Directing Adult Human Periodontal Ligament–Derived Stem Cells to Retinal Fate

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Purpose. To investigate the retinal fate competence of human postnatal periodontal ligament (PDL)-derived stem cells (PDLSC) through a directed differentiation mimicking mammalian retinogenesis.

Methods. Human teeth were collected from healthy subjects younger than 35 years old. Primary PDLSC were isolated by collagenase digestion and cultivated. PDLSC at passage 3 were cultured in the induction media containing Noggin (antagonist of bone morphogenic protein) and Dkk-1 (antagonist of Wnt/β-catenin signaling). Gene expression of neural crest cells, retinal progenitors, and retinal neurons, including photoreceptors, was revealed by RNA analyses, immunofluorescence, and flow cytometry. The neuronal-like property of differentiated cells in response to excitatory glutamate was examined by fluo-4-acetoxymethyl calcium imaging assay.

Results. Primary human PDLSC stably expressed marker genes for neural crest (Notch1, BMP2, Slug, Snail, nestin, and Tuj1), mesenchymal stem cell (CD44, CD90, and vimentin), and embryonic stem cell (c-Myc, Klf4, Nanog, and SSEA4). Under low attachment culture, PDLSC generated neurospheres expressing nestin, p75/NGFR, Pax6, and Tuj1 (markers of neural progenitors). When neurospheres were plated on Matrigel-coated surface, they exhibited rosette-like outgrowth. They expressed eye field transcription factors (Pax6, Rx, Lhx, Otx2). By flow cytometry, 94% of cells were Pax6+ and Tuj1+, indicative of retinal progenitors. At prolonged induction, they expressed photoreceptor markers (Nrl, rhodopsin and its kinase) and showed significant responsiveness to excitatory glutamate.

Conclusions. Primary human PDLSC could be directed to retinal progenitors with competence for photoreceptor differentiation. Human neural crest–derived PDL is readily accessible and can be an ample autologous source of undifferentiated cells for retinal cell regeneration.

Keywords: periodontal ligament–derived stem cells, neurogenesis, retinal progenitors, photoreceptors

Retinal degeneration is a common cause of visual impairment or even irreversible blindness associated with various eye diseases, including retinitis pigmentosa and age-related macular degeneration, in which retinal cells lose their function or die.1 Since human retinal neurons lack intrinsic regeneration capability, stem cell–based therapy may provide a strategy for the treatment of retinal diseases by rescuing or replacing damaged and malfunctioning cells.

Over the past decade, a variety of cell sources have been investigated for retinal fate specification and functional cell regeneration.2–6 Among them, embryonic stem (ES) and induced pluripotent stem (iPS) cells show potential for deriving retinal phenotypes.7,8 Human ES cells were directed to retinal progenitors through mimicking of the guiding signals along mammalian retinogenesis.9 Respective inhibition of bone morphogenic protein (BMP) and Wnt signaling and supplementation of insulin-like growth factor-1 (IGF-1) were shown to promote anterior neural plate development. Since then, a series of modified strategies using various cocktails of exogenous signaling modulators and growth factors for retinal cell differentiation have been attempted.10–14 Such development potentiates the future application of stem cell–based technologies to treat retinal degenerative diseases. Even though ES and iPS cells have shown their potential application in treating different retinal degenerative diseases in animal models,15,16 there are concerns when they are considered for clinical application. The use of ES cells provokes ethical controversy and the risk of immune rejection, whereas safety, especially with respect to tumorigenesis, is a major issue for the use of iPS cells. A continuing search for an accessible autologous source of stem cells for efficient retinal lineage specification and cell regeneration is needed for the future design of retinal cell
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therapy. The protocol must avoid the risk of tumorigenesis and immune rejection.

