. Expression of cell surface makers of SHED at passage 2. (A) SHED were positive for STRO-1, staining by immunocytochemistry. (B) No staining was detected in the negative control. (C–E) Flow cytometric analysis of *ex vivo*-expanded SHED revealed the expression of STRO-1 (25.4%), CD44 (99.9%) and CD90 (99.1%). (F) The negative control showed only 1.4% positive cells. Scale bar: 100 μm.

blue staining and safranin O staining and were processed for the immunocytochemical or immunocytofluorescence analysis of type II collagen and aggrecan.

Immunohistochemistry and immunocytofluorescence staining

The cells were sub-cultured into 6-chamber slides (3×10^4 cells/well). When grown to sub-confluence, the cells were fixed in 4% paraformaldehyde for 20 min and then endogenous peroxidase and non-specific reactions were blocked by pre-treatment with 3% H_2O_2 and goat serum. Then, the cells were incubated with primary antibodies overnight at 4°C. For enzymatic immunohistochemical staining, the SABC kit (Wuhan Boster Biological Technology Ltd, Wuhan, China) was used according to the manufacturer's protocol. For immunocytofluorescence analysis, after incubating with primary antibodies, the cells were subsequently incubated for 1 h at room temperature with goat anti-mouse secondary antibodies (1:100 dilution, Zhongshan Goldenbridge Biotechnology, Beijing, China) that were labelled with green fluorescence. The following primary antibodies were used: mouse antibody STRO-1 (1:50 dilution, Santa Cruz), anti-alkaline phosphatase (1:50 dilution, Santa Cruz), anti-osteocalcin (1:50 dilution, Santa Cruz), anti-bone sialoprotein (1:50 dilution, Santa Cru), anti-aggrecan (1:100 dilution, Wuhan Boster) and anti-type II collagen (1:100 dilution, Wuhan Boster). For the immunocytochemical analysis of STRO-1, the negative control was treated with PBS instead of primary antibodies.

In vitro analysis of SHED grown in β-TCP

The *β*-TCP particles (Shanghai Bio-lu Bio Materials Co., Ltd, Shanghai, China) were ~4 mm in diameter with a porosity of 70% and a pore diameter of 200 ~ 500 μm. The particles were sterilized by soaking in 75% alcohol and were then washed with PBS three

times, and incubated at 37°C for 24 h. Each particle was placed in a well of a 96-well plate. SHED at passage 3 were suspended in cell culture medium and were then seeded at a density of 2×10^5 cells/20 μL into each β-TCP particle for a 5-min incubation period. Then, 200 μL normal medium was added to the culture for further incubation. The medium was changed every 12 h. After culturing for 4 h and 7 days, the cell-seeded constructs were processed for scanning electron micrography (SEM) to observe their attachment and morphology. In brief, the samples were fixed with 2.5% glutaraldehyde in PBS for 24 h. Then, they were freeze-dried, coated with gold and examined with a SEM (S-3000N, Hitachi, Japan).

Transplantation

To further investigate the chondrogenic capacity of SHED, an *in vivo* transplantation assay was performed. The SHED/β-TCP constructs were considered as the study group and only the β-TCP as the control group. For the study group, SHED at passage 3 (2×10^5 cells/20 μL) were seeded into the scaffolds as described previously for the *in vitro* analysis. Then, the SHED/β-TCP constructs were kept undisturbed in the incubator for 4 h for better cell attachment. These constructs were ready for transplantation. For the control group, the β-TCP particles were also soaked with culture medium for 4 h. Five-week-old nude mice were purchased from the Center of Experiment Animal of Sun Yat-sen University. A total of 18 mice were randomly divided into three groups, with six in each group. The mice were anaesthetized and a dermal space was created by blunt lateral dissection from a single dorsal midline incision. Each mouse received two transplants, one SHED/β-TCP construct and one β-TCP particle, one on each side. The wounds were sutured for primary closure. The transplants were recovered at 2, 4 and 8 weeks post-transplantation, fixed with 4% formalin, decalcified with buffered 10% EDTA (pH 8.0) and processed for histological examination (H&E staining and immunohistochemistry). All animal procedures complied with the guidelines provided by the Animal Care Committee of Guangzhou Women and Children's Medical Centre (receipt and permission number: 2011032).

