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Generation and periodontal differentiation of human gingival fibroblasts-derived integration-free induced pluripotent stem cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) have been recognized as a promising cell source for periodontal tissue regeneration. However, the conventional virus-based reprogramming approach is associated with a high risk of genetic mutation and limits their therapeutic utility. Here, we successfully generated iPSCs from readily accessible human gingival fibroblasts (hGFs) through an integration-free and feeder-free approach via delivery of reprogramming factors of Oct4, Sox2, Klf4, L-myc, Lin28 and TP53 shRNA with episomal plasmid vectors. The iPSCs presented similar morphology and proliferation characteristics as embryonic stem cells (ESCs), and expressed pluripotent markers including Oct4, Tra181, Nanog and SSEA-4. Additionally, these cells maintained a normal karyotype and showed decreased CpG methylation ratio in the promoter regions of Oct4 and Nanog. *In vivo* teratoma formation assay revealed the development of tissues representative of three germ layers, confirming the acquisition of pluripotency. Furthermore, treatment of the iPSCs *in vitro* with enamel matrix derivative (EMD) or growth/differentiation factor-5 (GDF-5) significantly up-regulated the expression of periodontal tissue markers associated with bone, periodontal ligament and cementum respectively. Taken together, our data demonstrate that hGFs are a valuable cell source for generating integration-free iPSCs, which could be sequentially induced toward periodontal cells under the treatment of EMD and GDF-5.

Contents

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1. Introduction

Periodontal disease is dental plaque-induced infection and inflammation characterized by irreversible destruction of tooth supporting tissues [1]. Periodontal regeneration is extremely challenging as it requires restoration of alveolar bone, periodontal ligament (PDL) and cementum simultaneously with appropriate integration and interaction among the regenerated three types of tissues [2]. The use of traditional techniques, such as bone grafts replacement and guided tissue regeneration, has resulted in limited regeneration of periodontal tissues [3]. Therefore, novel therapeutic strategies are urgently needed, especially for complicated cases of periodontal defects. The recent advance of stem cell-based technologies has achieved great success in regenerative medicine and is likely to provide a new paradigm for periodontal regeneration, due to the multi-potential properties of the cells [3–5].

Induced pluripotent stem cells (iPSCs) are generated by introducing a group of defined reprogramming factors into somatic cells and have been recognized as an ideal cell source for regenerative therapy [6]. Akin to embryonic stem cells (ESCs) in self-renewal capability and pluripotency, iPSCs are advantageous for developing patient and tissue specific therapies, free from ethical and immune rejection concerns associated with ESCs [7,8]. Currently, iPSCs have been derived from an array of human adult somatic cells (e.g. fibroblasts, keratinocytes) [9,10]. However, the reprogramming efficiency varies among different cell types [11]. More importantly, emerging evidence has indicated that the reprogramming process prompts global epigenetic remodeling, which correlates with inherent epigenetic memory of iPSCs. The differentiation capability of iPSCs may be affected by the somatic cell sources and displays increased propensity to their original identity [6,12,13]. Therefore, a key issue of using iPSCs for periodontal regeneration is to identify optimum donor cells with oral origin and evaluate their differentiation potential into periodontal cells.

Human gingival fibroblasts (hGFs) are abundant in oral gingival connective tissues, characterized by fast proliferation and rapid turnover. Gingiva can be easily harvested by dentists with minimal discomfort to patients. Hence, hGFs represent a promising source for generation of patient specific iPSCs. It has been reported that iPSCs can be successfully generated from human and mouse GFs via genomic-insertion of reprogramming factors carried by virus vectors [14,15]. However, such reprogramming method is associated with a high risk of genome mutation and thus restricts the use of these iPSCs clinically [7,16]. Additionally, the differentiation capacity of the hGFs-derived iPSCs toward periodontal cells remains largely unknown. The development of periodontal tissues involves orchestrated dental cell lineage differentiation and an array of signaling molecules. Various biological mediators have been evaluated for their potential to mimic and enhance the periodontal development related cellular events. Enamel matrix derivative (EMD) is purified extract of enamel matrix proteins (EMPs) comprising mainly amelogenin. It has been demonstrated that EMPs are expressed during development of tooth supporting tissues and they are considered to play a pivotal role in root cementogenesis and formation of periodontal apparatus [17]. Moreover, EMD can promote cell proliferation, osteogenic differentiation and matrix formation of multiple cell types, especially PDL cells, osteoblasts and mesenchymal stem cells (MSCs) [18–20]. Emdogain, the commercialized product of EMD, has been used in periodontal therapy due to its capability of promoting bone and cementum formation in infrabony defects [18]. Growth/differentiation factor-5 (GDF-5), another type of differentiation factor, belongs to the bone morphogenetic protein family and plays an important role in the regulation of cellular differentiation and formation of bone, cartilage and ligament tissues [21]. GDF-5 expression has been associated with development of root, dental follicle and PDL [22]. Notably, GDF-5 is expressed in PDL fibers inserting into alveolar bone and cementum, indicating its critical role in development of periodontal tissues [23]. *In vivo* studies have demonstrated that GDF-5 administration can support the regeneration of periodontal tissues [21,22]. Both EMD and GDF-5 appear to have substantial potential to promote periodontal cell differentiation and tissue regeneration.

