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ORIGINAL PAPER

Generation of periodontal ligament stem cells from human iPSCs with a chemically defined condition

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Abstract

Human periodontal ligament stem cells (PDLSCs) play an important role in periodontal tissue regeneration. The generation of PDLSCs from human induced pluripotent stem cells (iPSCs) by simulating the development pattern of PDLSCs in vivo provided a new way to obtain a large and stable source of PDLSCs. However, animal-derived components were still necessary for current differentiation protocols, which could cause safety and ethical problems and hinder the clinical application of iPSCs-derived PDLSCs. Here, we established a novel protocol to induce iPSCs into PDLSCs by chemically defined conditions. We first induced iPSCs into neural crest-like cells by inhibiting TGF-β pathway, BMP pathway and Notch pathway using SB431542, LDN and DAPT, respectively. The iPSC-induced neural crest-like cells were further cultured in chemically defined medium containing recombinant human bFGF as well as the rho-associated protein kinase inhibitor Y27632 to generate PDLSCs. The characteristics of iPSCs-derived PDLSCs and the bi-potentiality of osteogenesis and adipogenesis differentiation were verified in vitro. The establishment of the chemically defined differentiation system breaks through the limitation brought from animal-derived components and enables us to obtain a large number of PDLSCs, which holds a significant value to the research and treatment of periodontal diseases.

Keywords Periodontal ligament stem cells · Induced pluripotent stem cells · Chemically defined differentiation system

Introduction

Periodontitis is a worldwide common disease related to the interaction between oral bacteria and host immunity, which can lead to irreversible destruction of periodontal tissues such as gingiva, periodontal ligament and alveolar bone and ultimately lead to tooth loss. Periodontitis is also widely reported to be associated with systemic diseases [2, 5]. The goal of periodontal treatment is to control inflammation, block disease progression and restore the appearance and function of periodontal tissue. Traditional periodontal basic therapy is competent to control inflammation and block disease progression. However, there is no good treatment to regenerate the periodontal tissue. Clinical experts have made many important attempts in tissue regeneration, such as tissue regeneration/guided bone regeneration (GTR/GBR) and growth factor-based therapy [8, 27]. However, the clinical prognosis is not ideal, so it is important to explore more effective tissue repair methods.

Previous studies have shown that periodontal ligament contains multifunctional stem cells, which were named periodontal ligament stem cells (PDLSCs). PDLSCs can differentiate into osteoblasts and cementoblasts in vivo and adipocytes in vitro and take part in the formation of cementum/periodontal ligament-like tissues and the regeneration of bone, cartilage and nerves [14, 17, 28]. PDLSCs are often functionally disordered under inflammation circumstance in periodontitis patients, and the loss of periodontal tissue directly reduces the number of PDLSCs. Therefore, PDLSCs were essential for the basic research and even the repair or transplantation of PDLSCs to study and treat periodontal diseases [4, 11]. However, PDLSCs are mostly isolated from extracted teeth, which limits the number of cells and exhibits difference in gene background. Furthermore, allotransplantation and xenotransplantation may cause serious safety and ethical problems. Therefore, it is important to find safer and more stable sources of PDLSCs.

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Human embryonic stem cells (hESCs) are cells isolated in the early embryonic stage with the ability of stable self-renew and multi-lineage differentiation [15, 25]. The differentiation potential of hESCs to PDLSCs has been proved [6, 10]. However, the application of embryonic-derived cells and the addition of animal-derived components in the differentiation system bring severe safety and ethical issues [19]. In 2006, Yamanaka group found that over-expression of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) in mouse fibroblasts could enable fibroblasts to acquire embryonic stem cell properties and turn them into mouse induced pluripotent stem cells (iPSCs) [24]. In 2007, Yamanaka group reported the generation of human iPSCs from human fibroblasts with the same four transcription factors, and the human iPSCs had indistinguishable proliferation and differentiation ability compared with hESCs [23]. iPSCs overcame the ethical problem of the embryonic stem cells, and the functional cells derived from iPSCs could be used in clinical application [16, 20]. In 2018, Hidefumi Maeda team successfully induced iPSCs into PDLSCs. However, the method used a large number of animal-derived components such as animal serum resulting in the instability of the differentiation system [6]. These uncertain components may also cause the spread of viruses, mycoplasma and prions [19]. Therefore, it is of great value to establish a differentiation system inducing iPSCs into PDLSCs with chemically defined condition for future clinical and research application. Here, we established a chemically defined strategy to differentiate iPSCs to PDLSCs, which did not include any serum or composition with non-determinacy. This system enabled us to obtain applicable PDLSCs, which held great values in the research and treatment of periodontal diseases.