Results

Colony-forming capacity of SHED

To isolate the stem cells, single-cell suspensions were derived from the remnant pulp and placed at low density in liquid culture. As shown in Figure 1B, single cells were observed, showing spindle and fibroblastic morphology. SHED were able to form adherent clonogenic cell clusters (Figure 1C), similar to those recorded for different mesenchymal stem cell populations. Approximately 20–30 single colonies were formed at day 10.

Cell surface markers of SHED

Immunocytochemistry staining demonstrated that SHED were positive for STRO-1, which is an early mesenchymal stem cell marker (Figure 2A). Flow cytometric analysis further revealed that SHED at passage 2 contained 25.4% STRO-1-positive cells and were positive for CD44 (99.9%) and CD90 (99.1%) (Figures 2C–E).

Odonto/osteogenic and adipogenic differentiation

To verify that isolated dental cells qualified as stem cells, we examined their odonto/osteogenic and adipogenic potential. Round Alizarin red-positive nodules formed in the SHED cultures after 3 weeks of odonto/osteogenic induction (Figure 3A), indicating calcium accumulation *in vitro*. Immunocytofluorescence staining demonstrated that these induced SHED expressed an array of odonto/osteogenic markers, including ALP, BSP and OC (Figures 3C–E). Similarly, after the same period of culture in adipogenic medium, intracellular lipid vacuoles appeared in SHED cultures, which were confirmed by Oil Red O staining (Figure 3G).

Chondrogenic differentiation

Cells changed the spindle into a polygon shape after 2 weeks of chondrogenic induction, with a small size and deeply stained nuclei (Figure 4A). The chondrogenic differentiation of SHED was further confirmed by toluidine blue staining (Figure 4B), safranin O staining (Figure 4C), type II collagen and aggrecan immunostaining (Figures 4D and F).

Growth of SHED in β-TCP

The β-TCP scaffolds were highly porous with uniform distribution of orifices and their inner surface was smooth (Figure 5A). Cells attached well onto the surface of β-TCP after seeding into the scaffolds for 4 h (Figure 5B). SHED developed and secreted a rich matrix overspreading the scaffolds at day 7 (Figure 5C).

Formation of cartilage-like tissue in vivo

Of the 18 mice, one died and three suffered wound infection during the observation; six out of 14 healthy mice were dissected at the 2nd week, five were dissected at the 4th week and another three were dissected at the 8th week for histological staining. The β-TCP scaffolds started to degrade at 2 weeks