To facilitate the effective application of iPSCs in periodontal regeneration, in this study, novel iPSCs were generated from hGFs with an integration-free episomal approach, and the differentiation capacity of the hGFs-derived iPSCs into periodontal cells under EMD and GDF-5 treatment was investigated.

2. Material & methods

2.1. Cell culture

Human gingival tissues were harvested from the extracted teeth of systemically and periodontally healthy donors (18–30 years old) who sought for orthodontic therapy or third molar removal surgery with informed consent in Peking University Hospital of Stomatology. The protocols were approved by the Biomedical Ethics Committee, Peking University Hospital of Stomatology (Approval No. PKUSSIRB-201414048). hGFs were isolated by explant technique [24]. The fresh tissues were sliced into small pieces (1 mm^3) and cultured in α-Minimum Essential Media (α-MEM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA) and penicillin/streptomycin (P/S, 100 U/ml penicillin and 100 μg/ml streptomycin; Hyclone) at 37 °C with 5% CO_2. The medium was replaced every two days. After confluence of monolayer cultures, hGFs were detached with 0.25% Trypsin-EDTA (Gibco).

2.2. Generation of iPSCs

Ten micrograms of expression plasmid (Oct4, Sox2, Klf4, L-myc, Lin28 and TP53 shRNA) mixture was electroporated into the second

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to fifth passage of hGFs (5.0×10^5 cells/60 mm dish) in Nucleofector II Device (Lonza, Basel, Switzerland) under program U20 using Amaxa Fibroblasts Nucleofector kit [25]. The transfected cells were then cultured in α-MEM supplemented with 10% FBS and P/S under feeder-free conditions on 0.01% matrigel (BD Biosciences, Franklin Lakes, USA)-coated dishes. The medium was aspirated the next day and replaced with Reproeasy reprogramming culture medium (Cellapy, Beijing, China). After that the medium was refreshed every other day. Four weeks after transfection, the numbers of ESCs-like colonies were counted, and the ESCs-like colonies were picked out and expanded. Afterwards, PSCeasy culture medium (Cellapy) was used to maintain the iPSCs.

2.3. Characterization of iPSCs

2.3.1. Alkaline phosphatase (ALP) staining

ALP staining was performed according to the standard protocol of BCIP/NBT Alkaline Phosphatase Color Development Kit (ZSGB-BIO, Beijing, China). Briefly, the iPSCs were washed with PBS for three times and fixed in 4% paraformaldehyde for 15 min. Subsequently, BCIP/NBT solution was added to stain the cells for 15 min and the reaction was stopped by washing with distilled water. Images were captured under an inverted microscope (Olympus CKX41, Tokyo, Japan).

2.3.2. Immunofluorescence staining

The iPSCs were fixed in 4% paraformaldehyde for 30 min and washed with PBS for three times. Cells were then treated with 0.5% Triton X-100 for 45 min, and washed with PBS for three times. Blocking buffer (3% BSA) was transferred to immerse the cells for 1 h at room temperature. After removal of the blocking buffer, cells were incubated with primary antibodies including anti-Oct4 (Abcam, Cambridge, UK), anti-Tra181 (Chemicon, Billerica, USA), anti-Nanog (Abcam) and anti-SSEA-4 (Chemicon) overnight at room temperature. Next, samples were incubated with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, USA) or Alexa Fluor 594 (Invitrogen) for 1 h. Nuclei were stained using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). Images were captured by confocal laser scanning microscope (CLSM; Leica Microsystems CMS GmbH, Wetzlar, Germany).