Neural crest cells are a transient population of multipotent and migratory cells that originate at the interface between the nonneural ectoderm and the neural plate during embryonic development of vertebrates. During neurulation, they undergo the epithelial-to-mesenchymal transition and migrate extensively along defined pathways to different regions of the embryo, and become cranial, cardiac, vagal, and trunk neural crest cells. They are capable of differentiating into diverse cell lineages with regard to positioning along the anterior-posterior axis.17 Besides specification to the cranial ganglia, craniofacial cartilage and bones, thymus, and middle ear bones and jaws, cranial neural crest cells migrating to the pharyngeal pouches and arches can contribute to tooth formation.18 They give rise to most dental tissues including odontoblasts, dental pulp, apical papilla, dental follicle, and periodontal ligament (PDL). PDL is derived from the cranial neural crest-derived ectomesenchymal cells and is the connective tissue located between the root of the tooth and alveolar bone socket. It contains a mixed population of fibroblasts and epithelial, ectomesenchymal cells and is the connective tissue located between the root of the tooth and alveolar bone socket. It contains a mixed population of fibroblasts and epithelial, mesenchymal, and undifferentiated cells, as well as bone and cementum cells, sitting in the hydrated extracellular ground substance with collagen-rich fibrils.19 Apart from affixing the tooth to the alveolar bone and withstanding compressive force during the chewing motion, PDL provides sensory, nutritive, and homeostatic support to the alveolar compartment.

PDL-derived stem cells (PDLSC) have been shown to differentiate into either cementoblasts or osteoblasts for guided dental tissue regeneration, bone grafting, and enamel matrix reconstruction.20,21 Enzymatic treatment of human PDL released an undifferentiated population of cells that are capable of clonal growth and express markers of mesenchymal stem cells (MSC) (STRO-1, scleraxis), ES cells (Oct4, Sox2, Nanog, and Klf4), and neural crest cells (nestin, Slug, p75, Sox10), reflecting their pluripotent characteristics, and differentiate to multiple cell fates, including neurogenic, cardiomyogenic, chondrogenic, and osteogenic lineages.22-25 Recently, rat adrenal pheochromocytoma PC12 cells, through the release of synergistic molecules such as nerve growth factor, could induce human PDL-derived neural crest-like cells to neurocytic differentiation and migration.26 Human PDL has also been shown to express IGF-1, an important cytokine for cell differentiation and migration. In this study, retinal fate competence was identified in human adult PDLSC. Our findings suggest that PDL would be a novel and crucial autologous cell source for retinal lineage differentiation and the future design of retinal repair and regeneration.

Materials and Methods

Tooth Collection, PDLSC Isolation, and Culture

Permanent teeth were collected from young adults from the Oral-Maxillofacial Surgery and Dental Unit, North District Hospital, Hong Kong. The study protocol was approved by the Ethics Committee of the Department of Health, Hong Kong (L/M 118/2011). It was performed in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from the study subjects after explanation of the nature and possible consequences of the study. All subjects had good oral hygiene and no history of smoking, previous radiotherapy in the head and neck region, periodontal disease, or other active dental infection. They had no major eye diseases except myopia. Teeth that were fractured during removal were excluded from collection. All informed written consents were retained by W-MT.

Immediately after extraction, the tooth was submerged in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Invitrogen, Carlsbad, CA) with 300 U/ml penicillin G, 300 μg/ml streptomycin sulfate, and 2% amphotericin B (Invitrogen). Within 6 hours, it was delivered to the culture facility, and PDL tissue was scraped mechanically from the root surface and finely chopped, followed by digestion with 0.1% collagenase I and III (Worthington, Lakewood, NJ) in DMEM/F12 medium containing 0.5% fetal bovine serum (FBS; Invitrogen) and antibiotics for 4 to 6 hours with agitation (100 rpm) at 37°C.23 After passing through a cell strainer (40 μm pore size; BD Biosciences, Franklin Lakes, NJ), the cells were cultured in DMEM/F12 medium supplemented with 10% FBS and antibiotics.