Materials and methods

Cell culture and induction

iPSCs were generated according to previous report using Epi5™ Episomal iPSC Reprogramming Kit (Invitrogen) [22, 23]. Briefly, human embryonic fibroblasts (HEFs) (Beijing ZhongKeZhiJian Biotechnology) were cultured in DMEM with 10% FBS (Gibco) on Geltrex™ matrix-coated dishes until 75–90% confluent on the day of transfection. 10 µL HEFs (10^7 cells/ml) were transfected with 1 µL Epi5™ Reprogramming Vectors (Invitrogen) and 1 µL Epi5™ p53 and EBNA Vectors (Invitrogen) using the Neon™ Transfection System (Neon™ Transfection kits) according to the protocol from manufacturer (Invitrogen). Transfected HEFs were further cultured on Geltrex™ matrix-coated dishes with DMEM supplemented with 10% FBS (Gibco) overnight. On the next day, transfected HEFs were cultured in N2B27 medium (DMEM/F12 supplemented with 1% N2, 2% B27, 1% MEM Non-essential Amino Acids, 1% GlutaMAX, 100 µM β-Mercaptoethanol and 10 µg/mL bFGF). After 14-day culture in N2B27 medium, cells were further cultured in TeSR™1 medium, and iPSCs clones were selected, transferred and proliferated within 15–21 days of transfection. All medium were replaced every day. iPSCs were passaged every 3–4 days. When cells are confluent approximately 80% of the well, iPSCs were digested with EDTA in 37 °C in incubator for 10 min. EDTA were then discarded, and TeSR™1 were added to resuspend the cells. iPSCs were passaged at a ratio of 1:8–1:10 to matrigel-coated plates. Medium were changed every day.

To initiate the differentiation of neural crest-like cells, iPSCs were digested with EDTA into single cell and plated into matrigel-coated 6-well-plates at a ratio of 1:6. After cultured in TeSR™1 for 1 day, iPSCs were cultured in Neurobasal medium (Gibco) for 6 days supplemented with ITS—X (100X, Gibco), MEM Non-Essential Amino Acids Solution (100X, Gibco), GlutaMAX (100X, Gibco), penicillin/streptomycin (PS, Gibco), 200 µM 2-phospho-L-ascorbic acid, 10 µM SB431542 (4-(5-benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1 h-imidazol-2-yl)-benzamide) (Selleck), 0.5 µM LDN193189 (4-{6-[4-(1-Piperazinyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl}quinoline) (Selleck) and 5 µM DAPT (*N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (Selleck). After the generation of neural crest-like cells, medium was changed to Neurobasal medium (Gibco) supplemented with penicillin/streptomycin (PS, Gibco), ITS–X (100X, Gibco), recombinant human bFGF (Origene) and Y27632 ((R)-(+)-trans-4-(1-Aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride) (Selleck) for 2 weeks to generate iPSC-PDLSCs.

Osteoblastic differentiation of iPSC-PDLSCs

iPSC-PDLSCs were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibco), 0.1 µM dexamethasone (Sigma), 200 µM 2-phospho-L-ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) for 4 weeks. To further identify the character of the differentiated cells, cells were lysed in TRIzol for RT-QPCR or fixed in 4% formaldehyde for alizarin red staining. The alizarin red staining was proceeded according to the protocol from manufacturer. Briefly, the alizarin red was dissolved in DPBS (no calcium, no magnesium, Gibco) in a final concentration of 0.1% and incubated with cells for 30 min, then discarded the solution, washed with PBS 3 times and took photographs.