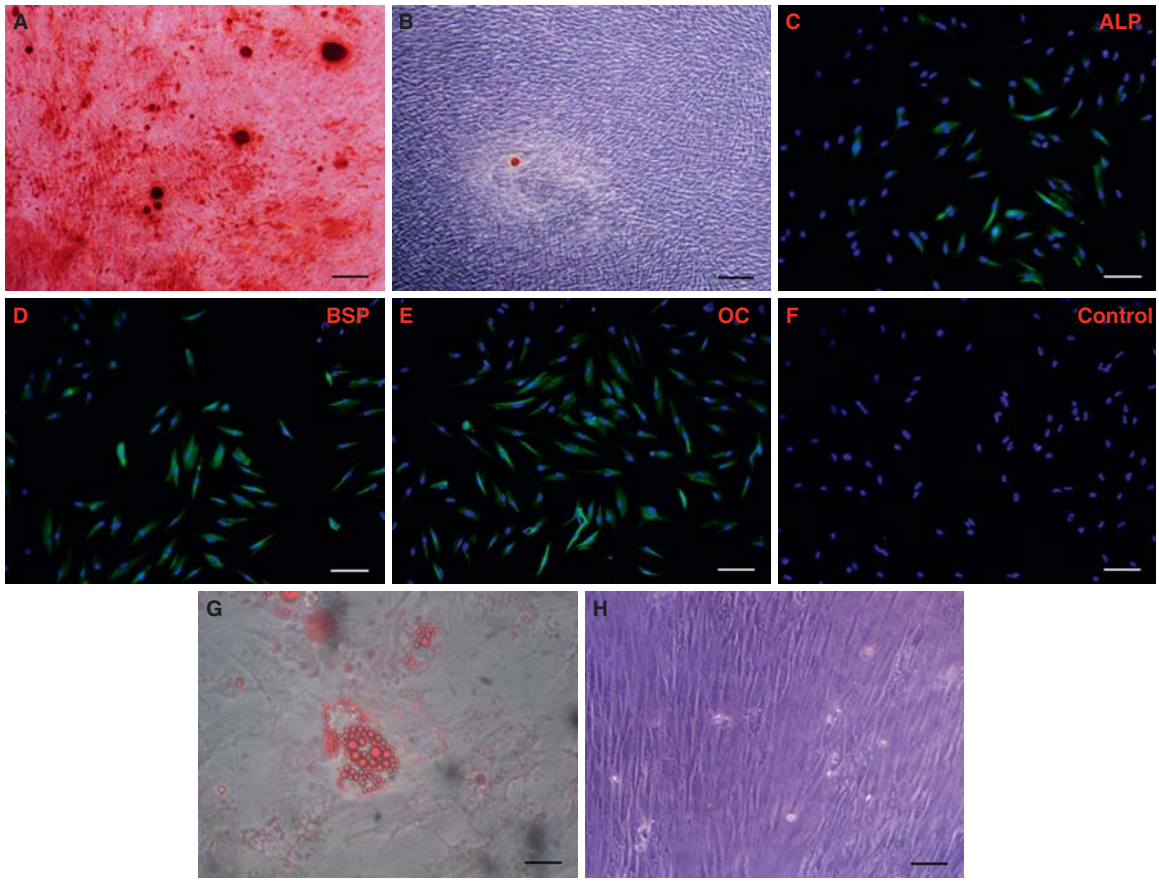

Figure 3. Odonto/osteogenic and adipogenic potential of SHED. (A) Calcium-specific Alizarin red staining of SHED after the induction of differentiation for 3 weeks. (B) No positive nodule was present in the uninduced (control) culture. (C–E) Odonto/osteogenic markers were shown by immunocytofluorescence staining. (F) No staining was detected in the uninduced (control) culture. (G) Oil Red O staining of the accumulated lipid droplets in cells. (H) No Oil Red O-positive lipid clusters appeared in the uninduced (control) culture. Scale bar: 100 μm.

post-transplantation. A few fibrous tissues were dispersedly distributed among these scaffolds (Figure 6A). More fibrous tissues were observed at 4 weeks post-transplantation (Figure 6B). No significant cartilaginification or osteogenesis was found in these two groups. However, cartilage-like tissue was observed in one of three samples (33.3%) in the experimental group after 8 weeks post-transplantation. The cartilage cells were located at the lacuna in the matrix, with an oval-shape cellular nucleus and cartilage capsule in heavy staining around the lacuna (Figure 6C). The newly formed cartilage was confirmed by type II collagen immunostaining (Figure 6D). In the control group, there was no cartilaginification or osteogenesis, but substantial fibrous tissues and few remaining scaffolds (Figure 6F).

Discussion

Cell morphology plays an important role in chondrogenesis [15] and a dramatic alteration in cell morphology was observed in our study. The majority of cells acquired a spherical morphology after 2 weeks in chondrogenic culture, which has been found to relate to the synthesis of extracellular matrix (ECM) components of cartilage [16]. Adult articular cartilage consists of a relatively sparse population of non-proliferating chondrocytes embedded within the ECM, mainly composed of two major types of macromolecules, collagens (90% of which is type II collagen) and proteoglycan (aggrecan), that together constitute 90% of the dry weight of healthy tissue [17]. Therefore, aggrecan and type II collagen were considered cartilage matrix-specific markers [18]. Aggrecan is a member of the family of large aggregating proteoglycans and binds strongly to hyaluronan [19]. It is abundantly expressed in cartilage during limb development and is maintained at high levels through adulthood in all cartilage types [19]. The expression of aggrecan and type II collagen indicated that SHED successfully differentiated into cartilage cells.

