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Molecules and Cells

Expression Profile of the Stem Cell Markers in Human Hertwig's Epithelial Root Sheath/Epithelial Rests of Malassez Cells

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Hertwig's epithelial root sheath/Epithelial rests of Malassez (HERS/ERM) cells are unique epithelial cells in the periodontal ligament. They remain in periodontal tissues throughout the adult life, and it is expected that their functional role is to maintain the homeostasis of the periodontium through reciprocal interactions with other periodontal cells. In this study, we investigated whether HERS/ERM cells have primitive stem cell characteristics: those of embryonic stem cells as well as of epithelial stem cells. Primary HERS/ERM cells had typical epithelial cell morphology and characteristics and they maintained for more than five passages. They expressed epithelial stem cell-related genes: ABCG2, ΔNp63, p75, EpCAM, and Bmi-1. Moreover, the expression of embryonic stem cell markers such as Oct-4, Nanog, and SSEA-4 were detected. Next, we investigated whether the expression of these stem cell markers was maintained during the sub-culture process. HERS/ERM cells showed different expression levels of these stemness genes at each passage, but their expression was maintained throughout the passages. Taken together, our data suggest that a primary culture of HERS/ERM cells contains a population of primitive stem cells that express epithelial stem cell markers and embryonic stem cell markers. Furthermore, these cell populations were maintained during the sub-culturing process in our culture conditions. Therefore, our findings suggest that there is a strong possibility of accomplishing cementum tissue engineering with HERS/ERM cells.

INTRODUCTION

Tooth development is a complex process that takes place through reciprocal interactions between dental mesenchymal and dental epithelial cells. After crown formation, the inner and outer enamel epithelial cells develop a bi-layered epithelial sheath called Hertwig's epithelial root sheath (HERS). HERS cells remain in the epithelial rests of Malassez (ERM) or undergo apoptosis (Kaneko et al., 1999; Wentz et al., 1950). These HERS/ERM cells are a unique population of epithelial cells in the periodontal ligament and are believed to have a crucial role in cementum repair (Spouge, 1980). Furthermore, it was recently reported that HERS/ERM cells could be differentiated into cementoblasts through epithelial-mesenchymal transition (EMT) (Sonoyama et al., 2007). However, the functional roles of HERS/ERM cells and their interplay with dental mesenchymal stem cells (MSCs) in the periodontium are not fully understood.

The periodontium is the specialized complex tissue that circumscribes and supports the teeth and maintains the position of the tooth in the bones. It also protects the tooth from infections, masticatory forces, and mechanical stresses throughout the adult life. It is anticipated that stem cells might be involved in the repair and regeneration of the periodontium. Five types of human dental stem cells have been identified: dental pulp stem cells, stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from the apical papilla, and dental follicle progenitor cells (Gronthos et al., 2000; Miura et al., 2003; Morsczeck et al., 2005; Seo et al., 2004; Sonoyama et al., 2006; 2008). These dental stem cells are all MSCs, which are able to form dentin- or cementum-like structures, and they have a proliferation and differentiation ability that is similar to bone marrow-derived MSCs. However, there has been no report of epithelial stem cells (EpSCs) in the periodontium, which might be involved in the formation of cementum- or enamel-like structures.

Several evidences indicated that HERS/ERM cells have crucial roles in maintenance of tooth and periodontium during whole life as well as their development (Foster et al., 2007). Particularly EMT seemed to be involved in these processes (Sonoyama et al., 2007). Consequently, these findings suggested that HERS/ERM cells might contain stem cell characteristics as well, even though HERS/ERM cells are primarily epithelial cells. In this study, we investigated the stem cell phe-

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notypes of HERS/ERM cells. Primarily isolated human HERS/ERM cells, which had typical epithelial characteristics, showed embryonic stem cell (EmSC) phenotypes as well as epithelial stem cells (EpSC) phenotypes. These results suggest that human HERS/ERM cells contain a primitive stem cell population that might be more primitive than epithelial stem cells. Therefore, it is expected that HERS/ERM cells play a role as an epithelial component for the repair or regeneration of cementum, and they will be able to contribute to the tissue engineering of teeth and periodontium.

