

Culture and characterization of human dental pulp-derived stem cells as limbal stem cells for corneal damage repair

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Abstract. Limbal stem cell deficiency (LSCD) is one of the leading causes of corneal damage. Injury or inflammation in the cornea causes LSCD, which may be unilateral or bilateral depending upon the cause. Limbal epithelial cell implants successfully improve vision in patients with chemical injury-induced LSCD. Transplantation of cultured epithelial stem cells has become a treatment of choice for numerous patients with LSCD. Bilateral LSCD is frequently observed in the general population, where no residual stem cells are available for *ex vivo* culture. Allografts are associated with a high risk of rejection, neoplasia, and disease transmission. In this respect, allogenic cell populations from other regions in the patient may substitute for allogenic material. In the present study, dental pulp stem cells were cultured in limbal stem cell media and these cells were characterized against limbal stem cells, revealing the significance of using dental pulp stem cell treatment in bilateral LSCD. The morphology and culture pattern of both limbal and dental pulp stem cells grown in limbal stem-specific media were similar. Polymerase chain reaction analysis revealed that stem cell markers were highly expressed in limbal stem cells compared to in dental pulp stem cells, regardless of the medium and scaffold in which they were grown. Although dental pulp stem cell molecular expression is quite low at the transcript level, the functional protein level according to immunocytochemistry and western blot analyses demonstrated that stem cells and corneal differentiation molecule levels were quite high, indicating their potential as limbal stem cells in the respective microenvironment.

Introduction

Approximately 10 million people worldwide suffer from corneal blindness (1,2). Limbal stem cell deficiency (LSCD) is among the leading causes of corneal damage since these cells are essential for maintaining the integrity of the corneal surface and transparency of the cornea (3,4). Injury or inflammation in the cornea causes LSCD (5), an extremely debilitating human eye disease that causes painful vision loss and other clinical manifestations including corneal neovascularization, chronic inflammation, erosions, ulceration, and stromal scarring (6,7). Hereditary aniridia is a primary LSCD, which is characterized by congenital erythrokeratoderma, keratitis, inadequate nutrition or cytokine supply, neurotrophic keratopathy, peripheral inflammation, and sclerocornea (8). LSCD may also be caused by secondary external factors such as trauma, chemical acid or alkali, or thermal injuries and radiation (9). Stevens-Johnson syndrome results in secondary LSCD (10), and LSCD may be unilateral or bilateral depending upon the cause.

LSCD is often diagnosed by clinical assessment or detection of conjunctival goblet cells on the corneal surface using impression cytology (3,9,11). Under conditions of limbal stem cell loss, replacement with healthy limbal cells is necessary, which can be achieved by transplanting healthy limbal tissue containing nourishing cells. Cells can be transplanted from the other eye of the patient if it is healthy (autograft) or from the eye of a living or cadaveric donor (allograft) to the eye with LSCD (12,13). When taking excess graft from other healthy eye or living donor, stem cell deficiency may occur in the donor eye. In the late 1990s, cultured, autologous, limbal epithelial cell implants were used successfully to improve vision in patients with chemical injury-induced LSCD (14). Since then, transplantation of cultivated epithelial stem cells has become a treatment of choice for numerous patients with LSCD. Bilateral LSC deficiency is frequently observed in the general population in which no residual stem cells are available for *ex vivo* culture. Allograft is associated with a high risk of rejection, neoplasia, and disease transmission (15). Thus, allogenic cell populations from other regions in the patient may replace the use of allogenic material. Various approaches have been developed for deriving limbal stem cells from different sources such as oral mucosal epithelial cells, conjunctival epithelial cells,

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hair follicle-derived epithelial stem cells, amniotic epithelial cells, human embryonic stem cells, induced pluripotent stem cells, umbilical cord lining epithelial stem cells, Wharton's jelly mesenchymal stem cells (16-19), and human immature dental pulp stem cells (DPSCs) (20,21). DPSCs are a relatively convenient resource, as teeth are easy to access and potentially superior to other types of adult stem cells (22). More than 20 years have passed since the first limbal stem cell transplantation, however, its standardization and application in India has not reached some populations, particularly those in rural regions. In the present study, DPSCs were cultured in limbal stem cell media and these cells were characterized against limbal stem cells, revealing the significance of using dental pulp stem cell treatment in bilateral LSCD.