Adipogenic differentiation of iPSC-PDLSCs

iPSC-PDLSCs were cultured in adipogenic induction medium (Cyagen) according to the protocol from

manufacturer. Briefly, cells were cultured in Adipogenic differentiation medium A (Cyagen) for 3 days and then in Adipogenic differentiation medium B (Cyagen) for another 1 day. Medium was changed periodically between medium A (3 days) and medium B (1 day) for 4 weeks. To further identify the character of the differentiated cells, cells were lysed in TRIzol for RT-QPCR or fixed in 4% formaldehyde for oil red staining. The oil red staining was proceeded according to the protocol from manufacturer. Briefly, oil red working solution was prepared by mixing oil red stock solution with water in a ratio of 3:2. Differentiated cells were incubated with oil red working solution for 30 min.

RT-QPCR assay

Total RNA was extracted by the TRIzol-chloroform (TRIzol; Invitrogen) method. Reverse transcription of total RNA into cDNA was performed using PrimeScript RT reagent Kit (Takara). SYBR Green PCR Master mix and RT-PCR detection system (Roche Diagnostics, Basel, Switzerland) were used and RPL13A as internal reference, the expression of related genes was detected and analyzed. The sequence of related primers is shown in Table 1. The reaction conditions were 94 °C 3 min; 35 cycles of 95 °C 10 s and 57 °C annealing for 30 s.

Statistical analysis

The statistical results for the purpose of group comparisons were calculated using one-way ANOVA followed by Tukey's multiple comparisons test or Student's *t* test (independent samples t test). The level of significance in all graphs is represented as follows: $*p<0.05$, $**p<0.01$, $***p<0.001$. Unless described otherwise, standard statistical analyses were performed with SPSS, GraphPad Prism 7 and Microsoft Excel using default parameters. All of the error bars represent SD. N represents the number of biological replicates.

Results

To establish a chemically defined protocol to generate human periodontal ligament stem cells (PDLSCs) from iPSCs, we first generated induced pluripotent stem cells (iPSCs) in vitro. iPSCs were generated from human embryonic fibroblasts with Epi5™ Episomal iPSC Reprogramming Kit and cultured in commercial TeSR™1 culture system, which was chemically defined and could maintain iPSCs without feeder layer cells. To identify these iPSCs, cells were first evaluated with immunofluorescence, and we detected the expression of *NANOG* and *OCT4* in these clones (Fig. 1a). Furthermore, RT-QPCR showed that the key pluripotent stem cell genes, including *NANOG* ($t(1)=9.278$, $p=0.068$), *OCT4* ($t(1)=21.324$, $p=0.030$), *SOX2* ($t(1.4)=240.9$, $p<0.001$) were up-regulated compared with HEFs analyzed by Student's *T* test (Fig. 1b). These results indicated the human iPSCs generated from HEFs acquired induced pluripotent stem cell identity.

Generation of neural crest-like cells from iPSCs

The evidence of the lineage differentiation system of pluripotent stem cells is mainly based on the developmental clues of humans and vertebrates. PDLSCs are finally obtained by adding small molecules to the iPSCs to simulate the developmental pathway in vivo. The first stage of PDLSCs development is to generate neural crest cells, which highly expressed *p75NTR* [10].

To generate neural crest cells from iPSCs with a chemically defined condition, we first selected the small molecules as well as chemically defined supplements to induce the robust expression of *p75NTR*. Several singling pathways have been found to be able to affect the generation of neural crest cells from iPSCs or ESCs, including TGF-β pathway, BMP pathway and Notch pathway [6, 18, 21]. We found that

Table 1 Primer sequences for human p75NTR, NANOG, OCT4, COL1, OPG, POSTN, FBN1, BMP2, CEBPA and RPL13A