MATERIALS AND METHODS

Primary isolation and culture of human HERS cells

Human third molars were delivered in Hank's balanced salt solution (HBSS; Welgene, Korea) supplemented with 3% antibiotics/antimycotics (Gibco, USA) at 4°C. Periodontal ligament tissues were extracted with fine forceps; they were minced and incubated in 1 mg/ml of collagenase type I and 2.4 mg/ml of Dispase (Gibco) at 37°C for 1 h. To isolate the HERS/ERM cells after inactivation of the enzymes, the cells were washed two times with serum-free keratinocyte basal medium (KBM; Lonza Rockland, USA). Single-cell suspensions were plated in serum-free keratinocyte growth medium (KGM; Lonza) with provided supplements. After colonies of the HERS/ERM cells were formed, the medium was changed every two days, and the cells were sub-cultured at 70% confluency. At each passage, the cells were counted and photographed; the population doubling level (PDL) was calculated.

FACS analysis

For FACS analysis, the cells were harvested 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; streptavidin-conjugated PE; biotin-conjugated HLA class I (all from BD Pharmingen, USA); APC-conjugated mouse anti-human CD105 (eBioscience, USA); APC-conjugated mouse anti-human EpCAM; PE-conjugated goat anti-Oct-3/4; and APC-conjugated mouse anti-SSEA-4 (all from R&D Systems, USA); mouse anti-human ABCG2 (ebioscience); mouse anti-human p63; rabbit anti-human p75 (both from Santa Cruz Biotechnology, USA); Alexa Fluor 488 conjugated goat-anti mouse IgG and Alexa Fluor 647 conjugated goat-anti rabbit IgG (both from Molecular Probes, USA). Each primary antibody was incubated with 10,000 cells for 30 min on ice. After washing, the secondary antibody was applied for 30 min on ice. After washing, the cells were fixed with 4% paraformaldehyde at 4°C before analysis. For intracellular staining, the cells were fixed with 1% paraformaldehyde for 10 min and permeabilized with ice-cold methanol for 10 min before incubation with primary antibody. The fluorescence intensity was measured on a FACS Calibur (Becton Dickinson, USA), and data were analyzed using FLOWJO software (Tree Star, Inc., USA).

RT-PCR

The total RNA was obtained from HERS/ERM cells using an RNeasy Mini Kit (Qiagen, USA). The total RNA (2 μg) was reverse-transcribed with M-MLV (Invitrogen™, USA) and oligo dT during a 1-h incubation at 42°C followed by a 10-min incubation at 90°C. The resulting cDNA was used as the template for the PCR. The PCR was performed with i-MAXII (Intron, Korea). The conditions used for the PCR and the oligonucleotide sequences of the gene-specific primer pairs that were used for the amplification of the EpSC-related genes (ABCG2, Bmi-1, ΔNp63, and p75), the EmSC-related genes (Oct-4 and Nanog), and three germ layer-related genes (α-cardiac actin, α-fetoprotein, and neurofilament) have been previously described (Cho et al., 2010; de Paiva et al., 2005; Milyavsky et al., 2003; Nam and Lee, 2009; Pyle et al., 2006). The PCR products were separated on a 1.5% agarose gel containing ethidium bromide.

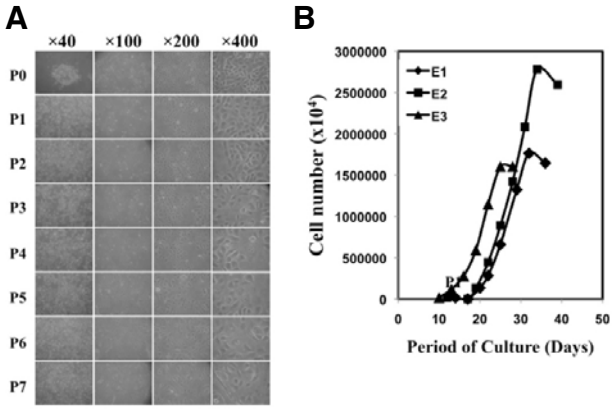

Fig. 1. Characterization of HERS/ERM cells. (A) HERS/ERM cells showed typical epithelial cell-like morphology and clonal growth. The morphology was maintained throughout the passages, but at later passages, the HERS/ERM cells became enlarged and flattened with many vacuoles. Magnifications are ×40, ×100, ×200, and ×400. (B) At each passage, 15,000 cells/cm^2 of HERS/ERM cells were plated, and after each passage, the PDL of HERS/ERM cells were calculated. The growths of three independent lines of HERS/ERM cells (E1, E2, and E3) are shown.

