Biochemical and Biophysical Research Communications 386 (2009) 135–139

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of novel epithelial stem cell-like cells in human deciduous dental pulp

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ARTICLE INFO

Article history:
Received 21 May 2009
Available online 6 June 2009

Keywords:
Dental pulp epithelial stem cells
Deciduous dental pulp
Epithelial stem cells
ABCG2
Bmi-1
ΔNp63
p75

ABSTRACT

It is well known that interactions between epithelial components and mesenchymal components are essential for tooth development. Therefore, it has been postulated that both types of stem cells might be involved in the regeneration of dental hard tissues. Recently, mesenchymal dental pulp stem cells that have odontogenic potential were identified from human dental pulp. However, the existence of epithelial cells has never been reported in human dental pulp. In the present study, we isolated and characterized epithelial cell-like cells from human deciduous dental pulp. They had characteristic epithelial morphology and expressed epithelial markers. Moreover, they expressed epithelial stem cell-related genes such as ABCG2, Bmi-1, ΔNp63, and p75. Taken together, our findings suggest that epithelial stem cell-like cells might exist in human deciduous dental pulp and might play a role as an epithelial component for the repair or regeneration of teeth.

Introduction

A series of reciprocal interactions between the oral epithelium and mesenchymal cells from the cranial neural crest regulate the process of tooth development: the initiation of tooth development, the differentiation of ameloblasts and odontoblasts, and the morphogenesis of teeth. Recently, dental pulp stem cells (DPSC) were identified from human dental pulp; these turned out to be adult stem cells, with the capability to differentiate into several types of cells. Furthermore, they had odontogenic potential as a mesenchymal component [1]. It has also been reported that mouse incisors contain epithelial stem cells that participate in the continuing growth of incisors that occurs throughout the murine lifespan [2,3]. Although it has been postulated that a second type of stem cell might be involved in tooth repair in human, cells of an epithelial lineage have never been reported in the human dental pulp.

It has been revealed that epithelial stem cells might be responsible for the continuous regeneration of tissues such as hair, skin, and gut. They reside in specific niches and proliferate very slowly [4–9]. Stimuli for repair or regeneration can cause them to proliferate actively and to differentiate into certain cell-types [2,3]. To identify epithelial stem cells, various stem cell markers have been proposed and can be categorized into three groups [10]: nuclear proteins such as the transcription factor p63; cell membrane or transmembrane proteins, including integrins (integrin β1, α6, and α9), receptors (epidermal growth factor receptor, CD71), and drug-resistance transporters (ABCG2); and cytoplasmic proteins such as cytokeratins (cytokeratin 19) and α-enolase. Although these markers are not sufficient to identify epithelial stem cells, their expression may serve as an important indicator.

Although human teeth do not grow continuously after maturity, they undergo various challenges such as attacks from pathogenic organisms and mechanical stress. Therefore, regeneration or repair might be necessary to maintain teeth. It has been postulated that human dental pulp contains various types of adult stem cells that serve this function. In this study, we isolated and cultured epithelial cell-like cells from human deciduous dental pulp (DPESC). Moreover, they expressed epithelial stem cell phenotype.

Materials and methods

Primary isolation and culture of human DPESCs. Deciduous teeth were delivered in Hank's balanced salt solution (HBSS; Welgene, Dae-gu, Korea) supplemented with 3% antibiotics/antimycotics (Gibco, Grand Island, NY) at 4 °C. To avoid the contamination of gingival tissues, early extracted deciduous teeth were selected. Dental pulp tissues were extracted with fine forceps and minced in 1 mg/ml of collagenase type I and 2.4 mg/ml of dispase (Gibco) at 37 °C for 1 h. After inactivation of enzymes, cells were washed

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.05.141

two times with keratinocyte basal medium (KBM; Lonza Rockland, Rock Island, ME). Single-cell suspensions were plated and maintained in serum-free keratinocyte growth medium (KGM; Lonza) with supplements provided. The medium was changed every 2 days, and cells were sub-cultured at 70% confluency. At each passage, cells were counted and photographed; the population doubling level (PDL) was then calculated.