Gene	Abbreviation	Primer sequence (5′–3′) forward	Primer sequence (5′–3′) reverse
Nerve growth factor receptor	p75NTR	CCGAGGCACCACCGACAACC	TGCTTGCAGCTGTTCCACCTCT
Nanog homeobox	NANOG	TCCAACATCCTGAACCTCAGCTA	AGGTTCCCAGTCGGGTTCAC
POU class 5 homeobox 1	OCT4	GCAATTTGCCAAGCTCCTGAA	GCAGATGGTCGTTTGGCTGA
SRY-box transcription factor 2	SOX2	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT
Collagen type I alpha 1 chain	COL1	CCCGGGTTTCAGAGACAACTTC	TCCACATGCTTTATTCCAGCAATC
TNF receptor superfamily member 11b	OPG	TGGCACCAAAGTAAACGCAGAG	CTCGAAGGTGAGGTTAGCATGTC
Periostin	POSTN	CATTGATGGAGTGCCTGTGGA	CAATGAATTTGGTGACCTTGGTG
Fibrillin 1	FBN1	AAGTGATCCACTGTGTGCCAACTC	TGCACCTATGGAACCATGTGATAG
Bone morphogenetic protein 2	BMP2	TCCACTAATCATGCCATTGTTCAGA	GGGACACAGCATGCCTTAGGA
CCAAT enhancer binding protein alpha	CEBPA	ACAAGAACAGCAACGAGTACCG	CATTGTCACTGGTCAGCTCCA
Ribosomal protein L13a	RPL13A	AGATGGCGGAGGTGCAG	GGCCCAGCAGTACCTGTTTA

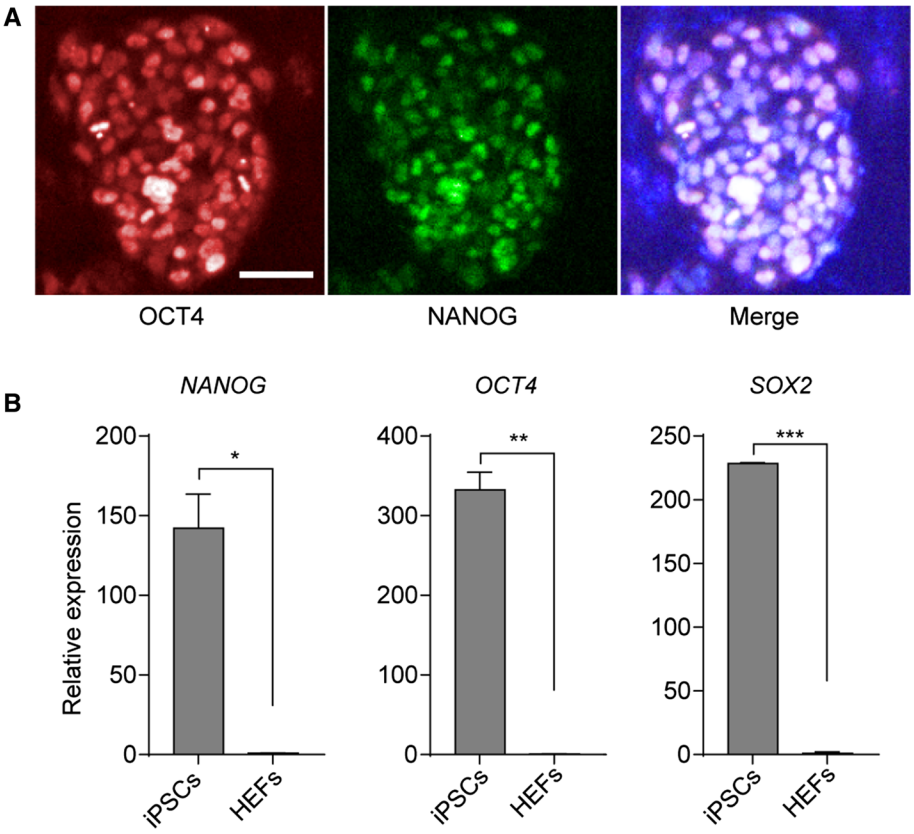
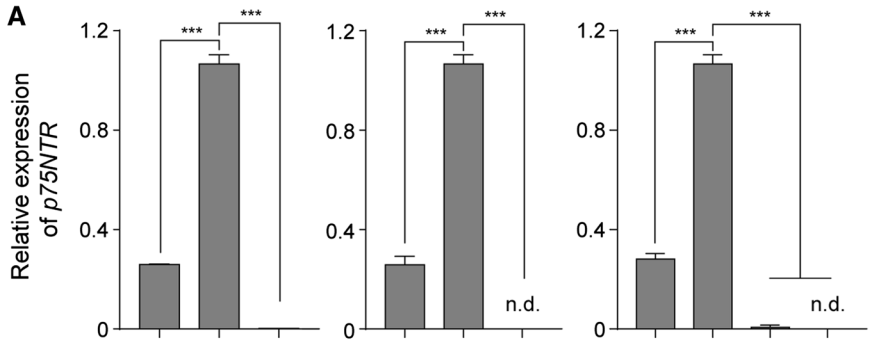