The cartilage cells express and maintain the characteristic phenotype of differentiated chondrocytes under the control of several signal molecules. Amongst them, the members of the transforming growth factor β superfamily (TGF-βs) play an important role [17]. Regarding the cartilage matrix components, their expression and synthesis can be obtained in the presence of TGF-β alone, which acts as an

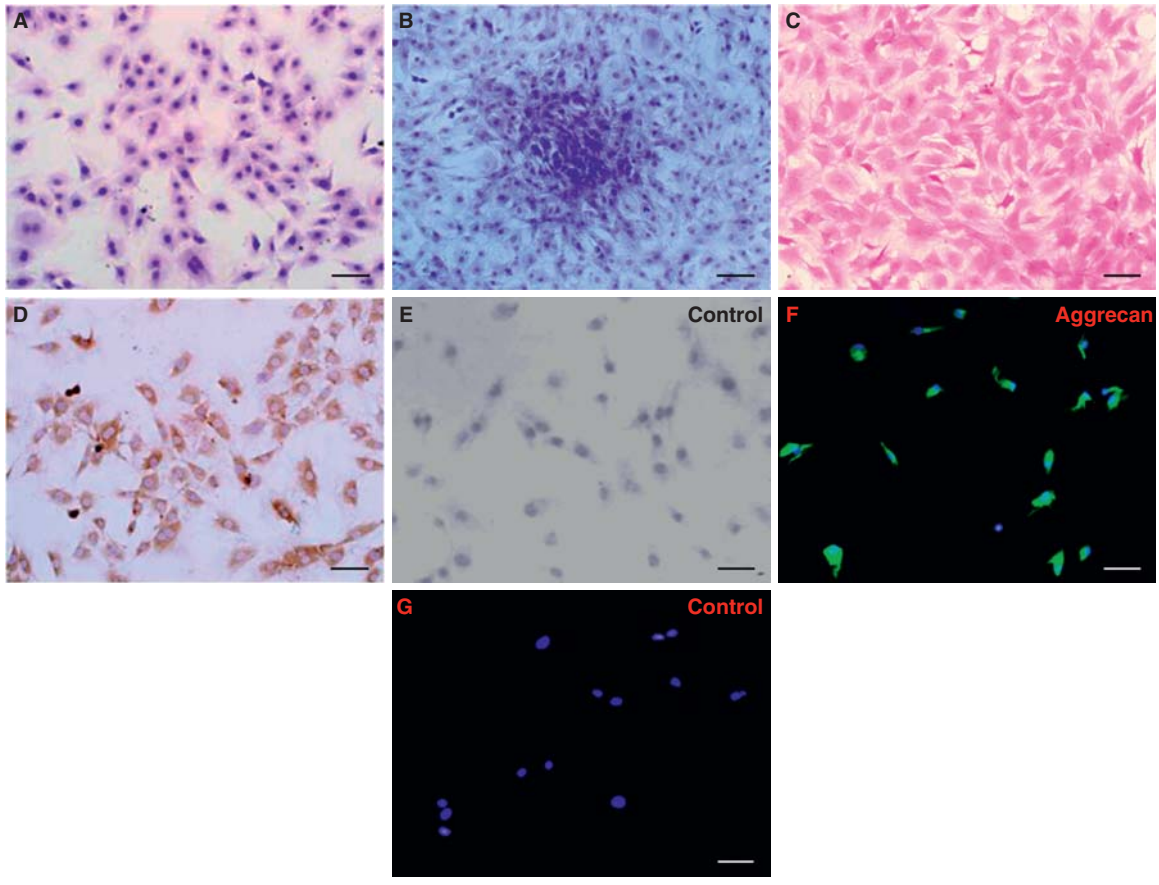

Figure 4. Chondrogenic potential of SHED. (A) H&E staining showed that SHED were induced to differentiate into chondrocytes, with a polygon appearance. (B) Toluidine blue staining was positive. (C) Safranin O staining showed positive results. (D) The induced SHED were positive for type II collagen, staining by immunocytochemistry. (F) Immunofluorescence staining showed positive expression of aggrecan. (E and G) No staining was detected in the uninduced (control) culture. Scale bar: 50 μm.

inducer of chondrogenesis of MSCs [17]. Although all three TGF-β sub-types have been shown to induce chondrogenesis, TGF-β2 and TGF-β3 are more effective than TGF-β1 in promoting chondrogenesis, causing a 2-fold greater accumulation of glycosaminoglycan and earlier and more extensive deposition of type II collagen [19]. Recently, TGF-β3 has been proposed as the initial stimulus of MSCs conversion