2.3.3. Teratoma formation

The iPSCs (5.0×10^6 cells) were suspended in 200 μl PBS and injected subcutaneously into bilateral hind legs of 4–6 week old male nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice (Vital River, Beijing, China). All animal experiments were approved by the Biomedical Ethics Committee of Peking University (Approval No. LA 2014163). After 8 wk, tumors were harvested and fixed in 4% paraformaldehyde. The tumor samples were embedded in paraffin and processed for hematoxylin/eosin (HE) staining.

2.3.4. Karyotyping and DNA methylation analysis

Standard G-banding chromosome analysis was performed to examine the chromosomes of the iPSCs in the Cytogenetics Laboratory, Peking University. Genomic DNA from hGFs, iPSCs and ESCs was extracted using FastPure DNA kit (Takara Biomedicals, Tokyo, Japan). DNA methylation analysis was carried out at Saijie Biotechnology Co., Ltd (Shanghai, China) to evaluate the CpG methylation of Oct4 and Nanog promoters.

2.4. Directed differentiation of iPSCs toward periodontal cells

To examine the directed differentiation of the iPSCs, embryoid bodies (EBs) were formed. In brief, the iPSCs were suspended in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12, Hyclone) containing 20% FBS, 0.1 mM β-mercaptoethanol, 1 mM L-glutamine, 1% non-essential amino acid and P/S on non-coated dishes, and cultured in suspension for 5 days. Then the EBs were transferred to 0.01% matrigel-coated dishes and cultured in the following media: i) EMD medium (DMEM/F12 containing 20% FBS together with 30, 60 or 90 μg/ml of Emdogain (Straumann, Basel, Switzerland)); ii) GDF-5 medium (DMEM/F12 containing 20% FBS together with 100, 200 or 400 ng/ml of recombinant human GDF-5 (Pepro Tech, Rocky Hill, NJ)). The EBs cultured in DMEM/F12 containing 20% FBS were used as controls. The media were replenished every other day.

2.4.1. Quantitative real-time PCR (qRT-PCR)

After 14 days of incubation, qRT-PCR was used to examine the expression of genes representative of bone (osteocalcin (OCN) and bone sialoprotein (BSP)), PDL connective tissues (periostin and vimentin) and cementum (cementum attachment protein (CAP) and cementum protein 1 (CEMP1)) [26–28]. Total RNA was extracted using RNAeasy kit (Qiagen, Hilden, Germany) and cDNA was synthesized using PrimeScript® II 1st strand cDNA synthesis kit (Takara Biomedicals). qRT-PCR was performed in an Mx3000P qPCR System (Agilent Technologies, Santa Clara, USA) using SYBR Premix Ex Taq II reagent (Takara Biomedicals). Relative quantification of gene expression was assessed with the comparative cycle threshold (Ct) method, using GAPDH as internal control. The primer sequences are listed in Table 1.

2.4.2. Immunofluorescence staining

After 14 days of incubation, immunofluorescence staining was used to evaluate the protein expression of OCN, periostin and CAP. The cells were stained with primary antibodies against OCN (Abcam), periostin (Abcam) or CAP (Santa Cruz, Dallas, USA) followed by secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594. Nuclei were stained using Vectashield Mounting Medium with DAPI. Images were captured under CLSM.

2.5. Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD) and analyzed by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Table 1
List of primer sequences used for qRT-PCR.

Primer	Sequence
GAPDH	Forward: 5′-AGGTCGGTGTGAACGGATTTG-3′
	Reverse: 5′-TGTAGACCATGTAGTTGAGGTCA-3′
OCN	Forward: 5′-GCGCTCTGTCTCTCTGACCT-3′
	Reverse: 5′-GCCGGAGTCTGTTCACTACC-3′
BSP	Forward: 5′-CAGGGAGGCAGTGACTCTTC-3′
	Reverse: 5′-AGTGTGGAAAGTGTGGCGTT-3′
Periostin	Forward: 5′-TGGAGAAAGGGAGTAAGCAAGG-3′
	Reverse: 5′-TTCAAGTAGGCTGAGGAAGGTG-3′
Vimentin	Forward: 5′-GGGACCTCTACGAGGAGGAG-3′
	Reverse: 5′-CTGTCCTACAACTGTTACGC-3′
CAP	Forward: 5′-CTGCGCGCTGCACATGG-3′
	Reverse: 5′-GCGATGTCGTAGAAGGTGAGCC-3′
CEMP1	Forward: 5′-GGGCACATCAAGCACTGACAG-3′
	Reverse: 5′-CCCTTAGGAAGTGGCTGTCCAG-3′