Fig. 1 Generation of iPSCs in vitro. **a** Immunofluorescence analysis of the expression of NANOG and SOX2 in iPSCs generated from HEFs. **b** The expression of *NANOG*, *SOX2* and *OCT4* in iPSCs compared with HEFs. Data are normalized to HEFs and housekeeping gene and are presented as Mean ± SD. Statistical difference is shown as $*p<0.05$, $**p<0.01$, $***p<0.001$. $N=2$

the combination of SB431542 (TGF-β pathway inhibitor), LDN193189 (BMP pathway inhibitor) and DAPT (Notch pathway inhibitor) combined with the chemically defined supplement ITS (Insulin,-Transferrin-Selenium-X) was able to induce the expression of *p75NTR*. The concentration of these small molecules can finely tune the expression of *p75NTR*. By adjusting the concentration of these three small molecules, we found that SB431542 at 10 μM, LDN193189 at 0.5 μM and DAPT at 5 μM can highly induce the expression of *p75NTR* (Fig. 2a). There is a significant decreased expression of *p75NTR* when the concentration of SB431542 was increased or decreased ($F(2,3)=1402$, $p<0.001$) analyzed by one-way ANOVA. The similar results were also observed when we increased or decreased the concentration of LDN193189 ($F(2,3)=750.4$, $p<0.001$) or DAPT ($F(2,3)=1077$, $p<0.001$) analyzed by one-way ANOVA (Fig. 2a). Furthermore, by evaluating of the cells we generated at different time points, we found that the expression level of *p75NTR* was time dependent. The highest expression level of *p75NTR* was detected at day 6 after induction of iPSCs, which is significantly higher than the expression of *p75NTR* in other group analyzed by one-way ANOVA ($F(6,7)=314.7$, $p<0.001$) (Fig. 2b). By evaluation of the iPSCs-derived neural crest-like cells (iPSC-NC) with RT-QPCR, we found that *p75NTR* was significantly up-regulated by 50-fold compared with iPSCs analyzed by Student's *T* test ($t(1.014)=40.32$, $p=0.015$) (Fig. 2c). Moreover, the expression of iPSCs-related genes *NANOG* and *OCT4* was down-regulated (Fig. 2c).

Generation of periodontal ligament stem cells from iPSC-NC like cells

To generate PDLSCs from iPSC-NC like cells in a chemically defined condition, we first replace fetal bovine serum (FBS) by B27 and ITS. Several signaling pathways or growth factors could affect the formation or maintenance of periodontal ligament stem cells (PDLSCs) [7, 26]. Therefore, we followed these clues and found that the recombinant human bFGF (FGF-2) combined with the rho-associated protein kinase inhibitor Y27632 were able to induce the formation of PDLSC-like cells. After 14-day induction, the expression of PDLSC-related markers, *COL1* ($F(2,3)=90.63$, $p=0.003$), *OPG* ($F(2,3)=104.9$, $p=0.002$), *POSTN* ($F(2,3)=2842$, $p<0.001$) and *FBN1* ($F(2,3)=27.94$, $p=0.036$) were significantly up-regulated compared with iPSC-NC like cells analyzed by one-way ANOVA, which indicated the generation of PDLSCs from iPSCs (Fig. 3). Among these genes, POSTN, was undetectable in iPSC-NC-like cells but was highly expressed in iPSC-induced PDLSCs (iPSC-PDLSCs). Furthermore, the expression of *OPG* in iPSC-PDLSCs



SB431542 (μM)	5	10	20		10	10	10		10	10	10	10
LDN (μM)	0.5	0.5	0.5		0.1	0.5	2		0.5	0.5	0.5	0.5
DAPT (μM)	5	5	5		5	5	5		1	5	10	20