We also isolated mesenchymal stem cells from deciduous dental pulp (DPSC); after enzyme digestion, single-cell suspensions were cultured in α-MEM (Hyclone, Road Logan, Utah) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% antibiotics/antimycotics. The medium was changed every 3 days.

FACS analysis. For FACS analysis, cells were detached and washed with PBS supplemented with 2% FBS. The following antibodies were used; FITC-conjugated mouse anti-human CD14, CD31, CD44, and CD45; PE-conjugated mouse anti-human CD29, CD73, and CD117; PE.Cy5-conjugated mouse anti-human CD90; APC-conjugated mouse anti-human CD34 and HLA-DR; streptoavidin conjugated APC (all from BD PharMingen, San Jose, CA); APC-conjugated mouse anti-human CD105 (eBioscience, San Diego, CA); and biotin-conjugated mouse anti-human HLA class I (BD PharMingen). Quantities of 100,000–500,000 cells were incubated with the primary antibody for 30 min on ice. After washing, cells were fixed with 4% paraformaldehyde at 4 °C before analysis. Fluorescence intensity was measured on a FACS Calibur (BD PharMingen), and data were analyzed using FLOWJO software (Tree Star, Inc., Ashland, OR).

Immunofluorescence staining. Cells were detached, spotted on gelatin-coated slides, and fixed with ice-cold methanol for 10 min at −20 °C. Cells were washed with PBS, and then blocked with 10% normal goat serum for 1 h at room temperature (RT). We used rabbit-anti-E-cadherin (1:200; Santa Cruz Biotechnology Inc., CA), rabbit-anti-N-cadherin (1:200; Santa Cruz); mouse anti-vimentin (1:300; DAKO, Glostrup, Denmark), anti-pan-cytokeratin (1:50; DAKO), anti-desmin (1:100; DAKO); Alexa 488-conjugated goat-anti-rabbit IgG (1:1000; Molecular Probes, Eugene, OR), and Texas Red-conjugated goat-anti mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Primary antibodies were applied overnight at 4 °C. After washing with PBS three times, secondary antibodies were applied for 1 h at RT and washed with PBS three times. DAPI (Sigma–Aldrich, St. Louis, MO) was used to stain nuclei, and slides were mounted in fluorescent mounting medium (DAKO). Cells were observed under a confocal laser scanning microscope (Fluoview FV 300, Olympus, Japan).

RT-PCR. Total RNA was obtained from DPESCs using TRIzol (Invitrogen, Carlsbad, California). Total RNA (5 μg) was reverse-transcribed with M-MLV (Invitrogen) and Oligo dT by 1 h incubation at 42 °C, followed by 10 min incubation at 90 °C, and the cDNA was used as a template for PCR. PCR was performed with i-MAXII (Intron, Sungnam, Korea). Conditions for PCR and the oligonucleotide sequences of the gene-specific primer pairs used for amplifica-