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3. Results

3.1. Generation of hGFs-derived iPSCs

The spindle-shaped hGFs isolated from gingival tissues of donors were shown in Fig. 1A. Three weeks after episomal transfection of the hGFs from the three donors, small ESCs-like colonies emerged and were further propagated to establish hGFs-derived iPSCs clones. Fig. 1A showed a representative iPSC colony. In our experiments, around 20 ESCs-like colonies per 1.0×10^5 hGFs were obtained, therefore the calculated reprogramming efficiency was approximately 0.02%.

3.2. Characteristics of hGFs-derived iPSCs

The iPSCs clones maintained morphology and proliferation characteristic of ESCs after passaging. All colonies exhibited positive ALP staining (Fig. 1B). The cells were then examined for expression of pluripotency markers by immunofluorescence staining, which showed strong expression of Oct4, Tra181, Nanog

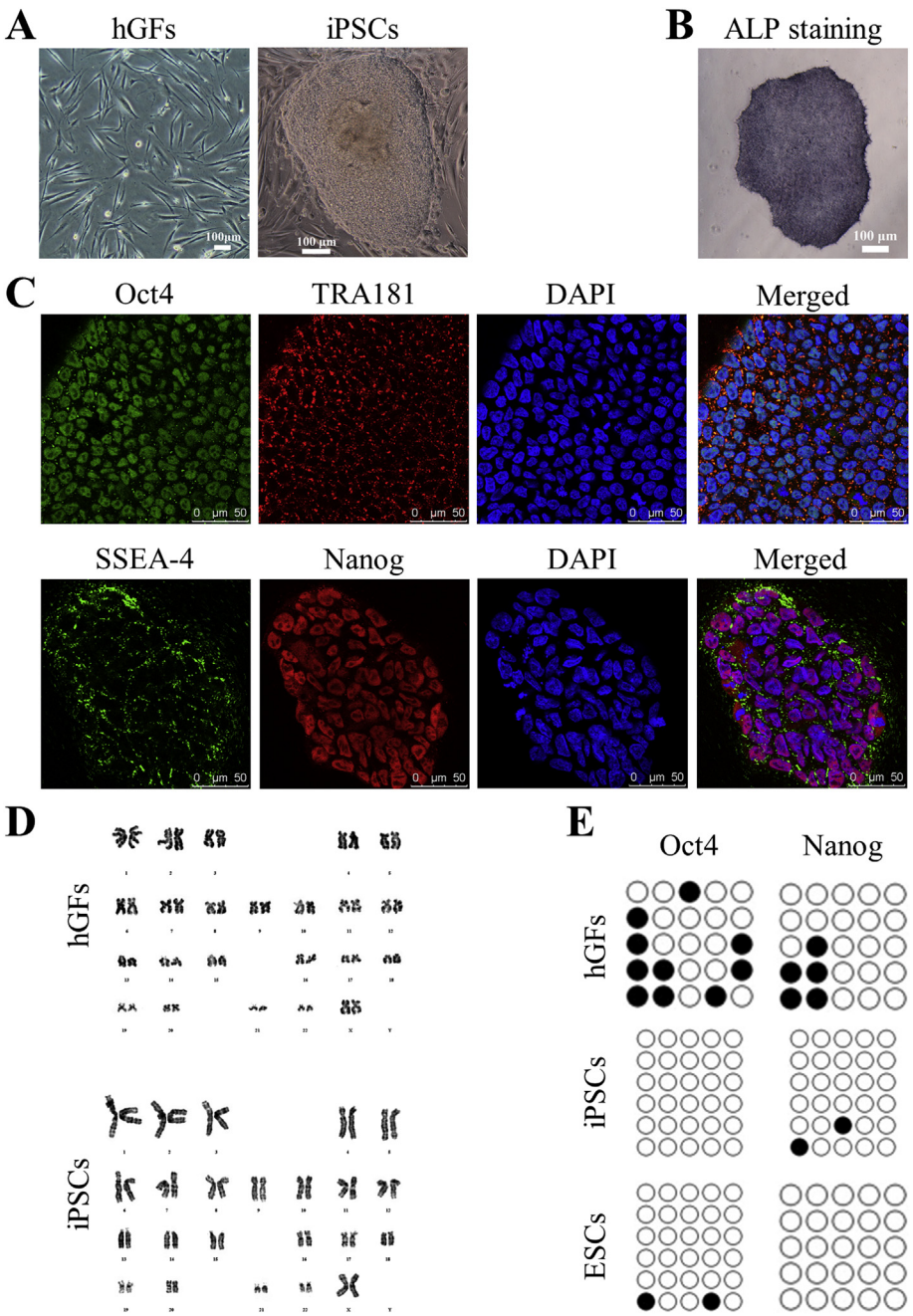

Fig. 1. Characterization of the integration-free iPSCs generated from hGFs. (A) Morphology of the parental hGFs in monolayer culture, and iPSCs at day 21 after transfection. (B) Positive ALP staining of the undifferentiated iPSCs. Scale bar, 100 μm. (C) Immunofluorescence