Retinal Fate Induction

PDLSC at passage 3 were recruited for a two-step retinal fate induction procedure. Step 1 was to obtain neurospheres by culturing cells with induction medium 1 (IM1), which was DMEM/F12 supplemented with 1% B27 (Invitrogen), 1 ng/mL mouse Noggin (R&D Systems, Minneapolis, MN), and 1 ng/mL human recombinant Dkk-1 (R&D Systems), on an ultra-low attachment culture plate (Corning, Tewksbury, MA) for 3 days. Step 2 was to plate neurospheres on culture surface coated with protein matrix secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (Matrigel; BD Biosciences) in induction medium 2 (IM2), to which IM1 was added with 10 ng/mL Noggin, 10 ng/mL Dkk-1, and N2 (Invitrogen). Fresh medium was replenished every 3 days and the culture maintained for up to 25 days. The cells were collected for characterization at different time intervals.

Cell Proliferation Assay

PDLSC at passage 2 were seeded at a density of 5 × 10^3 cells/well in a 96-well plate. Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method at different time intervals. MTT (0.5 mg/mL, Sigma, St. Louis, MO) was applied to cells in culture for 3 hours, and isopropanol was added to dissolve formazan crystals. The solution absorbance was determined by spectrophotometry with excitation wavelength at 590 nm (Power-Wave microplate reader; BioTek Instruments, Inc., Winooski, VT). Results from triplicate experiments were represented as mean ± SD.

Immunocytochemistry

Cells were fixed with freshly prepared 2% neutral buffered paraformaldehyde and permeabilized with 0.15% saponin (Sigma). After blocking, the samples were incubated with primary antibodies (Supplementary Table S1). The secondary antibodies were either Alexa 488 or Rhodamine Red-X conjugated (Jackson ImmunoResearch, West Grove, PA). All nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Results were visualized using confocal laser scanning microscopy (SP5; Leica, Wetzlar, Germany). A minimum of 10 fields viewed at ×20 magnification (objective) were obtained for cell quantification. The percentage of labeled cells in triplicate experiments was expressed as mean ± SD. Results were compared between control and treated samples and analyzed by paired Student's t-test. P < 0.05 was considered statistically significant.

Flow Cytometry

Trypsinized cells were washed and fixed with 2% paraformaldehyde in DMEM/F12 medium containing 2% bovine serum albumin. The cells were permeabilized in 0.1% saponin (Sigma) and stained with primary markers (Supplementary Table S1). Antigen expression was analyzed using a FACSCalibur (BD Biosciences) or a FACSCanto II (BD Biosciences). FlowJo (Treestar, Inc., Ashland, OR) was used for data analysis.
albumin (Sigma) for 20 minutes. After washes, they were incubated overnight with primary antibodies (Supplementary Table S1) or isotype control at 4°C, followed by either donkey antirabbit Alexa 488 or donkey antiamouse Alexa 488 secondary antibodies for another 2 hours at ambient temperature. Single-cell suspension after passage through a cell strainer (40 μm pore size; BD Biosciences) was analyzed by flow cytometry (LSR Fortessa flow cytometer; BD Biosciences). For each sample, a minimum of 10,000 events were recorded for data analysis by FACSDiva software (BD Biosciences).

Western Blotting
Cells were collected in lysis buffer containing 50 mM Tris-HCl, 150 mM sodium chloride, 1% Nonidet P-40 (Sigma), 0.25% sodium deoxycholate (Sigma), protease inhibitor cocktail (Complete; Roche, Basel, Switzerland), and 1 mM phenylmethyl sulfonylfluoride (PMSF; Sigma). The clear soluble lysate was then denatured with 2% sodium dodecylsulfate (SDS, weight/volume). The samples (equivalent to 3.5 × 10^4 cells) were resolved with SDS-PAGE (polyacrylamide gel electrophoresis), blotted, and immunolabeled with primary antibodies (Supplementary Table S1) followed by appropriate horseradish peroxidase–conjugated Ig secondary antibodies (Jackson Immunoresearch). Immunodetection was done by chemiluminescence (ECL; GE Healthcare, Pittsburgh, PA).