RESULTS

Primary isolation and characterization of HERS/ERM cells

To isolate human HERS/ERM cells from the periodontal ligaments, the tissues were minced and digested with collagenase and dispase. Single cell suspensions were plated and cultured in KGM. Initially, the primarily isolated cells showed two distinct morphologies: a bipolar fibroblast-like appearance and a rounded epithelial cell-like appearance. After passaging, the fibroblast-like cells disappeared from the culture. Consequently, HERS/ERM cells remained and formed colonies (Fig. 1A). The HERS/ERM cells grew well and continued to proliferate for more than five passages for four weeks. They became enlarged and showed a round morphology, and the number of vesicles was increased at later passages. Then they ceased to proliferate (Fig. 1B). The average population doubling time was approximately 81 h.

The immunophenotyping profile of HERS/ERM cells showed that the expression levels of the mesenchymal stem cell markers such as CD44, CD90, and CD105 were low but that CD29 and CD73 were similarly expressed; hematopoietic and endothelial markers were not detected (Fig. 2). Therefore, it was concluded that the HERS/ERM cells were neither mesenchymal cells nor hematopoietic or endothelial cells.

The expression of epithelial stem cell markers in HERS/ERM cells

To verify that HERS/ERM cells have epithelial stem cell-like characteristics, we examined the expressions of five EpSC markers in these cells. RT-PCR analysis of ABCG2, ΔNp63,

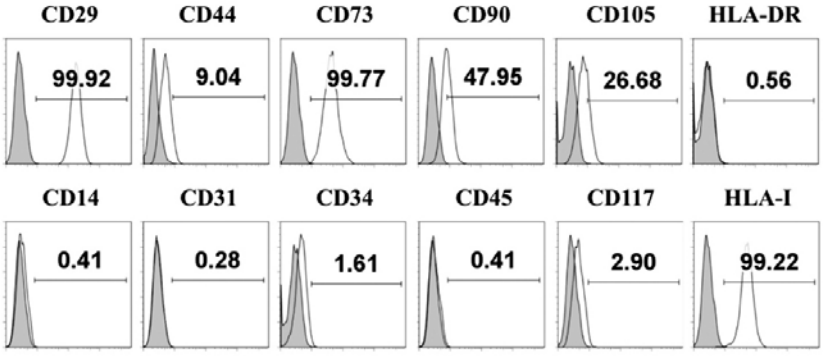

Fig. 2. Immunophenotyping profile of HERS/ERM cells. To determine immunophenotypes of HERS/ERM cells, they were analyzed with mesenchymal, endothelial, and hematopoietic markers by flow cytometry. The expression of CD44, CD90, and CD105 in the HERS/ERM cells was low. Endothelial and hematopoietic markers were not detected. A single representative example of three separate HERS/ERM cells is shown.

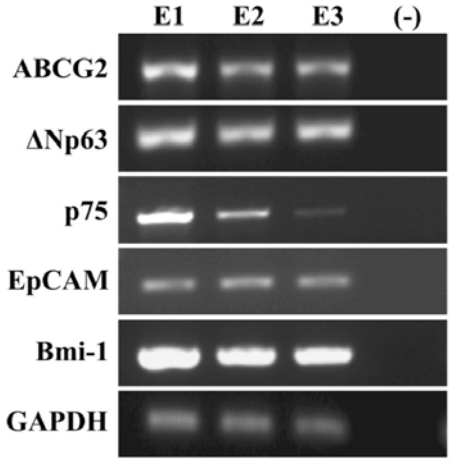

Fig. 3. EpSC-related gene expression of human HERS/ERM cells. Three independent lines of human HERS/ERM cells (E1, E2, and E3) at passage 2 were analyzed using RT-PCR. All samples were positive for ABCG2, ΔNp63, p75, EpCAM, and Bmi-1, which are epithelial stem cell markers. (-), no template as a negative control.

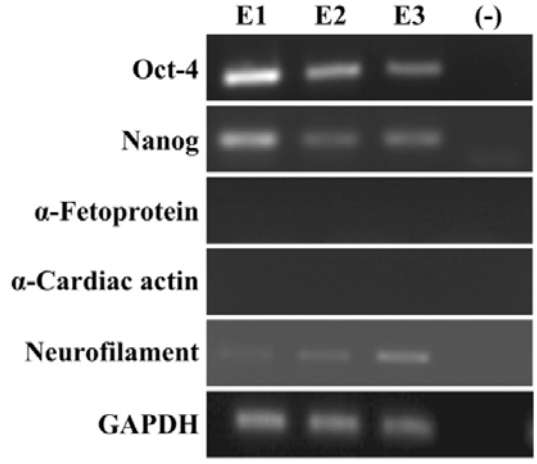

Fig. 4. EmSC- and three germ layer-related gene expression of human HERS/ERM cells. Three independent lines of human HERS/ERM cells (E1, E2, and E3) at passage 2 were analyzed using RT-PCR. All samples were positive for Oct-4, Nanog, and Neurofilament but negative for α-Fetoprotein and α-Cardiac actin. (-), no template as a negative control.

p75, EpCAM, and Bmi-1 expression showed interesting expression patterns. Our results demonstrate that these five EpSC markers are highly expressed in three independent HERS/ERM cell lines (Fig. 3). These findings suggest that the HERS/ERM cells have an EpSC phenotype.