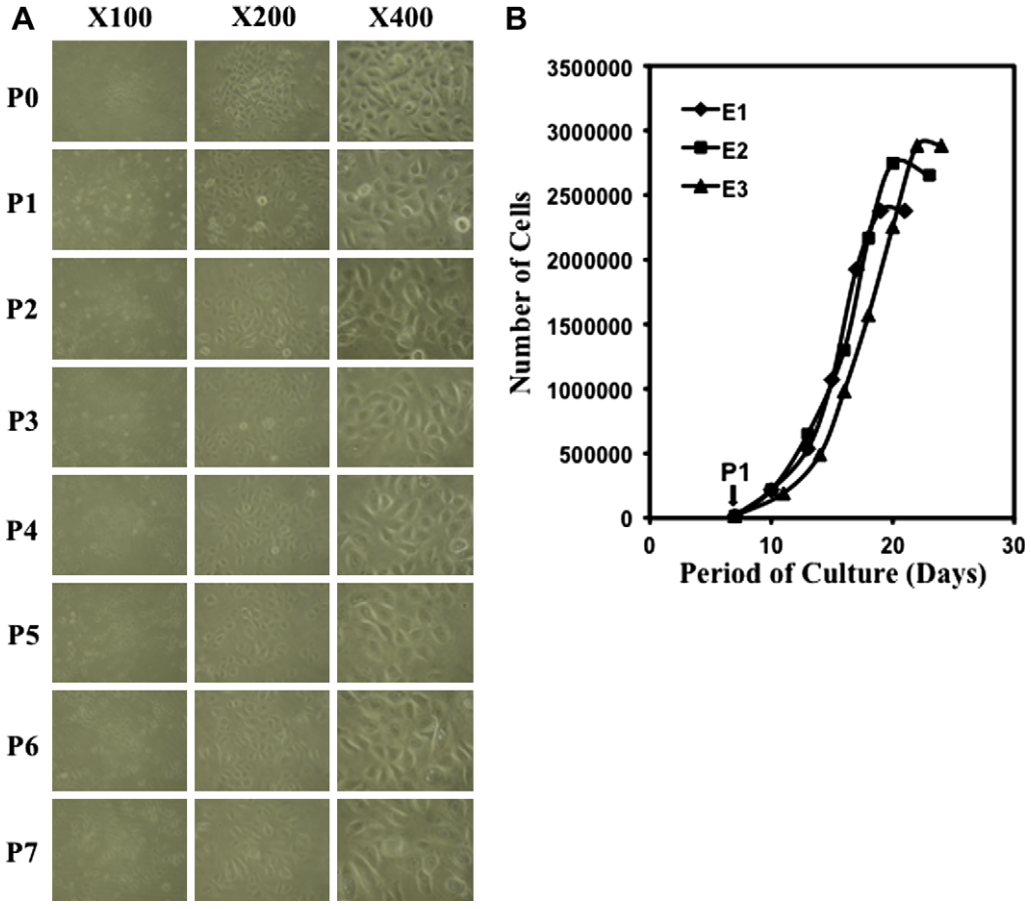

Fig. 1. Microscopic features and growth curves of human DPESCs. (A) DPESCs showed typical epithelial cell-like morphology and clonal expansion. The morphology was maintained through subculture, but at high passage numbers, DPESCs became flattened and contained many vacuoles. Magnifications are X100, X200, and X400. (B) Primary cells at P0 consisted of two distinct types of cells, and the fibroblast-like cells rapidly disappeared from the culture. At passage 1 (P1), 15,000 cells/cm^2 with an epithelial cell-like appearance were plated, and DPESCs were counted after passage 1. The growths of three independent lines of DPESCs (E1, E2, and E3) are shown.

tion of ABCG2, Bmi-1, ΔNp63, and p75 were as previously described [10–12]. PCR products were separated on a 1% agarose gel containing ethidium bromide.

Results

Primary isolation of human DPESCs

Human deciduous pulp tissue was digested by collagenase and dispase, and single-cell suspensions were cultured in serum-free KGM. Primary isolated cells initially had two distinct morphologies, one having a fibroblast-like appearance and the other with a more rounded and epithelial cell-like appearance. After passaging, the fibroblast-like cells rapidly disappeared from culture. The primarily isolated epithelial cell-like DPESCs formed adherent colonies that began to appear 3–5 days after the initial plating. The cells became almost confluent at about 7 days and were then sub-cultured. These DPESCs remained as a monolayer on the culture plate during the sub-culturing process. DPESCs showed typical epithelial cell-like, cuboidal- or polygonal-shaped appearances and colony-forming proliferation (Fig. 1A). The morphology of the cells was maintained through sub-cultures under normal epithelial cell culture conditions. To determinate proliferation properties, we cultured the cells for more than 3 weeks. The cells grew well and showed clonal expansion. Proliferation was continued until the sixth passage (Fig. 1B). Primary isolation of dental pulp cells at passage 0 was heterogeneous, but the fibroblast-like cells rapidly disappeared from the culture. Hence, at passage 1, 15,000 cells/cm^2 with an epithelial cell-like appearance were plated, and cell counting and PDL calculation were done after passage 1. The average population doubling time was approximately 68.3 h. After passage 5, the number and size of vesicles in the cytoplasm of DPESCs were increased, and the borders of cell–cell membranes began to disappear. At passage 7, DPESCs ceased to proliferate, but the attached cells were not dying (data not shown).