Gene Expression Analysis
Cells were collected in RLT buffer freshly added with 1% β-mercaptoethanol, and total RNA was extracted by RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. Reverse transcription of 1 μg total RNA was performed with SuperScript III RT-PCR kit (Invitrogen) using random primers. Gene expression was assayed by PCR using Master Mix (Invitrogen) and specific primers (Supplementary Table S2), and PCR products were resolved by agarose gel electrophoresis. Alternatively, quantitative real-time PCR (qPCR) was performed with Sybr Green Supermix (Applied Biosystems, Carlsbad, CA) in ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Experiments were run in triplicate. Relative gene expression of each sample was normalized by the mean CT value housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (CTGAPDH) and expressed as mean ± SD.

Calcium Imaging Assay in Response to Glutamate Stimulation
Intracellular calcium transient was evaluated using fluo-4-acetoxyethyl (Fluo-4AM) ester (Invitrogen). Cells were incubated in Hanks’ balanced salt solution (Ca^2+-Mg^2+-free; Invitrogen) containing 5 μM Fluo-4AM (Invitrogen) and 0.1% pluronic F-127 (Sigma) for 30 minutes at room temperature. Glutamate (1 μM; Sigma) was added to the culture, and fluorescence images were immediately captured using the Olympus Fluoview FV1000 laser scanning confocal system (Olympus, Melville, NY), with excitation wavelength at 495 nm and emission wavelength at 515 nm, every 5 seconds for a total of 2 minutes. Data were analyzed by Olympus FV10-ASW v1.7. Fluorescence intensity at specific time intervals was measured on a total of 50 cells in triplicate experiments (at least 10 cells in each sample) using FACSDiva software (BD Biosciences). The cellular change of fluorescence (F) (%ΔF/Fbaseline) was calculated as (Ftreated - Fbaseline)/Fbaseline × 100%. The Ca^{2+} fluorescence ratio was converted to Ca^{2+} concentration using the equation 27 [Ca^{2+}]_i = K_d/[K_i/(Ca^{2+})_{rest} + 1] - K, where KD is the dissociation constant of Fluo-4AM (400 nM; Invitrogen), K is the fluorescence ratio (ΔF/F_{baseline}), and [Ca^{2+}]_{rest} is the resting Ca^{2+} concentration (which is 10–100 nM in ordinary cells).28 Since the induced cells had neuronal gene expression, we assumed that the resting [Ca^{2+}] was 100 nM.

RESULTS
Neural Crest Features of PDLSC
We established 25 primary human PDL cultures from adult Chinese (age range, 13–33 years; male to female ratio, 2:3). Each primary PDLSC was derived from PDL tissue of a single donor. We did not pool PDL cells from different individuals as in other studies; our procedure would preserve individual variability. The retinal induction efficiency obtained from randomly chosen primary PDLSC is highly indicative of a unique property of PDL cells irrespective of individual variation. In our work, we collected a total of 45 teeth and successfully established 25 primary PDLSC. The success rate was 56%. The failure of cultures was due to yeast contamination of primary cells, absence of or insufficient PDL tissue on the tooth surface, and no growth of isolated PDL cells. Within 5 days of initial seeding, passage 1 PDLSC displayed clonal growth of six to eight cells. In our culture record, the longest time to the first appearance of cell clones with six to eight cells was 13 days. The adherent cells continued to proliferate and the colony size increased substantially in the next 3 to 4 days. Cells in the center of the colony were more densely packed than those in the periphery (Fig. 1A). We frequently observed more migratory cells in the peripheral region (data not shown). Most cells were slender and spindle shaped during early culture time. They displayed dendritic morphology with distinct cell nuclei and more intercellular connection at the ends of the dendritic processes at a later time in passage 1 culture (Fig. 1B).