Materials and methods

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), antibiotic and antimycotic solution, fetal bovine serum, Trypsin-EDTA, and tissue culture plastics were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Insulin, transferrin, selenium (ITS), epidermal growth factor (EGF), hydrocortisone, RNA extraction TRIzol®, and immunocytochemistry secondary fluorescence antibodies [Alexa Fluor® 647-conjugated goat-anti-rabbit immunoglobulin G (IgG; 2 mg/ml; cat. no. A21244) and Alexa Fluor 488-conjugated goat-anti-mouse IgG (2 mg/ml; cat. no. A11001)] were obtained from Molecular Probes (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Western blot protein markers, loading gel, chemiluminescence developers, and nitrocellulose membranes were procured from Bio-Rad Laboratories, Inc., Hercules, CA, USA). Antibodies (200 µg/ml) against limbal stem cell and corneal epithelial markers, including ATP-binding cassette super-family G member 2 (ABCG2; cat. no. sc-377176), cytokeratin 12 (cat. no. sc-25722), E-cadherin (cat. no. sc-7870), vimentin (cat. no. sc-32322) and loading control GAPDH (cat. no. sc-166574) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The polymerase chain reaction (PCR) primers were obtained from GeNoRime (Chennai, India), and PCR reagents were from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and Takara Bio, Inc. (Otsu, Japan).

Limbal stem cell separation and culture. The Institutional Ethics Committee Board of SDM College of Medical Sciences and Hospital (Manjushree Nagar, Dharwad, Karnataka, India; permit no. 065, ECR/683/INST/KA/2014) approved the study protocol. Informed consent was obtained from the donor patient family. After using the cadaveric donor eye for the graft, spare limbal tissues were collected from the SDM College of Medical Sciences Eye Bank (Dharwad, India) from May 2015 to March 2016. Concurrently, donor blood was collected and screened for hepatitis B and C and human immunodeficiency virus antigens. The corneo-scleral button of a cadaver was excised from fresh globes and limbal tissue with adjacent peripheral cornea was treated with trypsin to remove all epithelial cells from the peripheral cornea. The intact limbal basal epithelium was removed by incubation with Dispase II purchased from HiMedia Laboratories Pvt. Ltd. Concurrently,

an explant culture of limbal stem cells was prepared with the spare palisade of Vogt tissue in the respective medium.

Amniotic membranes used as scaffolds were harvested from the placenta of healthy women delivered by elective cesarean section at full-term. Consent for the use of the placenta was obtained from the mothers before delivery.

Dental pulp stem cell separation and culture. Human permanent teeth for dental pulp extraction were obtained during prophylactic orthodontic treatment or wisdom tooth extraction. Informed consent was obtained from the patients and their parents. The pulps were minced into small pieces 0.1-0.2 mm in diameter in DMEM. Small pieces of pulp tissue were dispersed in dispase II and digested by trypsin. These dispersed cells were seeded into 6-well plates containing DMEM. DPSCs were cultured at 37°C with 5% CO₂ and then characterized for mesenchymal stem cell surface markers Thy-1, homing cell adhesion molecule (HCAM), C-kit, cluster of differentiation (CD)-24 and CD-45 by western blot analysis (data not shown).