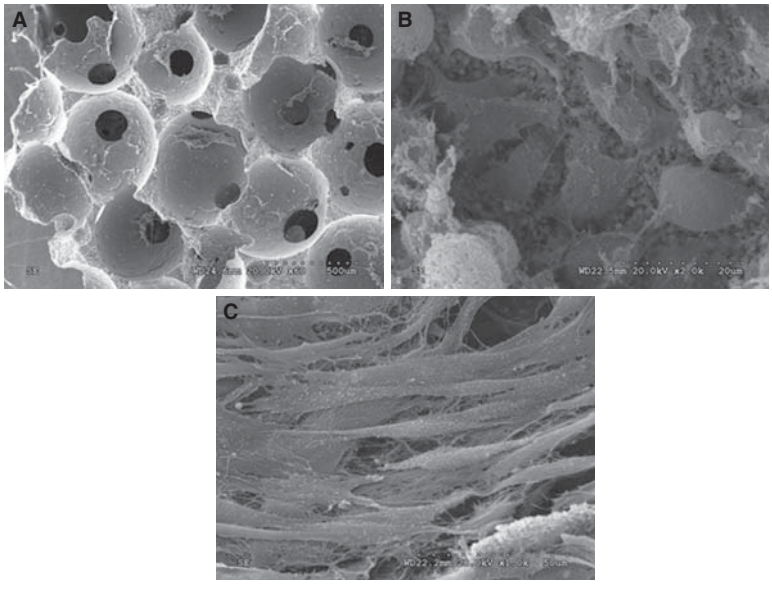

Figure 5. Cell attachment and growth in the β-TCP scaffold *in vitro*. (A) The SEM analysis showed that β-TCP scaffolds were highly porous. (B) Cells grew in a spreading way and adhered firmly to β-TCP at 4 h. (C) Rich extracellular matrix was secreted by SHED at day 7.

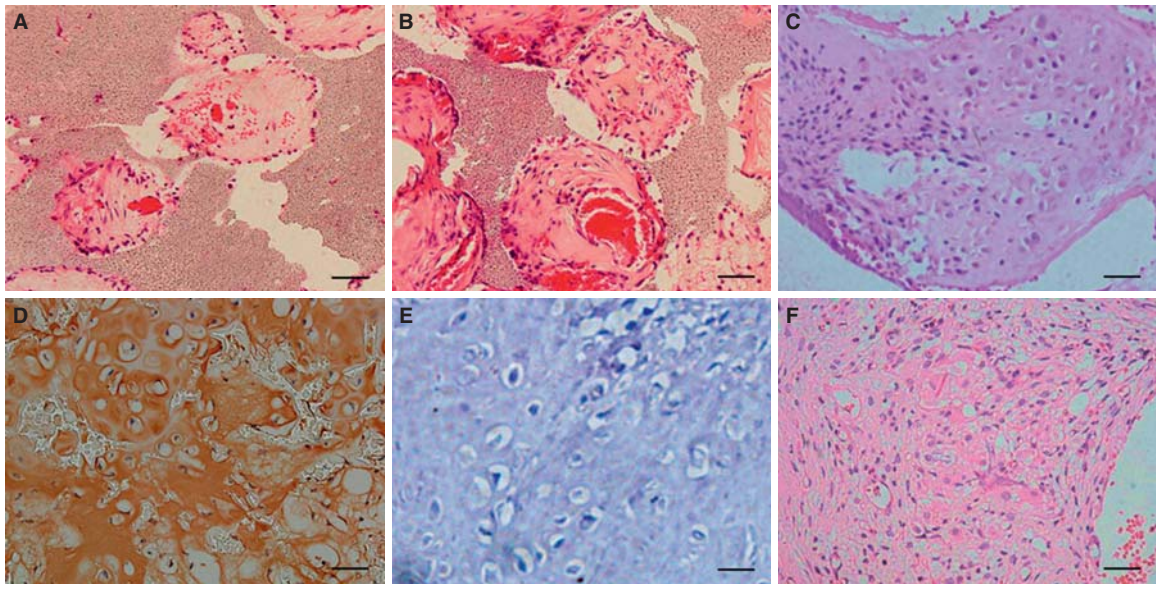

Figure 6. Histological analysis of *in vivo* SHED/β-TCP-mediated tissue regeneration. (A) The β-TCP started to degrade with a few fibrous tissues grown into it at 2 weeks post-transplantation. (B) More fibrous tissues grew into the β-TCP at 4 weeks post-transplantation. (C) Cartilage-like tissue was observed in the study group at 8 weeks post-transplantation. (D) Cartilage-like tissue showed positive staining for type II collagen. (E) No staining was detected in the negative control. (F) The β-TCP in the control group degraded and plenty of fibrous tissues grew into it at 8 weeks post-transplantation. Scale bar: 50 μm.