In this study, we randomly selected four primary PDLSC cultures from young subjects (PDL-8: female/21 years old; PDL-10: female/13 years; PDL-13: male/16 years; PDL-15: female/33 years). All cells at passage 2 had similar growth rates (Fig. 1C). The mean cell doubling time was 25 to 30 hours at the exponential growth phase. By RT-PCR, they stably expressed neural crest markers (Notch1, BMP2, Slug, Snail, nestin, and Tuj1) (Fig. 1D). The expression was similar to that in freshly isolated PDL tissue. This expression profile was consistent in all 25 primary PDLSC cultures, irrespective of age and sex of the subjects. Representative staining of PDL-8 cells with antibodies recognizing nestin, p75/NGFR, and Tuj1 is shown in Figures 2I, 2J, and 2K. Besides nestin expression in 89% of cells, >95% of cells were also immunopositive for p75 and Tuj1. In addition, they expressed MSC markers (CD44, CD90, and vimentin) as shown by immunofluorescence (Figs. 2A–C) and flow cytometry (Figs. 2E–G). PDLSC also expressed ES pluripotency genes at variable levels. The expression of c-Myc and Klf4 was similar to that in human ES H1 cells; but Oct4, Sox2, and Nanog were weakly expressed (Figs. 1E, 1F). SSEA-4 was expressed in a subset of PDLSC (Fig. 2D), and flow cytometry showed that 28% of cells were SSEA-4 positive (Fig. 2H).

Neural Retinal Fate Commitment From Human PDLSC
To test whether PDLSC could be induced toward a retinal fate, a two-step induction protocol involving initial neurosphere formation followed by propagation with adenovirus culture was undertaken. Human PDLSC were sensitive to low attachment culture and generated neurospheres within 24 hours (Fig. 3A).
Seeding of $10^5$ PDLSC per dish (60 mm diameter) gave rise to 14.6 ± 6.4 spheres/mm$^2$, indicating 4% efficiency. After 3 days, all neurospheres expressed markers identifying neural precursor (nestin, p75, Pax6, and Tuj1) and ES cells (ABCG2 and SSEA4) (Fig. 4). Peripheral cells coexpressed nestin and p75, whereas centrally located cells had more nestin staining. Also, ABCG2- and SSEA4-expressing cells were located in the periphery of spheres whereas Pax6 was expressed in both central and peripheral regions. Over 95% of cells expressed Tuj1 (Fig. 4M).

**Figure 1.** Primary human PDLSC culture and characterization. (A) A representative clonal expansion of primary PDLSC at day 4 of culture. (B) PDLSC culture at day 14. (C) Growth curves of PDL-8, -10, -13, and -15 cells at passage 2. Each point represents mean ± standard deviation from triplicate experiments. (D) RT-PCR analysis showing the expression of neural crest genes in three primary PDLSC cultures (at passage 2), compared to freshly isolated PDL tissue cells. (E) RT-PCR analysis showing the expression of ES pluripotency genes in three primary PDLSC cultures (at passage 2), compared to human ES H1 cells. (F) The relative expression fold changes of c-Myc and Klf4 among primary PDLSC cultures and H1 cells.

**Figure 2.** Characterization of primary human PDLSC. Representative confocal immunofluorescence images showed expression of MSC markers ([A], CD44; [B], CD90; [C], vimentin), ES marker ([D], SSEA-4), and neural crest markers ([I], nestin; [J], p75/NGFR; [K], Tuj1) in PDL-8 at passage 2. nuclei were counterstained with DAPI (blue). Scale bars: 50 μm. Flow cytometric histograms showed the event profiling of ([E]) CD44, ([F]) CD90, ([G]) vimentin, and ([H]) SSEA4. Open histograms denote the isotype controls.
When the neurospheres were plated on Matrigel-coated surface under IM2 condition (supplemented with 10 ng/mL Noggin and Dkk-1, as well as N2), they attached and exhibited rosette-like outgrowth. At day 3, Tuj1-immunopositive neurite-like processes extended from the spheres and formed an interlaced network with the neighboring spheres (Figs. 4M, 4N). Persistent neurite-like structures were frequently observed in prolonged IM2 culture. Representative phase-contrast images at different days of retinal fate induction are shown in Figure 3B.