staining of pluripotency markers of nuclear proteins Oct4 and Nanog, and surface antigens Tra181 and SSEA4. Nuclei were counterstained with DAPI in blue. Scale bar, 50 μm. (D) Chromosomal analysis using G-band demonstrated a normal karyotype in the iPSCs. (E) The CpG methylation ratios in the promoter regions of Oct4 and Nanog in the iPSCs were down-regulated compared to hGFs, and resembled those observed in human ESCs, confirming a demethylated state of the pluripotency genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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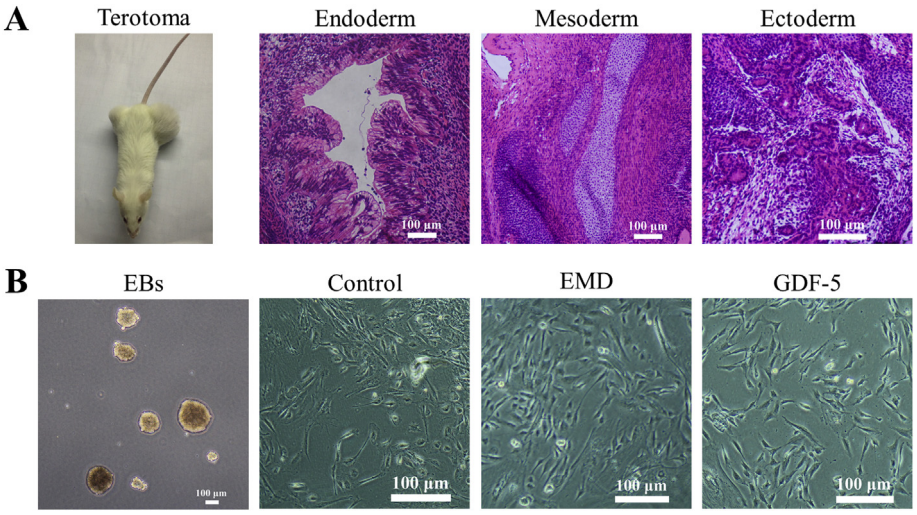

Fig. 2. Differentiation of the hGFs-derived iPSCs *in vivo* and *in vitro*. (A) Transplantation of the iPSCs gave rise to evident teratoma formation in NOD-SCID mice at week 8. HE staing of the teratoma samples showed formation of various types of tissues, including gut epithelium (endoderm), cartilage (mesoderm) and neural rosette (ectoderm). (B) *In vitro* spherical EBs formed 5 d after floating culture of the iPSCs. EMD and GDF-5 were then used to induce the directed differentiation of the iPSCs. After 14 d of treatment, the morphology of the iPSCs switched into a fibroblast-like shape. Scale bar, 100 μm.