towards the chondrogenic lineage [20]. Dexamethasone, a synthetic glucocorticoid, stimulates chondrogenesis directly via the glucocorticoid receptor [21]. It has been reported to be a potent stimulant for chondrogenesis in horses, rabbits and bovines [22]. Furthermore, dexamethasone potentiates the chondrogenic stimulation of TGF-β [21]. Basic fibroblast growth factor (bFGF) has been used to successfully expand MSCs while maintaining their chondrogenic differentiation capacity, allowing for the production of a large number of cells suitable for tissue engineering [23]. Although the mechanism by which bFGF maintains differentiation capacity is still under investigation, recent studies have drawn correlations between bFGF-treatment and decreased telomerase activity, suggesting that bFGF may play a protective role during cell proliferation [24]. In our study, the chondrogenic culture containing these growth factors made SHED differentiate into chondrocytes in 2 weeks.

An ideal scaffold is a critical component for cell-based cartilage tissue engineering. The β-TCP scaffolds have been shown to have many desirable properties, such as performance stability, non-toxicity, good biocompatibility, reasonable internal structure design, high mechanical intensity and a good degree of finish of the inner surface, which are favourable for tissue ingrowth [25]. A biocompatible scaffold providing structural support to MSCs and facilitating their chondrogenic differentiation to form three-dimensional (3D) cartilage-like tissues is needed. The β-TCP with orifices could provide such a 3D environment that favours cell condensation and cell–cell interactions analogous to the situation that occurs during embryonic skeletal development. The SEM analysis showed that, after 4 h culture, the cells adhered to the scaffold tightly and showed uniform distribution. Therefore, to avoid cells falling off the scaffolds, SHED/β-TCP constructs were incubated for 4 h *in vitro* for better cell attachment before transplantation. Furthermore, the SEM results demonstrated that SHED on the scaffolds could secrete a rich matrix, which is important for cartilage formation. The β-TCP scaffolds gradually degraded in the nude mice, providing proper time and space for cell ingrowth and cartilage formation. Guo et al. [26] successfully repaired articular cartilage defects with implants generated by seeding autologous MSCs into β-TCP. In our study, SHED recombined with β-TCP scaffolds were able to generate cartilage-like tissues as well. Therefore, the β-TCP may be a promising scaffold for cartilage tissue engineering.

At the developmental level, there are two mechanisms by which bone can be formed: intramembranous ossification and endochondral ossification [27]. Most bones develop through a process known as endochondral ossification, the initial stage of which is the formation of a cartilage model. The cartilage model once formed is invaded first at its centre and later at each end by a mixture of cells that establish the primary and secondary centres of ossification. These centres of ossification gradually encroach on the remaining cartilage, ultimately replacing it completely by the time skeletal maturity is achieved [28]. In contrast to the endochondral bone formation that ensues from a cartilaginous template, craniofacial

skeletal elements develop by intramembranous ossification, characterized by the direct differentiation of osteogenic mesenchyme into osteoblasts [29]. In previous studies, SHED were shown to generate bone-like tissue after 8 weeks of transplantation in an animal model [10]. However, using the same model, no significant cartilaginification was found at 2 or 4 weeks post-transplantation, but cartilage of definite morphological characteristics was formed at the 8th week in our study. Due to limited time and lack of samples, only one sample generated cartilage-like tissue. Whether the newborn cartilage-like tissue keeps a stable form or is just a special manifestation of endochondral ossification still needs further study. SHED are of craniofacial origin and are supposed to undergo intramembranous ossification. If the newborn cartilage-like tissue is a manifestation of endochondral ossification, what changed the SHED ossification model? This question still needs to be further investigated. Extending the observation time with a larger quantity of samples will be the next step. Combined with 3D scaffolds and microenvironment, however, we can draw a solid conclusion that SHED can form cartilage-like tissue *in vivo*.