At various time intervals, the cells showed upregulated gene expression associated with eye field specification, including DCX and transcription factors Lhx2, Chx10, Rx, Sox2, and Otx2 (Fig. 5A). In triplicate qPCR assays, Rx was upregulated by 110-fold (Fig. 5B). Chx10 expression was slightly increased, by less than 10-fold, when compared to that in untreated cells at day 25 (data not shown). Neural crest gene expression (Tuj1, nestin, Snail, and Slug) was unchanged during the induction process (data not shown).

Nuclear Translocation of Pax6

In untreated PDLSC, Pax6 had a cytoplasmic localization (Fig. 6A). After induction for 25 days, Pax6 was shifted to predominantly nuclear expression (Figs. 6B, 6C). Concomitantly this translocation was associated with Rx induction. Rx was exclusively expressed in Pax6 nuclear cells (Figs. 6G-I). Cells without nuclear Pax6 had negative Rx expression (arrow in Figs. 6D-F). By cell counting analysis of >500 cells, retinal fate induction resulted in 94.6 ± 4.7% of cells becoming Pax6 nuclear Rx+ (Fig. 6M), compared to 0% in control cells (Figs. 6J-M). This indicated an efficient conversion of PDL cells to Pax6 nuclear Rx+ retinal progenitors. A representative picture showing Pax6 nuclear Rx+ cells at day 17 of induction was shown in Figure 6N.

Acquisition of Photoreceptor Phenotypes

PDLSC-derived retinal progenitors could attain photoreceptor phenotype under retinal fate induction. Nrl and rhodopsin gene expression was detected by RT-PCR as early as day 14 of treatment (Figs. 5A, 5B). At day 25, both were upregulated, by 180- and 110-fold, respectively (Fig. 5B). Compared to 0% before treatment, 5.1 ± 0.5% cells expressed rhodopsin (Figs. 7A, 7B). Rhodopsin kinase (GRK1), an enzyme involved in phosphorylation of photo-transactivated rhodopsin in rod photoreceptors, was exclusively expressed in these cells (Fig. 7C).

Figure 3. Retinal fate induction procedure for PDLSC. (A) Primary human PDLSC at passage 3 were cultivated in IM1 under low attachment culture for 3 days to generate neurospheres, which were subsequently plated on Matrigel-coated surface in IM2 for adherent culture up to 25 days. (B) Representative phase-contrast images showing PDLSC culture at different days of retinal fate induction. The bottom row shows images at higher magnification. Scale bars: 200 µm.

Figure 4. Characterization of PDLSC-derived neurospheres. Neurospheres at day 3 of low attachment culture were assayed for expression of (A) nestin, (B) p75/NGFR, (E) ABCG2, (F) Pax6, (I) SSEA4, and (J) Tuj1. (C, G, K) Nuclei were counterstained with DAPI. (D, H, L) Merged images of respective stained images. (M) Tuj1-stained neurite-like processes interconnecting between neurospheres. (N) Phase-contrast micrograph of neurospheres attached on Matrigel-coated surface at day 3 of culture. The insert shows neurites between spheres. Scale bars: 50 µm.
photoreceptors, was also upregulated (Fig. 7E). Such significant elevation was similarly observed in the treatment of PDLSC samples ($P < 0.05$, paired Student’s $t$-test, $n = 3$ for each PDLSC sample) (Fig. 7F). In culture maintained in IM2 up to 45 days, the cells appeared with small and round cell body and extended very slender neurite-like processes (Fig. 7G). The thin cytoplasm contained abundant rhodopsin granules (Fig. 7D).