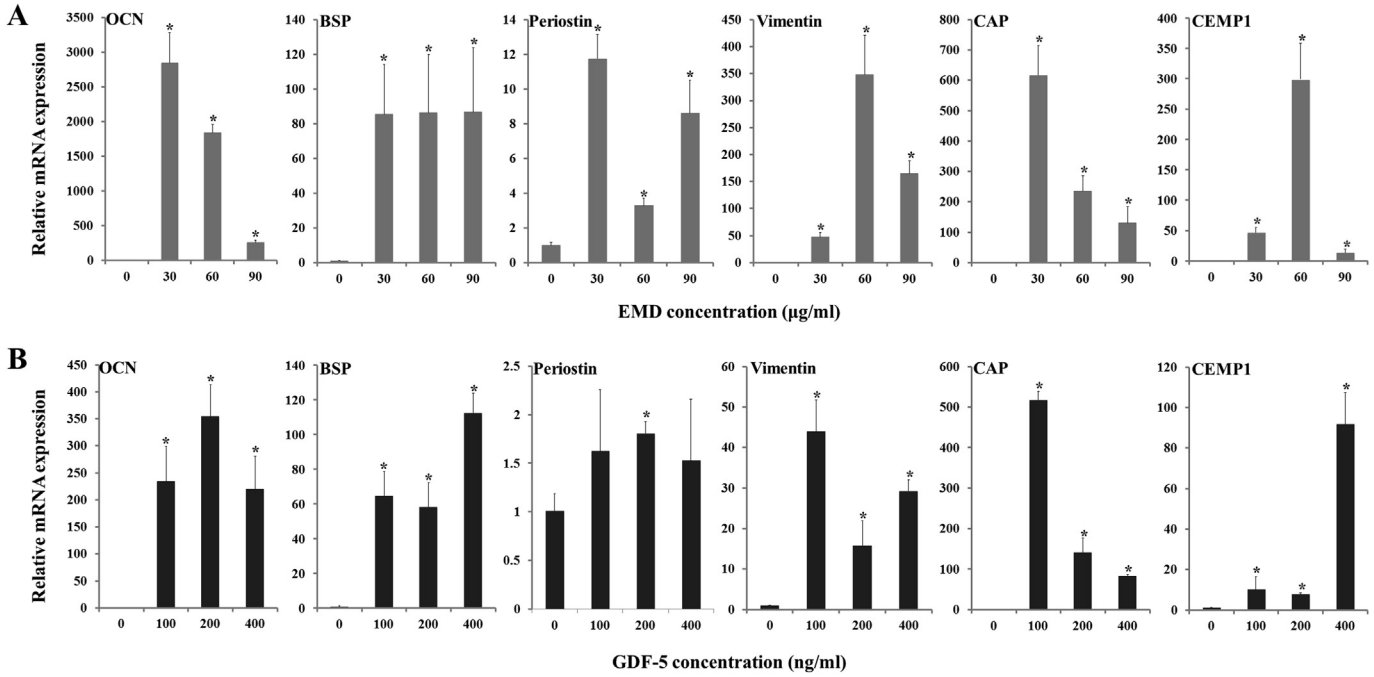

Fig. 3. qRT-PCR analysis of periodontal tissue specific markers in the hGFs-derived iPSCs with EMD and GDF-5 treatments. The relative expression of genes associated with bone (OCN and BSP), PDL (periostin and vimentin) and cementum (CAP and CEMP1) in the iPSCs treated with different concentrations of EMD (A) and GDF-5 (B) at day 14. (*$p < 0.05$ compared to untreated control).

and SSEA-4 (Fig. 1C). Chromosomal G-band analysis confirmed that the hGFs-derived iPSCs exhibited a normal karyotype (Fig. 1D). The CpG methylation ratios in the Oct4 and Nanog promoter regions of the iPSCs were 0 and 6.7% respectively, which were comparable to low ratios of 6.7% and 0 in human ESCs. In contrast, the ratios were 40% and 20% respectively in hGFs prior to reprogramming (Fig. 1E). The spontaneous differentiation of the iPSCs *in vivo* was evaluated by teratoma formation. There was evident formation of teratomas in NOD-SCID mice 8 wk after injection of the iPSCs. H&E staining of the teratomas sections showed tissues representative of three germ layers, including gut epithelium (endoderm), cartilage (mesoderm) and neural rosette (ectoderm) (Fig. 2A).

