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Are Hertwig's epithelial root sheath cells necessary for periodontal formation by dental follicle cells?

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ABSTRACT

Objective: The role of Hertwig's epithelial root sheath (HERS) cells in periodontal formation has been controversial. This study aimed to further clarify whether HERS cells participate in formation of the periodontium, and the necessity of HERS cells in differentiation of dental follicle cells (DFCs) for periodontal regeneration.
Design: HERS cells and DFCs were isolated and identified from post-natal 7-day Sprauge-Dawley rats. *In vitro*, direct co-culture of HERS cells and DFCs as well as the individual culture of HERS and DFCs were performed and followed by alizarin red staining and the quantitative real-time polymerase chain reaction analysis. For *in vivo* evaluation, the inactivated dentin matrix (iTDM) was fabricated. HERS cells and DFCs were seeded in combination or alone on iTDM and then transplanted into the rat omentum. Scanning electron microscope and further histological analysis were carried out.
Results: In vitro, mineral-like nodules were found in the culture of HERS cells alone or HERS + DFCs either by alizarin red staining or scanning electronic microscope. The mineralization and fiber-forming relevant mRNA expressions, such as bone sialoprotein, osteopontin, collagen I and collagen III in HERS + DFCs were significantly higher than that of the HERS or DFCs alone group. After transplantation *in vivo*, cementum and periodontal ligament-like tissues were formed in groups of HERS + DFCs and HERS alone, while no evident hard tissues and attached fibers were found in DFCs alone.
Conclusions: Hertwig's epithelial root sheath cells directly participate in the formation of the periodontium, and they are essential for the differentiation of dental follicle cells to form periodontal structures. The combination use of Hertwig's epithelial root sheath cells and dental follicle cells is a promising approach for periodontal regeneration.

1. Introduction

Tooth development is a complicate process of the reciprocal interactions between epithelial and ectomesenchymal cells. After completion of the tooth crown development, the inner and outer epithelium of enamel organ proliferate at the cervical loop and migrate apically forming the double-layer epithelium, known as the Hertwig's epithelial root sheath, which marks the initiation of the root development (Tummers & Thesleff, 2003). It is believed that the Hertwig's epithelial root sheath acts as the signal center and is indispensable during the development of the root (Huang, Bringas, Slavkin, & Chai, 2009; Jung et al., 2011). However, the functional roles of the Hertwig's epithelial root sheath and its interaction with mesenchymal cells are not fully understood (Nam et al., 2011), and it has been controversial for decades whether Hertwig's epithelial root sheath cells directly participate in the formation of the periodontium. Some researchers reported that Hertwig's epithelial root sheath cells underwent epithelial-mesenchymal transition to differentiate into cementoblasts and periodontal ligament

Abbreviations: HERS, Hertwig's epithelial root sheath; DFCs, dental follicle cells; iTDM, inactivated treated dentin matrix

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fibroblasts, participating in the formation of the periodontium (Bosshardt, 2005; Huang & Chai, 2012; Huang et al., 2009; Nam et al., 2011), while other researchers suggested that Hertwig's epithelial root sheath cells indirectly involved in the development of the periodontium by regulating differentiation of dental follicle cells which harbor precursors that give rise to the supporting structures of a tooth (Diekwisch, 2001; Sonoyama, Seo, Yamaza, & Shi, 2007; Zeichner-David, 2006). There were also studies claiming that Hertwig's epithelial root sheath cells involved in neither epithelial-mesenchymal transition nor formation of periodontal structures (Yamamoto & Takahashi, 2009; Yamamoto et al., 2015). Hence, further investigations are needed to clarify the role of Hertwig's epithelial root sheath cells in the development as well as the regeneration of periodontal structures.

Dental follicle cells, which surround the developing tooth germ, are the major cell source for periodontium formation. They are able to differentiate into cementoblasts, periodontal ligament fibroblasts and osteoblasts, which give rise to cementum, periodontal ligament fibers and alveolar bone, respectively. They are characterized by a high proliferation rate, multi-differentiation ability, high viability, easy accessibility and opportunity to be safely cryopreserved (Maxim, Soritau, Baciut, Bran, & Baciut, 2015; Yao, Pan, Prpic, & Wise, 2008). The characterization of dental follicle cells has increased their potential and excellence for use in tissue engineering applications, including periodontal regeneration (Chalisserry, Nam, Park, & Anil, 2017; Guo, Chen et al., 2012). It has been reported that the differentiation of dental follicle cells is controlled by a complex network of regulatory molecules and signal pathways (Bai et al., 2011; Jung et al., 2011; Orimoto et al., 2017; Yang et al., 2014). Studies have also suggested that Hertwig's epithelial root sheath cells are responsible for the differentiation of dental follicle cells (Jung et al., 2011; Zeichner-David, 2006). Nevertheless, the mechanisms that direct the differentiation of dental follicle cells to form periodontal structures remains unclear to date.