FACS analysis and immunofluorescence staining of human DPESCs

To characterize the DPESC cells, immunophenotyping profiling was performed at passage 3 in order to detect specific cell surface antigen sets for mesenchymal stem cells using flow cytometry. DPESCs had a different expression profile than the DPSCs. In DPSCs, which are adult mesenchymal stem cells, mesenchymal markers such as CD29, CD44, CD73, CD90, and CD105 were positive, and hematopoietic and endothelial markers such as CD14, CD31, CD34, CD45, and HLA-DR were negative (Fig. 2B). This pattern of expression is commonly found in mesenchymal stem cells. In DPESCs, the expression of CD44, CD90, and CD105 was decreased, but other mesenchymal markers were similarly expressed by both DPESCs and DPSCs. Also, hematopoietic and endothelial markers were still negative (Fig. 2A). Therefore, we confirmed that DPESCs were not mesenchymal-like cells and that they did not originate from hematopoietic or endothelial cells.

To further characterize DPESCs, we conducted immunofluorescence microscopy. The expression of E-cadherin and pan-cytokeratin as epithelial markers, and that of N-cadherin, desmin, and vimentin as muscle and mesenchymal markers, was investigated. DPESCs were positive for E-cadherin and pan-cytokeratin but negative for N-cadherin and desmin. Vimentin was weakly detected. We concluded that DPESCs were a different cell population from DPSCs and that they had epithelial cell characteristics (Fig. 3).

Gene expression of epithelial stem cell markers in DPESCs

To verify that DPESCs have epithelial stem cell-like characteristics, we examined the expressions of four epithelial stem cell markers in those cells. RT-PCR analysis of ABCG2, Bmi-1, ΔNp63,

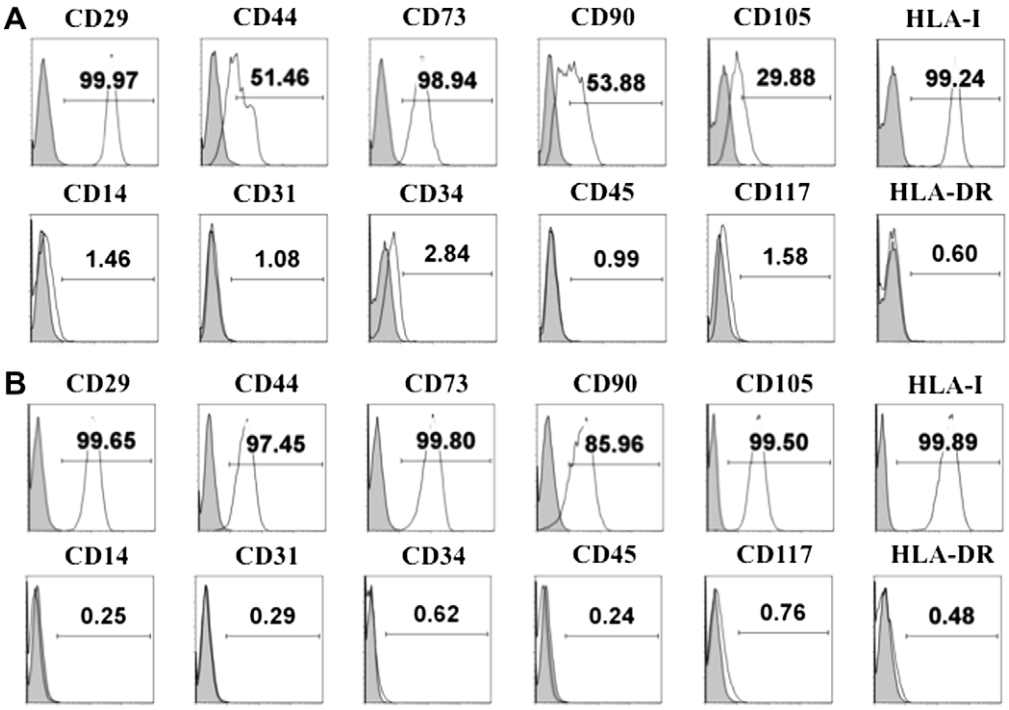

Fig. 2. Immunophenotypical characterization of human DPESCs and DPSCs. DPESCs and DPSCs were trypsinized, labeled with antibodies against the surface antigens indicated and analyzed by flow cytometry. The expression of CD44, CD90, and CD105 was decreased in DPESCs (A) compared to DPSCs (B). A single representative example of three separate samples of DPESCs and DPSCs is shown.