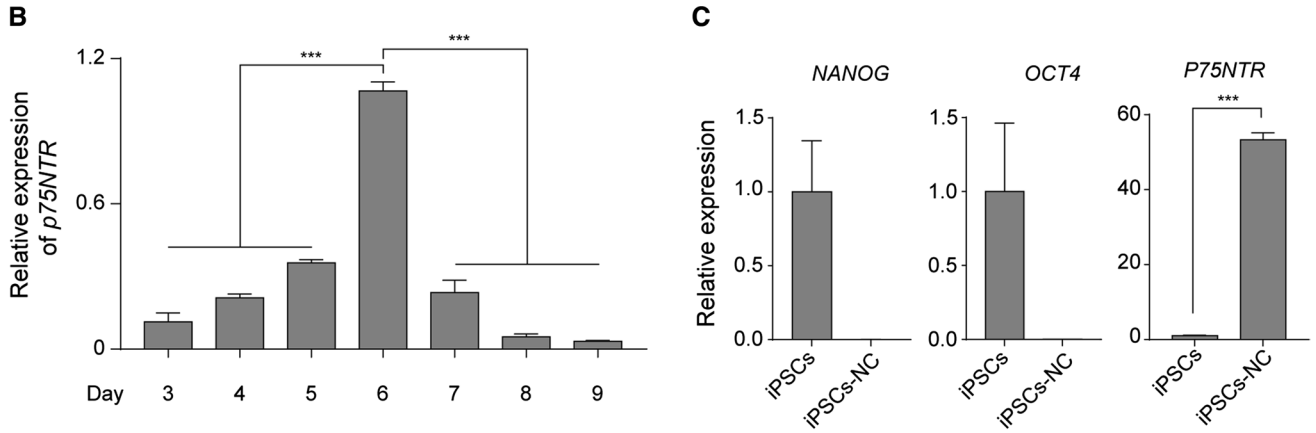


Fig. 2 Generation of neural crest-like cells from iPSCs with chemically defined medium. **a** The expression of *p75NTR* reveals the dose-dependent generation of neural crest-like cells from iPSCs at 6 days of post-induction. **b** Time-dependent expression of *p75NTR*. **c** The expression of iPSC-related genes and neural crest-related gene in iPSCs and iPSC-NC. Data are normalized to housekeeping gene and are presented as Mean ± SD. Statistical difference is shown as $*p<0.05$, $**p<0.01$, $***p<0.001$. $N=2$

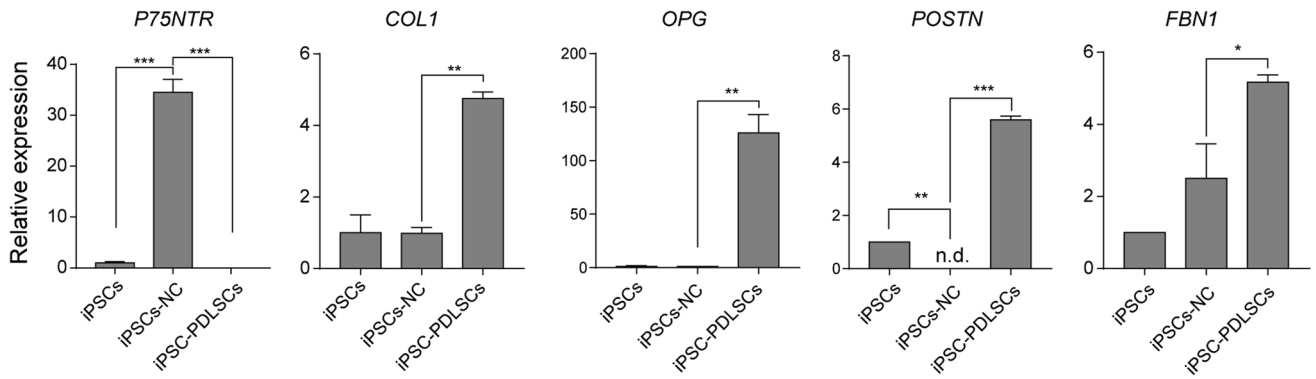


Fig. 3 Generation of PDLSCs from iPSC-NC with chemically defined medium. The expression of neural crest-related gene *P75NTR* and PDLSC-related genes, including *COL1, OPG, POSTN* and *FBN1* in iPSC-NC and iPSC-PDLSCs. Data are normalized to housekeeping gene and are presented as Mean ± SD. Statistical difference is shown as $*p<0.05$, $**p<0.01$, $***p<0.001$. $N=2$

was approximately 100-fold higher than iPSC-NC-like cells. Moreover, we noticed that the expression of NC-related marker *P75NTR* was significantly down-regulated compared with that in iPSC-NC-like cells analyzed by one-way ANOVA ($F(2,3) = 356.9$, $p < 0.001$), which indicated the loss of iPSC-NC-like cells identity.