In these PDLSC-derived retinal progenitors, $\text{Ca}^{2+}$ transient in response to glutamate insult was detected by Fluo-4 AM assay. The majority of cells (mean, 92% from triplicate experiments) treated for 25 days showed a rapid and robust increase of fluorescence intensity within the first 40 seconds after addition of 1 $\mu$M glutamate (Figs. 7G, 7H). The $\text{Ca}^{2+}$ spark peaked at 60 to 80 seconds and then decayed slowly. The response kinetics in time course and stimulus intensity varied among cells (Fig. 7I shows five representative PDL-10 cells after retinal fate induction). Whole-cell $\text{Ca}^{2+}$ imaging recorded in a total of 50 cells in triplicate experiments (at least 10 cells per test) showed a consistent increase of peak fluorescence in all three tested PDLSC cultures after retinal fate induction (191 ± 21% for PDL-8, 313 ± 39% for PDL-10, and 262 ± 19% for PDL-15) compared to untreated cells. All these values were significantly greater than in untreated samples ($P < 0.001$; Fig. 7I). Assuming that the resting $[\text{Ca}^{2+}]$ of untreated cells was 100 nM, we estimated that the peak $\text{Ca}^{2+}$ concentration of the $\text{Ca}^{2+}$ spark after induction was approximately 247 nM for PDL-8, 669 nM for PDL-10, and 440 nM for PDL-15 cells, respectively.

DISCUSSION

In this study, we induced four randomly selected human primary PDLSC cultures to retinal fate by chemically inhibiting Wnt and BMP signaling, a protocol to promote anterior neural plate development and retinogenesis. The four primary PDLSC cultures showed similar efficiencies in generating retinal progenitor-like cells. The treated cells expressed genes associated with eye field specification (Rx, Lhx2, Dcx, Chx10, Sox2, Otx2) and photoreceptors (Nrl and rhodopsin), in addition to neural crest (Tuj1, nestin, Snail, Slug, and vimentin) and neural crest stem cells (nestin, Snail, Slug, p75, and Tuj1), which is consistent with previous reports of postnatal PDLSC.30,31 These markers are regarded as the key indicators or regulators of multipotency, hence highlighting the plasticity and differentiation potential of PDLSC.

Development of neural retina from the anterior neural plate is associated with multiple signaling pathways.34–36 Antagonizing Wnt and BMP signaling can promote neural plate development anteriorly and medially.37–40 Importantly, this well-defined developmental program can potentially be recapitulated in vitro for retinal lineage differentiation from human ES and iPS cells with propensity.11–14 Our work repeated the induction methodology with optimization in postnatal human PDLSC. Similar formation of neurospheres and propagation of rosette-like outgrowth with Noggin and Dkk-1 are highly reminiscent of the embryoid bodies and formation of optic cup-like structures during the retinal differentiation process observed in iPS cells.14,44 Therefore our protocol to derive retinogenesis in pluripotent cells may be applicable to differentiate postnatal PDLSC toward the retinal lineage, largely according to their embryonic neural crest features.

Retinogenesis occurs as a stepwise process, beginning with the designation of the eye field within the anterior neural plate, followed by the formation of optic vesicle and optic cup.45 Each stage is defined by various transcription factors.42 Our results revealed that PDLSC under induction acquired the expression of transcription factors for anterior neural specification (Otx2 and Dcx), eye field specification (Rx and Lhx2), and retinal precursor (Pax6, Chx10, and Sox2). This expression pattern resembles that previously observed in differentiating human ES and iPS cells.44,45 Of note, the homeobox PAX6 gene is a master transcription regulator in the specification and maintenance of eye field development and retinal progenitors.44–46 The neural crest-derived PDLSC had intrinsic PAX6 expression; however, the protein was mainly located in the cytoplasm. Upon retinal fate induction, Pax6 protein was found shuttled to the nucleus in the majority of cells. This highlighted the transcription competence of Pax6 after induction. Nucleocytoplasmic shuttling and nuclear retention