3.3. Induced differentiation of hGFs-derived iPSCs toward periodontal cells

Next, we assessed the dose-dependent effects of EMD and GDF-5 on periodontal differentiation of the iPSCs. After 5 days of floating cultivation, the iPSCs formed spherical EBs *in vitro* (Fig. 2B). After 2 days of attachment in petri-dish, the cells were treated with EMD or GDF-5. The morphology of the differentiated cells switched into a fibroblast-like shape at day 14 (Fig. 2B). The 14 d treatments of EMD

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at 30, 60 and 90 μg/ml significantly enhanced the expression of genes indicative of bone (OCN and BSP), PDL (periostin and vimentin) and cementum (CAP and CEMP1), compared to untreated control ($p < 0.05$). With the increased EMD concentrations, the inductive effect on the expression of OCN and CAP was reduced, whereas no significant difference was observed for BSP and periostin expression. The induction of vimentin and CEMP1 reached the peak when treated with 60 μg/ml of EMD (Fig. 3A). Similarly, the treatment with GDF-5 at 100, 200 and 400 ng/ml for 14 d showed significantly higher expression of OCN, BSP, vimentin, CAP and CEMP1, compared to untreated control ($p < 0.05$). The expression of periostin was slightly up-regulated by GDF-5 treatment only at 200 ng/ml ($p < 0.05$). The increase of GDF-5 concentrations augmented the expression level of BSP and CEMP1, while reduced CAP expression. The OCN expression displayed the highest level when treated with 200 ng/ml of GDF-5 (Fig. 3B).

Immunofluorescence staining confirmed that the iPSCs treated with EMD (60 μg/ml) or GDF-5 (200 ng/ml) for 14 d presented strong expression of OCN and CAP, compared to negligible staining of untreated controls (Fig. 4A, C). However, positive staining of periostin was observed in both the treated groups and untreated control (Fig. 4B), which is consistent with the observed gene expression profile.

4. Discussion

The destructive periodontal disease has given rise to a significant healthcare burden globally, making development of more effective regenerative therapeutics a pressing issue for dentists and researchers. Stem cell therapy is expected to be a promising strategy for periodontal regeneration [29]. In particular, after the rollout of iPSCs, extensive studies have been focused on their potential to generate patient specific stem cells and clinical utility in regenerative medicine. It has been recently reported that the transplantation of non-odontogenic iPSCs and iPSCs-derived MSCs could promote tissue regeneration in rat periodontal fenestration defect models [30,31]. Nevertheless, there are still many obstacles to be addressed before the application of iPSCs in periodontal regeneration. The safety issues of reprogramming approaches and epigenetic memory of donor cells are important considerations. The genomic integration of exogenous genes by viral vectors has been the most commonly used reprogramming method, whereas it may cause insertional mutagenic effect and thus not suitable for clinical application [32]. Virus-based iPSCs reprogramming have been used to generate iPSCs from hGFs and PDL fibroblasts [14,15,33]. To avoid the risk of viral genome integration and associated genotoxicity, in this study, novel integration-free hGFs-derived iPSCs were successfully established through delivery of reprogramming factors with episomal plasmid vectors. Meanwhile, feeder-free culture conditions were adopted during the generation and maintenance of the iPSCs to avoid contamination of animal components. The reprogramming efficiency was comparable to those of iPSCs generated from human dermal fibroblasts with similar episomal approach reported by Okita and co-workers [25]. The newly generated iPSCs from hGFs displayed a normal karyotype and acquired hallmarks of pluripotent cells, including expression of typical ESCs markers and ESCs-like DNA demethylation pattern. The teratoma formation assay demonstrated the capability of the iPSCs to give rise to all three germ layers *in vivo*.