Although dental follicle cells are considered as the promising cell candidate for periodontal regeneration as recent studies have shown that cementum and periodontal ligament-like structures were formed *in vivo* without epithelial cells (Guo, Chen et al., 2012; Guo, Gong et al., 2012; Yang et al., 2012), this does not necessarily mean the role of Hertwig's epithelial root sheath cells can be ignored in differentiation of dental follicle cells during periodontal formation. Studies reported by Morsczeck et al. manifested that after transplantation of dental follicle cells into immuno-compromised mice, there was not any sign of periodontal tissue formation (Morsczeck et al., 2005). Bai et al. also reported that dental follicle cells stimulated by indirect co-culture with Hertwig's epithelial root sheath cells had a significant potential for mineralization *in vitro* and formed cementum and periodontal ligament-like tissues *in vivo*, while dental follicle cells alone produced only fibrous tissues (Bai et al., 2011). Nevertheless, Hertwig's epithelial root sheath cells are closely adjacent to and surrounded by dental follicle cells in the developing tooth germs. Interactions between Hertwig's epithelial root sheath cells and dental follicle cells are not only indirect but also direct cell-to-cell communication (Bosshardt, Stadlinger, & Terheyden, 2015). Thus, to recapitulate the interactions between Hertwig's epithelial root sheath cells and dental follicle cells during periodontal development and further clarify the role of Hertwig's epithelial root sheath cells and the necessity of them in dental follicle cell differentiation to form periodontal tissues, the direct co-culture of Hertwig's epithelial root sheath cells and dental follicle cells *in vitro* and the combined transplantation *in vivo* were performed in the present study. The implementation of the study would also provide potential strategies and insights for periodontal regeneration.

2. Materials and methods

2.1. Primary cell culture and identification

All the experimental protocols and animal procedures employed in this study were conducted in accordance with the guidelines of the Ethics Committee of West China School of Stomatology, Sichuan University.

Hertwig's epithelial root sheath (HERS) cells and dental follicle cells (DFCs) were harvested from the first molar germs of post-natal 7-day Sprauge-Dawley rats referring to a modified method as previously described (Ge et al., 2013). HERS cells were cultured in epithelial cell medium (ScienCell, USA) consisting of basal medium, 2% fetal bovine serum (ScienCell, USA), 1% epithelial cell growth supplement (ScienCell, USA), and 1% penicillin/streptomycin solution (ScienCell, USA), while DFCs were cultured with α-minimum Eagle's medium (Hyclone, USA) supplemented with 15% fetal bovine serum (Hyclone, USA) and 1% penicillin/streptomycin (Solarbio, China). After cells grew and fused by 70%, HERS cells and DFCs were purified referring to a differential digestion method using trypsin/EDTA (Millipore, USA) according to the procedures described previously (Chen et al., 2014). HERS cells were further identified by positive immunofluorescence staining with mouse anti-cytokeratin 14 antibody (1:800; Abcam, UK) and negative staining with mouse anti-vimentin antibody (1:1000; Millipore, USA), while DFCs were assessed for negative anti- cytokeratin 14 staining and positive anti-vimentin staining. Cells that met these criteria were used in the subsequent experiments.

2.2. Co-culture of HERS cells and DFCs

For direct co-culture, HERS cells of passage 2 and DFCs of passage 3 were simultaneously seeded into a culture plate with the same initial density of $5 \times 10^3/cm^2$ and cultured with the mixed medium of supplemented epithelial cell medium and α-minimum Eagle's medium in a ratio of 1:1. HERS cells and DFCs were also cultured alone with the mixed medium. Cells were routinely observed and photographed under a phase-contrast inverted microscope (Leica DMI 6000, Germany). Alizarin red staining and the analysis were performed to detect the mineralization after 9-day culture.

2.3. Quantitative real-time polymerase chain reaction analysis

Quantitative real-time polymerase chain reaction analysis was used to detect the relative mRNA expressions in the co-cultured and the individually cultured HERS cells and DFCs. After 9-day culture, cells were harvested and total RNA extraction was performed using RNAiso plus (TaKaRa, Dalian). The complementary DNA was synthesized with SYBR® Premix Ex Taq II (Perfect Real Time kit; TaKaRa, Dalian) according to the manufacturer's instructions. Sequences of the gene-specific primers synthesized by TaKaRa are listed in Table 1. The results were analyzed using the $2^{-\Delta\Delta CT}$ relative quantitative method with β-actin as an internal control.

2.4. Preparation of the inactivated treated dentin matrix (iTDM)

For *in vivo* evaluation of the differentiation of HERS cells and DFCs, the iTDM was used as the scaffold for transplantation in the present study. The iTDM was prepared referring to a method modified based on procedures as described previously (Guo, Gong et al., 2012; Guo et al., 2018; Ji et al., 2015; Li et al., 2011). Briefly, ten premolars were collected from five healthy patients requiring their removal for orthodontic treatment. Periodontal ligament, cementum, dental pulp tissues, predentin and a thin layer of the root dentin were removed. The resulting root dentin matrix was cut into pieces and grinded into regular shape as shown in Fig. 3. De-mineralization of the dentin matrix was then performed by treating with EDTA solution of gradient concentrations. Since it has been reported that the living dentin matrix contains many active growth factors, such as collagen I, transforming growth factor β, decorin, biglycan, dentin matrix protein 1 and bone sialoprotein, which are critical in inducing cell proliferation and differentiation (Li et al., 2011), we prepared the treated dentin matrix and