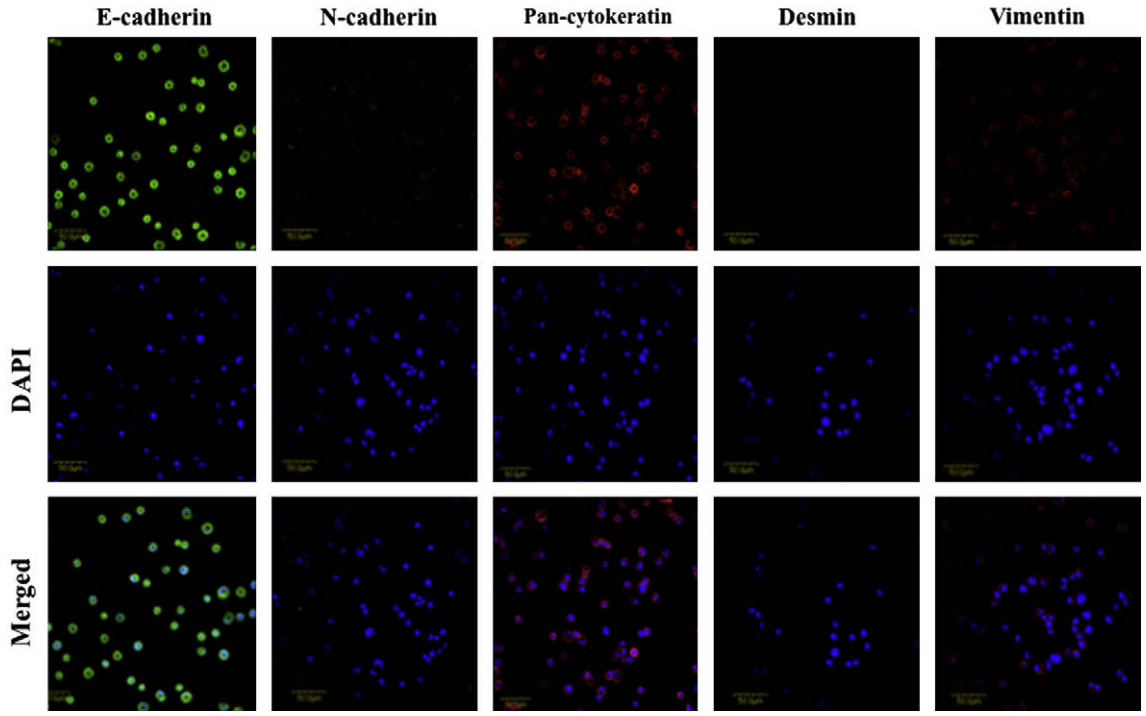

Fig. 3. Immunofluorescence microscopy of human DPESCs. The human DPESCs were stained with E-cadherin, N-cadherin, pan-cytokeratin, desmin, and vimentin. They were positive for E-cadherin and pan-cytokeratin but negative for N-cadherin and desmin. Vimentin was weakly detected. Nuclei were labeled with DAPI (blue). Magnification is X400. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and p75 demonstrated that these four epithelial stem cell markers were highly expressed in three independent DPESC lines (Fig. 4). These findings suggest that the DPESCs might have an epithelial stem cell phenotype.

Discussion

Human dental pulp contains nerves, blood vessels, and a wide variety of cells such as fibroblasts, odontoblasts, macrophages, granulocytes, mast cells and plasma cells. These cells and tissues combine with the extracellular matrix to constitute the three-dimensional dental pulp structure. The dental pulp is surrounded by dental hard tissues that must be sectioned or decalcified to identify or isolate cells in dental pulp. Partly due to these difficulties in isolating cells from human dental pulp, the existence of epithelial cells in human dental pulp has not been reported.