**FIGURE 5.** Retinal gene expression of human PDLSC after retinal fate induction. (A) RT-PCR analyses showed upregulation of gene markers identifying retinal progenitors (Lhx2, DCX, Chx10, Rx, Sox2, Otx2) and photoreceptors (Nrl and rhodopsin) at time intervals of IM2 treatment. (B) Expression fold changes of Rx, rhodopsin, and Nrl assayed by quantitative PCR analysis.
of Pax6 protein for its gene transcription activity are mediated by Smad3/SPARC interaction and TGFβ signaling.47–49 In our work, whether Pax6 translocation in treated PDLSC is caused by changes in retinogenesis signaling or other factors is yet to be determined. Notably, we observed unique coexpression of Rx with Pax6 nuclear, suggesting that Pax6 activation could switch on Rx and that the two function synergistically to specify retinal progenitor fate and identity. In other studies, the number of cells coexpressing Pax6 with Rx or Chx10 formed an estimate of the generation efficiency of retinal progenitors.7,50,51 Using a similar calculation approach, we showed that postnatal PDLSC under retinal fate induction had 94% cells expressing Pax6 nuclear/Rx+, which is greater than the 80% efficiency (Pax6’Chx10+) in Lamba’s study using human ES cells but similar to the 95% efficiency (Pax6’Rx+) in Meyer’s study on the iPS cell model.52,53

With such a high propensity to differentiate to retinal fate, PDLSC could be useful as replacement cell therapy for retinal cell loss and dysfunction. Also, they have the advantages that they can be obtained from an autologous source and from easily accessible tissue. Karyotyping, soft agar assay, and xenografting to SCID mice have shown that no chromosomal abnormalities related to tumorigenicity or tumor-related diseases occurred in cultured PDL cells.52,53 Hence, PDLSC might be less tumorigenic and are not genetically modified, as is the case with iPS cells. However, before consideration for
clinical application, further studies are required to determine the efficacy of ex vivo expanded cells to repair retinal defects. The growth and differentiation profiles for retinal cell lineage commitment have to be established. In addition, suitable carriers and inductive factors helping implants to integrate into the retinal neuron circuitry will need to be developed. Compared to observations in human ES cells, considerably reduced expression of Chx10 was detected in differentiating human PDLSC to retinal fate. Such low expression might be related to the lack of basic FGF in our induction media. This cytokine has been shown to mediate the retinal fate decision. Further optimization to achieve a better outcome and higher efficiency in generating retinal-like cells will be needed in future studies.

In addition, we confirmed the generation of functional retinal-like neurons in our study. Functional neurons possess sensory receptors for neurotransmission and can be evoked by excitatory stimuli. By Fluo-4 AM calcium imaging, we observed robust and effective fluorescence intensity increases evoked by glutamate insult. This represents calcium influx in PDLSC-derived retinal-like cells, and is consistent with previous observations in retinal progenitors and photoreceptor precursors. It is suggested that the induced PDLSC attain an excitable membrane property corresponding to developing retinal neurons. We noticed that the glutamate response is not
restricted to photoreceptors. It can be found in various retinal neurons and is mediated by ionotropic glutamate receptors (NMDA and non-NMDA receptors). Functional NMDA receptors are found in retinal ganglion cells and some amacrine cells, whereas non-NMDA receptors are found in horizontal, OFF-bipolar, amacrine, and retinal ganglion cells. Moreover, glutamate transporters have been identified on photoreceptors, ON-bipolar, and Müller cells. Once glutamate is incorporated into the transporter, this elicits cotransport of Na	extsuperscript{+} of undifferentiated cells for retinal cell generation.

PDL could be a readily accessible and ample autologous source with competence for photoreceptor differentiation within these cells have the propensity for retinal fate specification. Glutamate additionally supports the neuronal features. On the basis of the rationale presented above, we conclude that PDLSC could be induced to retinal cell types other than photoreceptor-like cells under similar treatment conditions.

In conclusion, human PDL tissue contains a population of postnatal stem cells with primitive attributes originating from the embryonic neural crest. We reported for the first time that these cells have the propensity for retinal fate specification with competence for photoreceptor differentiation within a short period of induction. Hence, human neural crest-derived PDL could be a readily accessible and ample autologous source of undifferentiated cells for retinal cell generation.

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