Total cell extraction and western blotting. For the stem cell surface marker expression analysis of limbal and DPSCs, cells were collected and lysed in extraction buffer (25 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, and 0.1 mM PMSF) with several freeze-thaw cycles. The lysates were then centrifuged at 12,000 x g for 15 min at 4°C, and the supernatants were collected. The protein content was measured via a bicinchoninic acid protein assay using various concentrations of serum albumin as standards as previously described (23). Total protein (40 µg/lane) was separated on a 10% Bis-Tris polyacrylamide gel in Tris-HCl buffer. Separated proteins were transferred to nitrocellulose membranes, blocked with 1% bovine serum albumin (BSA) from HiMedia Laboratories Pvt. Ltd. at room temperature for 1 h. The membranes were hybridized with limbal stem cell and corneal epithelial marker antibodies (diluted at 1:1,000 in 1% BSA) against ABCG2 (cat. no. sc-377176), vimentin (cat. no. sc-32322), cytokeratin 12 (cat. no. sc-25722), E-cadherin (cat. no. sc-7870), Thy-1 (cat. no. sc-59396), HCAM (cat. no. sc-65265), c-kit (cat. no. sc-13508), CD24 (cat. no. sc-70598), CD45 (cat. no. sc-1178) and the loading control GAPDH (cat. no. sc-166574; all from Santa Cruz Biotechnology, Inc.) overnight at 4°C. The appropriate horseradish peroxidase-conjugated goat-anti-mouse (cat. no. 170-6516; Bio-Rad Laboratories, Inc.) or goat-anti-rabbit (cat. no. 1706515, Bio-Rad Laboratories, Inc.) secondary antibodies (1:3,000) were incubated with the respective membranes for 2 h at room temperature. The membranes were developed using ECL Plus (Bio-Rad Laboratories, Inc.), and images were obtained by autoradiography using a G:BOX ChemiXX9 system from Syngene Europe (Cambridge, UK). GeneSys V1.6.5.0 software (Syngene, Frederick, MD, USA) was used to quantify protein expression.

Immunocytochemistry. Cells were detached from 100-mm plates with 0.25% trypsin-EDTA (Hi Media) and washed twice with PBS. Cover slips sterilized with methanol were placed into 6-well plates; 1-3x10⁵ cells were seeded into each well and grown overnight in respective growth media at 37°C with 5% CO₂. Cells were fixed and permeabilized with 100%

Table I. List of primers and their sequences used in the study.

Gene name	Primer sequence (5'-3')	Length (bp)
E-Cadherin	FP: TGGGCTGGACCGAGAGAGTT	20
	RP: ATCTCCAGCCAGTTGGCAGT	20
Connexin 43	FP: GCGTGAGGAAAGTACCAAAC	20
	RP: GGGCAACCTTGAGTTCTTCC	20
Keratin 12	FP: CTACCTGGATAAGGTGCGAGCT	22
	RP: TCTCGCATTGTCAATCTGCA	20
Keratin 14	FP: CATGAGTGTGGAAGCCGACAT	21
	RP: CCCTCTCAGGGCATTCTCTC	21
Pax 6	FP: GTGTCCAACGGATGTGTGAG	20
	RP: CTAGCCAGGTTGCGAAGAAC	20
GAPDH	FP: GAGCGAGATCCCTCCAA	18
	RP: ACTGTGGTCATGAGTCCTTC	20

FP, forward primer; RP, reverse primer.

methanol for 15 min at room temperature. The cells were then washed twice with PBS and blocked with 1% BSA in PBS. After 30 min of incubation on ice, the aforementioned antibodies against ABCG2, E-cadherin, cytokeratin 12 and vimentin were added at 1:50 dilution in 1% BSA, and the cells were incubated at 4°C overnight. The following steps were performed in the dark. Subsequently, secondary antibodies (Alexa Fluor 647 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG) was added at 1:200 dilution in 1% BSA, incubated for 1 h at room temperature and then washed three times with PBS for 5 min in the dark. Counterstaining was performed using DAPI (1 µg/ml) for 2 min in room temperature at dark. The cover slips were transferred upside down onto glass slides with one drop of polyvinyl alcohol in PBS, and coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). Images were acquired using a Zeiss LSM 510-META confocal microscope (Zeiss AG, Oberkochen, Germany).

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After DNase treatment, 1 µg total RNA was reverse-transcribed using a RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific, Inc.) in a total volume of 20 µl reaction mixture according to the manufacturer's instructions. qPCR was performed with Verso SYBR Green I dye (Thermo Fischer Scientific, Inc. A) as described by the manufacturer in a final volume of 25 µl in a Cepheid SmartCycler II. qPCR was conducted as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and primer melting temperature for 30 sec (Connexin 43, 63°C; Keratin 12, Keratin 14 and PAX6, 60°C; E-Cadherin, 55°C; and GAPDH, 54°C), and a final step of 68°C for 30 sec. The expression of each gene was normalized to that of GAPDH, which served as a loading control. The $2^{-\Delta\Delta C_q}$ method was used to determine the relative gene

expression (24). The primer sequences used to amplify each gene are listed in Table I.