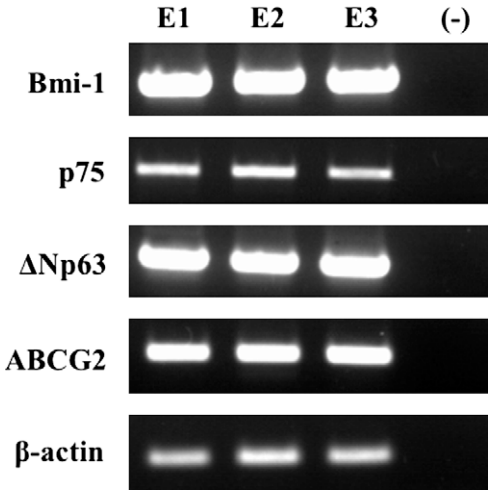

Fig. 4. Epithelial stem cell-related gene expressions of human DPESCs. Three independent lines of human DPESCs (E1, E2, and E3) at passage 3 were analyzed using RT-PCR. All samples were positive for Bmi-1, p75, ΔNp63, and ABCG2, which are epithelial stem cell markers. (−), no template as a negative control.

To identify epithelial components in human dental pulp, we carried out enzymatic digestion of deciduous dental pulp and primary cell cultures in serum-free KGM. The yield of primary epithelial cells was very low, suggesting that epithelial components were rare and sensitive to environmental or culture conditions. To rule out the contamination of epithelial cells from the outside of the deciduous teeth, we carefully peeled off the tissue remnants from the outside of the teeth and cleaned the surfaces of the teeth. Although fibroblast-like cells were not able to proliferate well in our culture conditions, it was initially difficult to completely remove fibroblast-like cells. However, these fibroblast-like cells rapidly disappeared from the culture. Therefore, serum-free KGM was efficient for isolation of epithelial cells from human dental pulp. Our immunofluorescent data and FACS analysis showed that these were epithelial cell-type cells and different from DPSCs.

The most interesting result was the expression of epithelial stem cell-related genes in DPESCs. RT-PCR showed that they highly expressed ABCG2, Bmi-1, ΔNp63, and p75. ABCG2, a member of the ABC transporter family and formally known as breast cancer resistance protein 1 (BCRP1), has been identified as a molecular determinant of bone marrow stem cells. Recently, it has been reported that expression of ABCG2 is a conserved feature of stem cells from a wide variety of tissues [13–15]; thus, this molecule has been proposed as a universal stem cell marker [13,16]. The p75 molecule, a low-affinity neurotrophin receptor, is a member of the tumor necrosis factor receptor superfamily [17]; there is evidence that p75 is involved in controlling the fate of murine keratinocyte stem cells [18] and that it is a marker for esophageal

keratinocyte stem cells *in vitro* [19]. Recently, the nuclear transcription factor p63, a member of the p53 family, was proposed to be a marker of epithelial stem cells. The p63 protein is highly expressed in the basal cells of many human epithelial tissues, and its truncated dominant-negative isoform ΔNp63 is the predominant form in these cells [20–22]. It has been reported that p63 expression is associated with proliferative potential in human keratinocytes [23,24]. Bmi-1 is a member of the polycomb group of genes, which have an essential role in embryogenesis and regulation of the cell cycle and lymphopoiesis [25–27]. It is known that Bmi-1 is expressed in hematopoietic stem cells and in stem cells from other tissues [28,29]. Although the molecular markers and their specific roles for epithelial stem cells has not been fully elucidated, it has been postulated that the expression of ABCG2, Bmi-1, ΔNp63, and p75 in DPESCs may be important for their proliferation and differentiation.

There is currently no evidence for the existence of epithelial cells in the dental pulp. Our results demonstrate that epithelial cells can be isolated from human deciduous dental pulp and that they can be cultured in serum-free KGM. These cells had characteristic epithelial morphology and expressed epithelial markers. Moreover, they had an epithelial stem cell phenotype. Although it is necessary to elucidate functional roles for DPESCs, these findings suggest that epithelial stem cell-like cells might exist in human deciduous dental pulp and that they could play a role as an epithelial component for the repair or regeneration of teeth. This study may be the first report of epithelial components in human deciduous dental pulp.

Acknowledgments

We thank Dr. Jae Cheoun Lee, Children's Dental Center and CDC Baby Tooth Stem Cell Bank, Korea, for tooth samples used in this study. This work was supported by the Korea Science and Engineering Foundation grant funded by Ministry of Education, Science and Technology, Republic of Korea (No. M10646010001-08N4601-00110).

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