Embryonic stem cell characteristics in HERS/ERM cells

We then further studied whether HERS/ERM cells have more primitive stem cell characteristics than EpSCs and whether they share similarities with EmSCs. To address these questions, we investigated the expression of stemness-related genes by RT-PCR: undifferentiated EmSC markers (Oct-4 and Nanog) and differentiated three germ layer markers (α-fetoprotein, α-cardiac actin, and neurofilament). As shown in Fig. 4, the HERS/ERM cells uniformly expressed the undifferentiated EmSC markers: Oct-4 and Nanog. Of the differentiated three germ layer markers, they did not express α-fetoprotein (endoderm marker) or α-cardiac actin (mesoderm marker), but they did express neurofilament as an ectoderm marker. Therefore, a primitive stem cell population might be included in the HERS/ERM cell culture, which shared similarities with the EmSC and they only expressed the ectoderm marker of the three germ layer markers.

The expression profiles of stem cell markers in HERS/ERM cells during the sub-culturing process

Next, we investigated whether the expression of epithelial and embryonic stem cell markers would be maintained during the sub-culturing process. In an early passage, the primary HERS/ERM cells were small in size and showed little cytoplasmic complexity; they gathered around the low forward- and side-scattering region. After serial passages, the cell size and cytoplasmic complexity were increased, and they spread out over a broad area (Fig. 5). This pattern indicated that the degree of differentiation and the heterogeneity of the cells were increased during the sub-culture. We then focused on the cells in the low forward- and side-scattering region and investigated the expression profiles of the epithelial and embryonic stem cell markers ABCG2, ΔNp63, p75, EpCAM, Oct-4 and SSEA-4. HERS/ERM cells showed different expression levels of these genes at each passage, but their expression was maintained throughout the passages (Fig. 5). Interestingly, at later passages, only a small number of cells remained in the low forward- and side-scattering region; however, those cells highly expressed the EpSC and EmSC markers. On the other hand, the cells, which were increased in cell size and complexity, showed dramatically decreased expression of the EpSC and

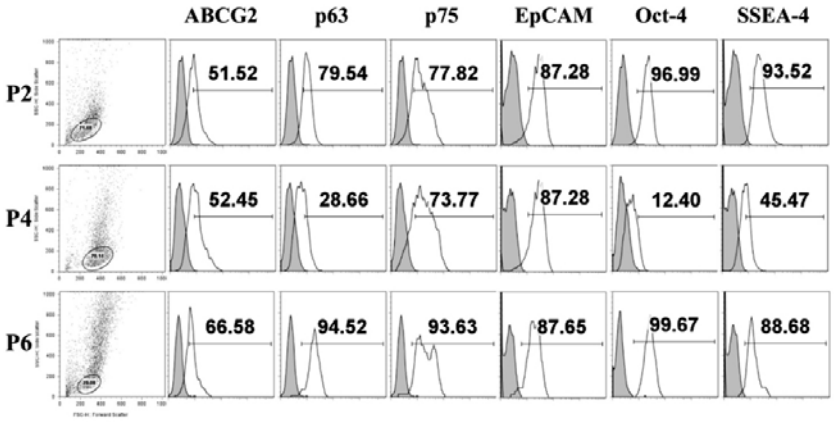

Fig. 5. FACS analysis of EmSC markers and EpSC markers during the sub-culturing process. HERS/ERM cells at passage 2, 4, and 6 were analyzed by EmSC markers and EpSC markers using flow cytometry. Within the gated population, the expression of EmSC markers and EpSC markers was maintained throughout the passages.

EmSC markers, as expected (data not shown). These data suggest that the primary culture of HERS/ERM cells contained more primitive cell populations and were maintained throughout passages in our culture conditions.

DISCUSSION

Human periodontium contains ligament, mucosa, alveolar bone, cementum and a wide variety of cells. HERS/ERM are unique epithelial cell remnants that are present in the periodontium throughout life. It is not clear why the HERS/ERM cells are maintained and what role they play *in vivo*. This study may partially provide evidence for the stemness of HERS/ERM cells: they expressed EmSC markers and EpSC markers, and they did not express all three of the germ layer markers but rather only the ectoderm marker. Hence, they seem to be more primitive stem cells than other adult epithelial stem cells, and they have ectodermal characteristics. Moreover, in our culture conditions, the primitive stem cell-like population was maintained during the sub-culturing process, although the size of this population might have decreased.