Statistical analysis. qPCR analysis results are presented as mean values, with error bars representing the 95% confidence intervals. Immunocytochemistry and western blot quantification analyses are presented as averages, with error bars representing the standard deviation. Where applicable, the results were compared using Tukey's and Sidak's multiple comparison tests following two-way analysis of variance (ANOVA) with $P < 0.05$ as the level of significance. ImageJ 1.52h (National Institutes of Health, Bethesda, MD, USA) was used to analyse and quantify the immunocytochemistry data and western blot data was quantified using densitometric scanning. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used to compare the data sets.

Results

Morphological characteristics of limbal and dental pulp stem cells. Morphologically, limbal and dental pulp stem cells formed an epithelial-like migrated monolayer of cells in different regions of the culture flask within a few hours after seeding. The shape and culture pattern of both cell phenotypes were similar (Fig. 1A and B). The cells growing on the culture flask in DMEM became confluent within three weeks of seeding; however, cells growing in limbal stem cell (LSC) media with an amniotic membrane scaffold (Fig. 1C) took longer to become confluent, but exhibited the same morphological phenotypes (Fig. 1D and E). The approximate cell yield after three weeks of growth was 1×10^4 cells/mm³ (counted manually using Neubauer's chamber).

Molecular characterization of limbal and dental pulp stem cells. To verify the potential of DPSCs to function as limbal stem cells in the respective microenvironment, the relative expression of specific molecular markers such as e-cadherin,

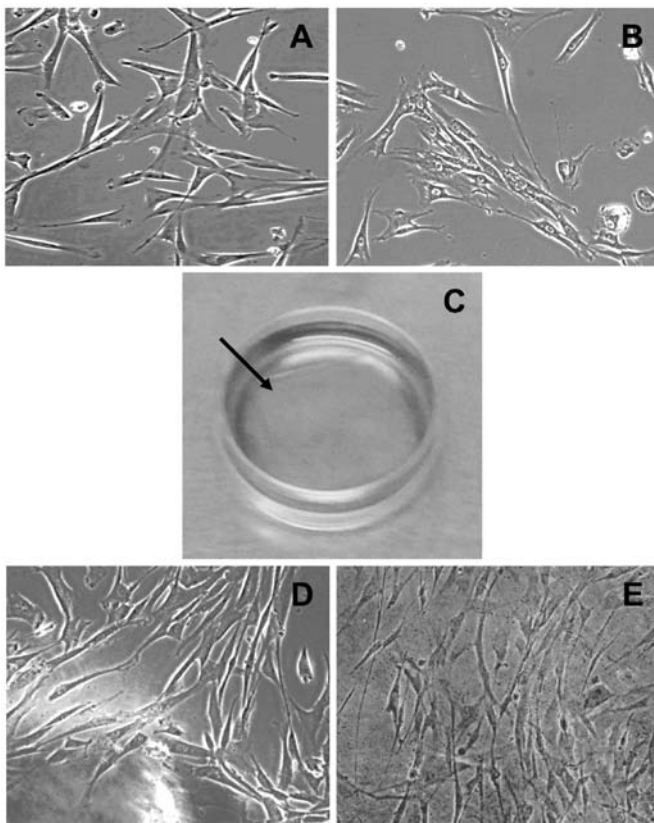


Figure 1. Homogenous population of cultured (A) limbal and (B) dental pulp stem cells on the cell culture flask. (C) Cured amniotic membrane scaffold on the glass slide, which was used to grow limbal stem cells. Growth lineage of (D) limbal and (E) dental pulp stem cells on the amniotic membrane.