Osteoblastic and adipogenic differentiation ability of iPSC-PDLSCs

As the PDLSCs are multipotent cells, we next proceed osteoblastic and adipogenic differentiation of iPSC-PDLSCs. iPSC-PDLSCs were cultured in osteoblastic differentiation medium or adipogenic differentiation medium for 4 weeks. After osteoblastic differentiation, iPSC-PDLSCs were tested to be alizarin red-positive and mineralized nodule formation was observed, and the mRNA level of osteogenic gene *BMP2* was also up-regulated analyzed by Student's *T* test ($t\ (1) = 4.582$, $p = 0.1368$) (Fig. 4a, b). These results indicated the differentiation of iPSC-PDLSCs toward to osteoblasts.

After 4 weeks of adipogenic differentiation of iPSC-PDLSCs, we detected the formation of lipid droplets in these cells determined by oil red staining (Fig. 4d). We also evaluated the mRNA level of adipogenic gene *CEBPA* which confirmed the adipogenic differentiation. *CEBPA* was significantly up-regulated compared with iPSC-PDLSCs analyzed by Student's *T* test ($t(1.1) = 15.12$, $p = 0.0314$) (Fig. 4c). All these results indicated that the PDLSCs we generated were multipotent cells.

Discussion

The chemically defined condition differentiation system of iPSCs enables us to obtain cells that are applicable in basic research and clinical therapeutic research which is a major goal in the field of regenerative medicine [13, 23]. Feeder cells or animal-derived components were no longer essential for the culture of embryonic stem cells and iPSCs since the establishment of chemically defined condition which could maintain the human ESCs and iPSCs. This chemically defined condition promotes the application of human iPSCs on regenerative medicine [1, 12, 19]. Furthermore, researchers have developed several chemically defined differentiation system on multiple lineages [3, 13]. The establishment of these differentiation protocols have greatly promoted the application possibility of these cells in clinical therapy and research.

Human ESCs or iPSCs could differentiate to PDLSCs [6, 10]. For example, the neural crest cells differentiation system established by Studer et al. [10] used traditional mouse feeder layer cells to maintain stem cell properties and initiate differentiation. Moreover, animal-derived component, such as fetal bovine serum, were also used to promote cell differentiation toward PDSCs [10]. In the differentiation system established by Maeda et al. [6], not only feeder cells and fetal bovine serum were used, but also the extracts of primary human cells were used [6]. These uncertainties greatly hinder the future application in clinical treatment of PDLSCs.

In this study, we have established a chemically defined strategy to proliferate iPSCs and differentiate iPSCs to