Our results show that HERS/ERM cells express the EpSC-related genes ABCG2, ΔNp63, p75, EpCAM and Bmi-1. ABCG2 (drug-resistance ABC transporter) (Ding et al., 2010), ΔNp63 (truncated dominant-negative isoform of p63) (Barbareschi et al., 2001; Parsa et al., 1999; Pellegrini et al., 2001; Yang et al., 1998; 1999), and p75 (a low-affinity neurotrophin receptor) (Nakamura et al., 2007; Okumura et al., 2003) are widely accepted as epithelial stem cell markers. EpCAM is a pan-epithelial differentiation antigen that is expressed in undifferentiated pluripotent stem cells; it is a calcium-independent cell adhesion molecule that is related to increased epithelial proliferation and negatively correlates with cell differentiation (Balzar et al., 1999; Sundberg et al., 2009). Bmi-1 has an essential role in embryogenesis regulation of the cell cycle and lymphopoiesis (Jacobs and van Lohuizen, 1999; Raaphorst et al., 2001), and it is expressed in hematopoietic stem cells and in stem cells from other tissues (Molofsky et al., 2003; Park et al., 2003). Although the molecular markers and their specific roles for epithelial stem cells have not yet been fully elucidated, it has been postulated that the expression of ABCG2, ΔNp63, p75, EpCAM and Bmi-1 may be important for their proliferation and differentiation.

The most interesting result was the expression of EmSC markers such as Oct-4, Nanog, and SSEA-4 in HERS/ERM cells. The transcription factors Oct-4 (Rosner et al., 1990; Scholer et al., 1990) and Nanog (Chambers et al., 2003; Mitsui et al., 2003) are highly expressed in EmSCs and play a central role in the regulation of their pluripotency and self-renewal. Additionally, SSEA-4, a positive marker of human embryonic stem cells (Thomson et al., 1995; 1998) that has recently been used for isolating primitive stem cells from bone marrow and periodontium (Gang et al., 2007; Kawanabe et al., 2010), was also expressed in the HERS/ERM cells. The expression of these genes suggests that HERS/ERM cells might contain more primitive stem cell characteristics. Recently, the possibility that adult stem cells (ASCs) may contain populations of cells with varying degrees of stemness has been suggested (Shin et al., 2010; Stripp, 2008; Verstappen et al., 2009). The hierarchi-cal organization of stem cells was described for various organs and tissues including the breast, intestine, limbus, lung, skin, and intestine. These hierarchical models suggested the cellular and molecular mechanisms regulating the tissue regeneration and repair: the process of symmetrical or asymmetrical cell division leads to the hierarchy of ASCs. Additionally, it is known that epithelial stem cells are small in size and have little cytoplasmic complexity, and that the size and complexity of these cells increases in a differentiated state (De Paiva et al., 2006; Izumi et al., 2007; Romano et al., 2003). In this study, during the sub-culturing process, the size and complexity of the HERS/ERM cells were clearly increased, and the expression of stemness-related genes showed diverse patterns: the number of cells residing in increased cell size and complexity were highly increased but they showed the dramatically decreased expression of the EpSC and EmSC markers (data not shown). Therefore, at passage 6, the expression of the stemness-related genes in whole cell population seemed to be decreased. However, the gated small group of the cells still showed high expression levels of these genes (Fig. 5) and it seem that the number of the cells, which highly expressed these genes was not changed. These findings suggest that HERS/ERM cells have hierarchical populations. For further studies on their functional roles and basic characteristics, clonal analyses of HERS/ERM cells will be required.

Both mesenchymal component and epithelial component are considered essential for tissue engineering of teeth as the process of tooth development (Volponi et al., 2010). Because HERS/ERM cells are a unique epithelial population in adult teeth, establishment of their expansion technique and characterization of their stemness are a matter of the utmost importance in the tooth regeneration. In conclusion, our data suggest that HERS/ERM cells may contain primitive stem cells that express EpSC and EmSC markers; this expression was maintained during the sub-culturing process in our culture conditions.

Although the functional roles of HERS/ERM cells have yet to be elucidated, these findings indicate that primitive stem cells within the primary culture of HERS/ERM cell might play a role as an epithelial component for the repair or regeneration of cementum and periodontal tissues.

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