connexin 43, keratin 12, keratin 14, and pax6 genes (25,26) to GAPDH gene in limbal and dental pulp stem cells grown in DME medium, LSC media, and LSC media with an amniotic membrane as scaffold (LSC-AM) was evaluated by RT-PCR (Fig. 2). As expected, irrespective of the medium in which the cells were grown, DPSCs expressed all stem cell markers at similar levels as in LSCs. However, dental pulp and limbal stem cells grown in LSC media with the AM exhibited good expression of stem cell markers compared with cells grown in DMEM and LSC media only, with the exception of keratin 12 and E-cadherin. Immunofluorescence analysis of DPSCs grown in LSC media revealed positive expression for limbal-specific epithelial stem cell markers such as vimentin, ABCG2, and keratin 12 (Fig. 3A) (25). Similarly, expression of all stem cell markers in limbal and dental pulp stem cells was confirmed by western blot analyses (Fig. 3B). Although molecular expression is quite low at the transcript level in DPSCs, the functional protein level expression according to immunocytochemistry and western blot analyses revealed that stem cell and corneal differentiation molecule levels were significantly ($P \leq 0.05$) high. Thus, these cells can function as limbal stem cells in the appropriate microenvironment.

Discussion

Stem cell deficiency in the limbus region of the eye can reduce vision and seriously affect the quality of life. Several

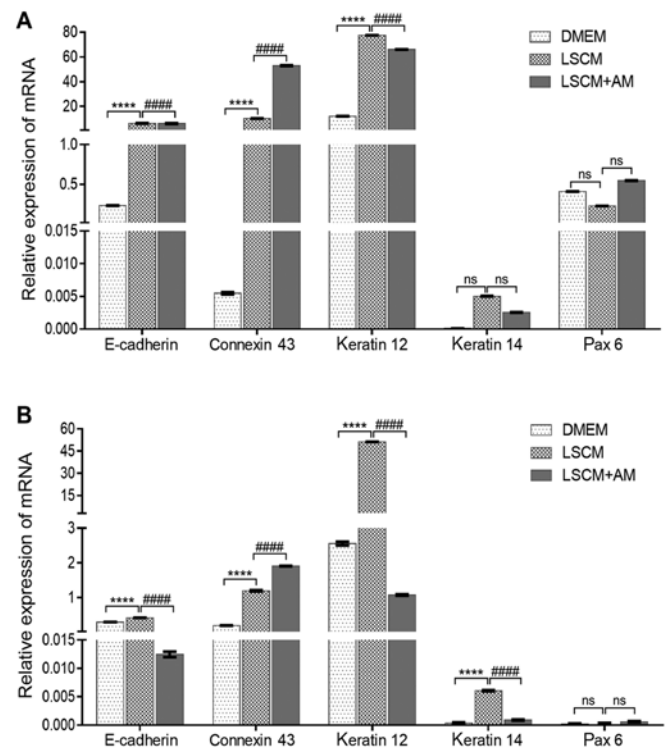


Figure 2. Relative message expression of stem cell markers in (A) limbal and (B) dental pulp stem cells grown in DMEM, LSC media, and LSC media with amniotic membrane. Three simultaneous experiments were conducted and pooled data is presented. Error bars represent the 95% confidence interval of data. Significant differences were determined using Tukey's multiple comparison test. **** $P < 0.0001$; #### $P < 0.0001$. ns, not significant; DMEM, Dulbecco's modified Eagle's medium; LSC, in limbal stem cell.

treatment modalities have been developed for limbal stem cell deficiency (LSCD). The development of methods for *ex vivo* engineering of stem cells provided better treatment options for LSCD patients involving transplantation of *in vitro* cultured LSCs (14,27). Studies have revealed that cells with high *in vitro* self-renewal and multi-potential differentiation capacity are good candidates for cell therapy. Therefore, various tissues including adipose, umbilical cord blood, amniotic fluid, and placental tissues, peripheral blood, Wharton's jelly, dental pulp, oral mucosa, corneal epithelium, dermal fibroblasts, cartilage and bone marrow, have been evaluated for deriving stem cells for autogenic transplantation (16-19). Amniotic membrane transplantation (AMT) has become a popular therapy for ocular surface damage and can be used to treat partial LSCD on its own or to treat total LSCD by limbal allografting (13). Drug therapy can be combined with AMT, such as the use of antivirals and steroid therapies to arrest inflammation and treat ocular herpes (28). Additionally, the development of a suitable culture system using different carriers of sheet, culture medium, or feeder layers are essential to avoid immune reactivity and produce high-quality stem cells.