To facilitate the repair of periodontal defect with iPSCs, it is imperative to direct and control the differentiation of the pluripotent cells into functional periodontal cell types that enable further formation of periodontal tissues. We investigated the effect of EMD and GDF-5 treatment on the directed differentiation of iPSCs. The results indicate that both EMD and GDF-5 can induce the differentiation of the iPSCs toward periodontal cells. After 14 d of treatment, EMD and GDF-5 could substantially up-regulate the mRNA expression level of the key markers associated with bone

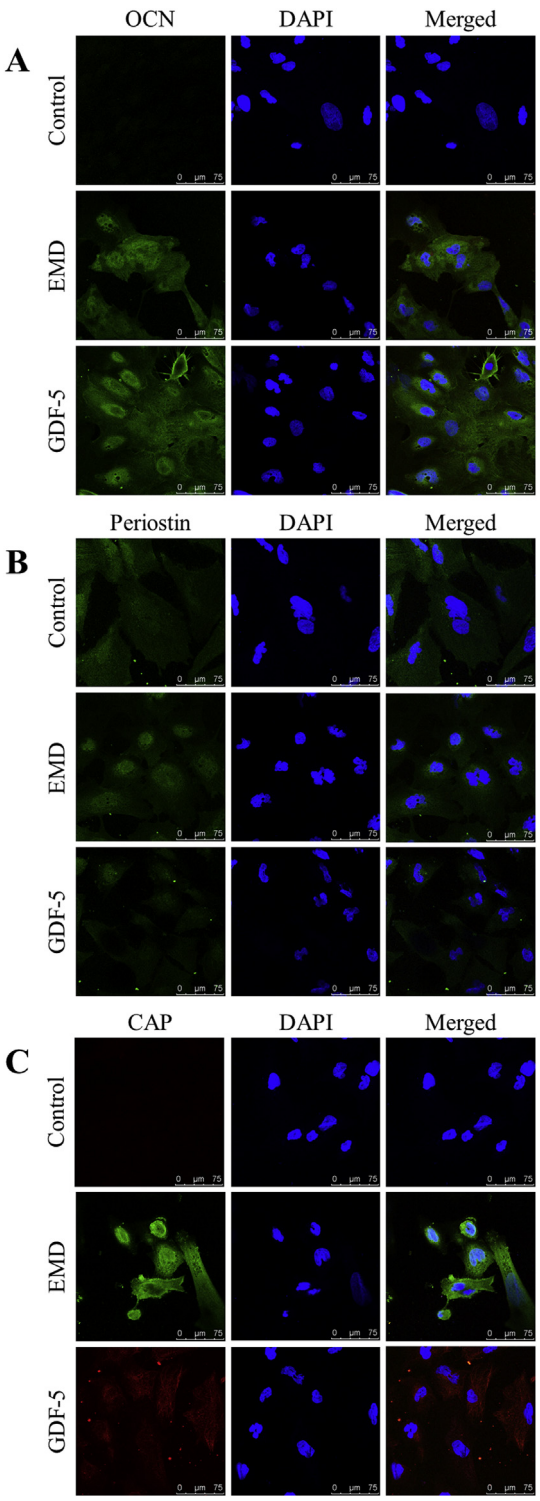

Fig. 4. Confocal images displaying the expression of periodontal tissue specific markers in the hGFs-derived iPSCs treated with EMD (60 μg/ml) or GDF-5 (200 ng/ml) for 14 d. (A) Increased expression of bone marker OCN in EMD or GDF-5 treated iPSCs compared to untreated control. (B) Positive staining of PDL marker periostin in EMD or GDF-5 treated iPSCs and untreated control. (C) Increased expression of cementum marker CAP in EMD or GDF-5 treated iPSCs compared to untreated control. DAPI (blue) was used to stain nuclei. Scale bar, 75 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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(OCN and BSP), PDL (vimentin) and cementum (CAP and CEMP1), and moderately enhance the expression level of PDL marker periostin. Previous studies have reported that EMD can enhance osteogenic differentiation of iPSCs and MSCs by activating expression of bone-associated genes [19,30,34]. Likewise, GDF-5 has been shown to induce osteogenic differentiation of MSCs [21]. Our study further demonstrated that EMD and GDF-5 can induce PDL and cementogenic differentiation of the iPSCs. Therefore, these two factors are promising in inducing the complicated periodontal cellular differentiation and tissue formation, although the optimized formulations remain to be explored.

Taken together, our study has successfully established novel iPSCs from hGFs via the integration-free episomal approach under feeder-free conditions. EMD and GDF-5 may serve as promising candidate factors for directing the differentiation of the iPSCs into periodontal cells. These findings suggest that hGFs represent a practical starting cell source for generating patient and tissue-specific integration-free pluripotent stem cells, and would further promote the clinical application of iPSCs in periodontal tissue regeneration.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.10.012.

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