In summary, our study provides evidence of the chondrogenic potential of SHED. With their accessibility, SHED may be an ideal resource for cartilage tissue engineering.

Acknowledgements

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***Declaration of interest*:** The authors declare that there is no conflict of interest.

References

[1] Redman SN, Oldfield SF, Archer CW. Current strategies for articular cartilage repair. Eur Cell Mater 2005;9:23–32.

[2] Wang WG, Lou SQ, Ju XD, Xia K, Xia JH. *In vitro* chondrogenesis of human bone marrow-derived mesenchymal progenitor cells in monolayer culture: activation by transfection with TGF-beta2. Tissue Cell 2003;35:69–77.

[3] Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.

[4] Breinan HA, Minas T, Hsu HP, Nehrer S, Sledge CB, Spector M. Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. J Bone Joint Surg Am 1997;79:1439–51.

[5] Rodriguez-Lozano FJ, Insausti CL, Iniesta F, Blanquer M, Ramirez MD, Meseguer L, et al. Mesenchymal dental stem cells in regenerative dentistry. Med Oral Patol Oral Cir Bucal 2012;17:e1062–7.

[6] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.

[7] Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–4.

[8] Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 2001;3:778–84.

[9] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Proc Natl Acad Sci USA 2000;97:13625–30.

[10] Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 2003;100:5807–12.

[11] Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004;364:149–55.

[12] Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, et al. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One 2006;1:e79.

[13] Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, et al. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol 2005;24:155–65.

[14] Zheng Y, Liu Y, Zhang CM, Zhang HY, Li WH, Shi S, et al. Stem cells from deciduous tooth repair mandibular defect in swine. J Dent Res 2009;88:249–54.

[15] Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. Biochem Biophys Res Commun 2002;290:763–9.

[16] von der Mark K, Gauss V, von der Mark H, Muller P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 1977;267:531–2.

[17] Freyria AM, Mallein-Gerin F. Chondrocytes or adult stem cells for cartilage repair: the indisputable role of growth factors. Injury 2012;43:259–65.

[18] Cancedda R, Descalzi CF, Castagnola P. Chondrocyte differentiation. Int Rev Cytol 1995;159:265–358.

[19] Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001;268:189–200.

[20] Mehlhorn AT, Niemeyer P, Kaiser S, Finkenzeller G, Stark GB, Sudkamp NP, et al. Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. Tissue Eng 2006;12:2853–62.

[21] Derfoul A, Perkins GL, Hall DJ, Tuan RS. Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular matrix genes. Stem Cells 2006;24:1487–95.

[22] Csaki C, Schneider PR, Shakibaei M. Mesenchymal stem cells as a potential pool for cartilage tissue engineering. Ann Anat 2008;190:395–412.

[23] Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun 2001;288:413–19.

[24] Yanada S, Ochi M, Kojima K, Sharman P, Yasunaga Y, Hiyama E. Possibility of selection of chondrogenic progenitor cells by telomere length in FGF-2-expanded mesenchymal stromal cells. Cell Prolif 2006;39:575–84.

[25] Lu JX, Flautre B, Anselme K, Hardouin P, Gallur A, Descamps M, et al. Role of interconnections in porous bioceramics on bone recolonization *in vitro* and *in vivo*. J Mater Sci Mater Med 1999;10:111–20.

[26] Guo X, Wang C, Zhang Y, Xia R, Hu M, Duan C, et al. Repair of large articular cartilage defects with implants of autologous mesenchymal stem cells seeded into beta-tricalcium phosphate in a sheep model. Tissue Eng 2004; 10:1818–29.

[27] Jones GN, Pringle DR, Yin Z, Carlton MM, Powell KA, Weinstein MB, et al. Neural crest-specific loss of Prkar1a causes perinatal lethality resulting from defects in intramembranous ossification. Mol Endocrinol 2010;24: 1559–68.

[28] Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 2008;40:46–62.

[29] Deckelbaum RA, Majithia A, Booker T, Henderson JE, Loomis CA. The homeoprotein engrailed 1 has pleiotropic functions in calvarial intramembranous bone formation and remodeling. Development 2006;133:63–74.