Many procedures have been developed for treating LSCD and restoring at least some functional vision. In the present study, DPSCs were cultured in LSC media supplemented with an amniotic membrane to induce regeneration of limbal stem cells. DPSCs can be easily accessed during tooth extraction or minimally invasive pulpectomy. Thus,

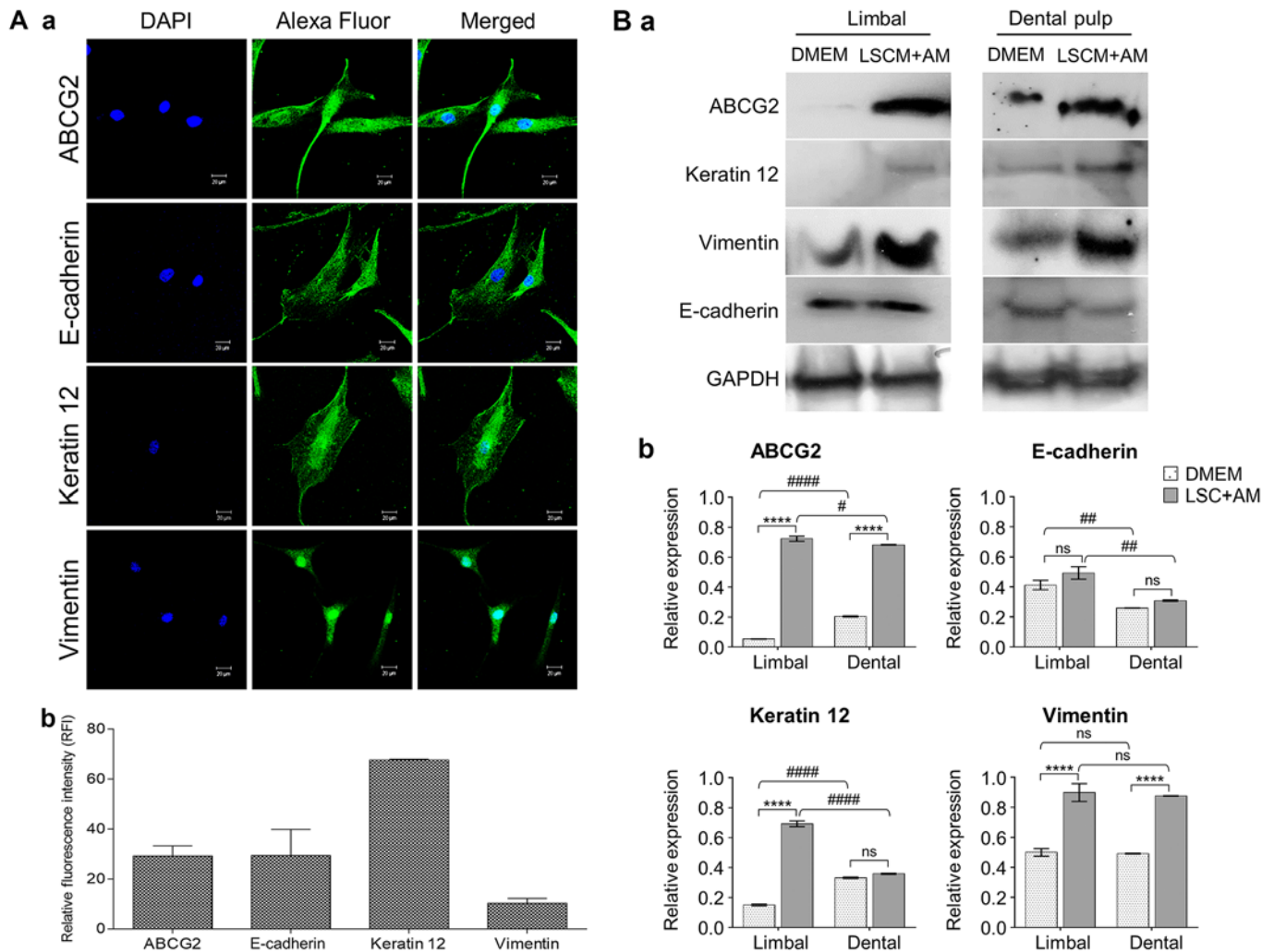


Figure 3. (A-a) Immunofluorescence labeling revealing the expression of stem cell markers in dental pulp stem cells grown in LSC media with amniotic membrane. (A-b) Quantitative expression analysis of immunofluorescence data. Error bars represent the 95% confidence interval of data. (B-a) Western blot analysis revealing the expression of stem cell markers in limbal and dental pulp stem cells grown in LSC media with amniotic membrane. (B-b) Quantitative data corresponding to western blot expression of respective stem cells and corneal epithelial differentiation markers. The data presented are from a total of three experiments. The epithelial differentiation markers Keratin-12 and E-Cadherin expression in dental pulp stem cells were statistically significant (two-way ANOVA, Sidak's multiple comparison test) compared with limbal stem cells grown in LSC media with amniotic membrane scaffold. **** $P < 0.0001$; # $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$. ns, not significant; Error bars represent the 95% confidence interval of data. LSC, in limbal stem cell; ANOVA, analysis of variance.

there is no risk of limbal grafting and harvesting epithelial/limbal cells from a healthy eye is unnecessary (29). Previously, Karaöz *et al* (30) revealed that DPSCs have properties of epithelial stem cells and that the expression of several markers was similar to that in bone marrow stem cells. Furthermore, they found that DPSCs have a higher rate of proliferation and stronger neural and epithelial stem cell properties, including the expression of corneal epithelial specific cytokeratin 12 (31). Spath *et al* (32) revealed that the explant method of DPSC isolation enhanced the cell differentiation abilities compared to the enzymatic digestion method, whereas Kerkis and Caplan (33) revealed no difference between the two methods. The methods of isolation and selection are key determinants in the transdifferentiation success of stem cell lines (34,35). Therefore, the explant protocol was used successfully and demonstrated the differentiation of DPSCs to limbal stem cells in the present study. DPSCs grown in the presence of the amniotic membrane revealed similar morphological