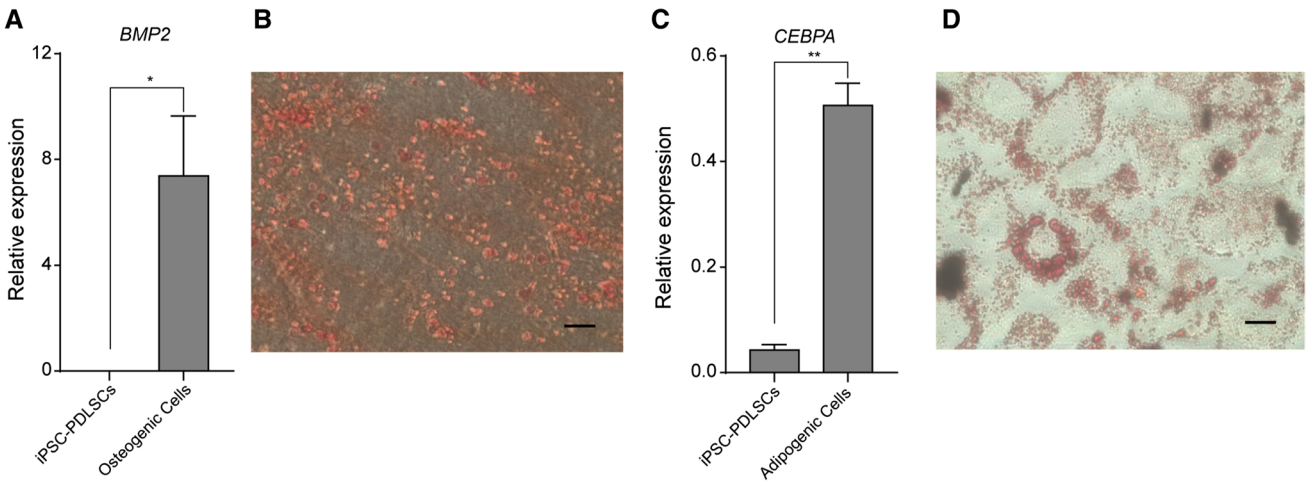


Fig. 4 Differentiation potential of iPSC-PDLSCs. **a** The expression of osteogenic cell-related gene, *BMP2*, after osteoblastic differentiation of iPSC-PDLSCs. **b** Alizarin red staining reveals the further differentiation of iPSC-PDLSCs to an osteoblastic fate. **c** The expression of adipogenic cell-related gene, *CEBPA*, after adipogenic differentiation of iPSC-PDLSCs. **d** Oil red staining reveals the further differentiation of iPSC-PDLSCs to an adipogenic fate. Scale bar: 10 μm. Data are normalized to housekeeping gene and are presented as Mean ± SD. Statistical difference is shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. $N = 2$

PDLSCs, which did not include any serum or composition with non-determinacy. To precisely initiate the differentiation toward a neural crest fate, we selected three signaling pathways, TGF beta, Notch and BMP signaling pathways [9]. All these signaling pathways were reported to contribute to the formation of neural crest on ESCs or iPSCs. To generate iPSC-NCs with a chemically defined medium, we manipulated these signaling pathways by small molecules and finely tuned their concentrations as well as the differentiation periods. Any changes in concentration of this condition will affect the outcome of differentiation. We noticed that in our chemically defined protocol to generate neural crest, there was an approximately 30-fold increase of p75NTR expression related to iPSCs. This result is comparable to the result reported by Maeda et al. [6], where an approximately tenfold increase of p75NTR expression was observed. This indicated that this chemically defined protocol is sufficient to generate neural crest-like cells in vitro. According to the reported signaling pathways and factors which could promote the formation or maintenance of PDLSCs, we combined Y27632 and bFGF to generate iPSC-PDLSCs from iPSCs-NCs. We do find the up-regulation of several PDLSC-related genes; however, the expression level of some of these genes related to iPSCs was not comparable with the results reported by Maeda et al. [6]. These results indicated that our chemically defined medium was able to generate PDLSCs form iPSCs, but fetal bovine serum was still a crucial component for the PDLSCs induction, which could only be partially replaced by the chemically defined medium. Furthermore, PDLSCs obtained by this method are with bidirectional differentiation potential. After 4 weeks of osteogenesis and adipogenesis induction, PDLSCs showed a strong potential for osteogenesis and adipogenesis differentiation. The similar results were also reported by other established differentiation systems containing a large number of animal-derived components [6, 10]. A large number of iPSC-PDLSCs could be generated stably by this chemically defined differentiation protocol, which held great promising in the field of basic research and clinical treatment.

Conclusion for future biology

In this study, in order to obtain PDLSCs with clinical application prospects, we established a chemically defined differentiation system without using any animal-derived components which induced iPSCs cells into PDLSCs by selecting small molecules and their combinations, regulating the concentration. The induced PDLSCs by this method can stably express key functional genes and have the potential of bidirectional differentiation in vitro. Importantly, the chemically defined condition could reduce the unsteadiness and safety concerns brought from animal-derived components. It is of great significance to clinical application of PDLSCs for periodontal diseases in the future.

Author contributions YW contributed to conception, design, data acquisition, analysis, interpretation, drafted and critically revised the manuscript; YH contributed to conception, design and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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