and molecular characteristics as limbal stem cells. In the present study, the dental pulp stem cells were characterized with the respective marker analysis by western blot assay. However, fluorescence-activated cell sorting should be conducted to ensure the homogeneous cell population of DPSCs, which may further enhance specific epithelial regeneration. Multipotency of isolated DPSCs through differentiation along adipogenic, chondrogenic, and osteogenic lineages is not elucidated as it is out of the scope of the present preliminary study. Although the data presented evaluates the use of dental pulp stem cells in bilateral LSCD cases, further additional information concerning the functionality of LSC is required before its application for potential therapeutic use. In recent studies, researchers used different media factors and in other studies different scaffolds such as contact lenses were used (30,32). Although use of amniotic membrane in corneal treatment is an old practice, combination of amniotic membrane as scaffold to grow and tune these dental pulp stem cells to limbal stem

cells is unique and an easy standardization protocol, which was adapted in the present study (31,36).

In summary, it was demonstrated that stem cells can be easily isolated from dental pulp and used for limbal stem cell differentiation. Additionally, these transdifferentiated DPSCs could provide a good barrier between corneal and conjunctival epithelia and prevent conjunctivalization of the cornea compared to other cell sources.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SP, MP and PS conceived and planned the experiments. SP, PS, CD, AB, MP and SK conducted the experiments. PP, SP, VP, VK and PS contributed to the analysis and interpretation of the results. SP and PS investigated and supervised the findings of this study. PS wrote the manuscript with support from SP, PP and VK. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study protocol was approved by SDM College of Medical Sciences and Hospital (Manjushree Nagar, Dharwad, Karnataka, India; permit no. 065, ECR/683/INST/KA/2014). Informed consent was obtained from the donor patient family. Consent for the use of the placenta was obtained from the mothers before delivery. Informed consent was obtained from the patients and their parents for dental pulp extraction.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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