Indirect Co-Culture of Stem Cells from Human Exfoliated Deciduous Teeth and Oral Cells in a Microfluidic Platform

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Oral epithelial-mesenchymal interactions play a key role in tooth development and assist differentiation of dental pulp. Many epithelial and mesenchymal factors in the microenvironment influence dental pulp stem cells to differentiate and regenerate. To investigate the interaction between oral cells during differentiation, we designed a microfluidic device system for indirect co-culture. The system has several advantages, such as consumption of low reagent volume, high-throughput treatment of reagents, and faster mineralization analysis. In this study, stem cells from human exfoliated deciduous teeth were treated with media cultured with human gingival fibroblasts or periodontal ligament stem cells. When human exfoliated deciduous teeth was incubated in media cultured in human gingival fibroblasts and human periodontal ligament stem cells under the concentration gradient constructed by the microfluidic system, no remarkable change in human exfoliated deciduous teeth mineralization efficiency was detected. However, osteoblast gene expression levels in human exfoliated deciduous teeth incubated with human gingival fibroblasts media decreased compared to those in human exfoliated deciduous teeth treated with human periodontal ligament stem cells media, suggesting that indirect co-culture of human exfoliated deciduous teeth with human gingival fibroblasts may inhibit osteogenic cytodifferentiation. This microfluidic culture device allows a co-culture system set-up for sequential treatment with co-culture media and differentiation additives and facilitated the mineralization assay in a micro-culture scale.

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Key Words: Microfluidic device; Indirect co-culture; Stem cells from human exfoliated deciduous teeth; Osteogenic differentiation

INTRODUCTION

Postnatal stem cells, which are also called adult stem cells, are found in all adult tissues and play a supportive role due to their regenerating power. Stem cell niches have also been found in dental pulp of permanent teeth [1] and exfoliated deciduous teeth [2], in periodontal ligaments [3], in the apical papilla [4], in dental follicles [5], and in the periosteum of the maxillary tuberosity [6]. Multipotent stem cells isolated from these human dental tissues are capable of differentiating into diverse mesenchymal lineages, including adipocytes, chondrocytes,

osteoblasts, and odontoblasts, as well as myocytes, neurons, and angiogenic endothelial cells. Specifically, stem cells located in dental pulp of the human adult third molar can regenerate dentin and are a good source to remedy a damaged tooth or regenerate bone [1,7,8]. In addition, other peripheral tissues of teeth are excellent sources of ectomesenchymal stem cells, which are transformed from neural crest cells after undergoing the epithelial-mesenchymal transition [1,9-11]. The sequential interaction between mesenchymal and epithelial cells in the oral region during embryonic development regulates the differentiating power of odontoblasts. The initiation of tooth development is regulated by basement membrane components [12], and commitment and differentiation of odontoblasts are regulated by interactions with mesenchymal and epithelial cells in the oral environment during terminal differentiation. Moreover, soluble factors in the extracellular matrix, such as growth factors in the dental microenvironment during development, are important for osteogenic/odontogenic differentiation and to determine the fate of human dental pulp stem cells (hDPSCs) [13-16]. Platforms for co-culturing dental stem cells have been

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developed to mimic cell-cell interactions. Co-culture of stem cells from the dental follicle and apical papilla induces the formation of a bone-like structure [17]. Co-culture of DPSCs with epithelial cells and non-dental mesenchymal stem cells (MSCs) induces epithelial invagination of DPSCs and bone differentiation of MSCs, respectively [12,18]. Additionally, tooth germ cell-conditioned media induces odontogenic differentiation of DPSCs and creates an odontogenic microenvironment very close to that observed *in vivo* [19]. It is important to understand the communication between cells through cytokine gradients, such as juxtacrine and paracrine actions, to investigate stem cell niches [20-22]. Microfluidics is an emerging technology for cell culture [23,24], single cell isolation [25,26], cell metabolism analysis [27,28], stem cell biology [29], and tissue engineering [30,31]. This technology has many advantages, including a small reagent volume, high-throughput platform, reproducibility, and ease of integration [32]. The traditional co-culture system of mixing two cell types randomly or a using strainer to assess indirect communication between cells in the same media is not suitable for discriminating paracrine and juxtacrine effects. Additionally, this type of system is inefficient for high-throughput applications of cytokine and growth factor sequential gradients. In this study, we designed a microfluidic co-culture platform, which is useful for seeding and attaching cells, flushing media, and mineralization assay in a single device. We used this device to examine whether secretory factors from human gingival fibroblasts (hGFs) or periodontal ligament stem cells (hPDLSCs) offer an osteogenic niche for stem cells from human exfoliated deciduous teeth (SHED).

MATERIALS AND METHODS

Cell culture

Discarded impacted third molars were collected from children aged 6–13 years under guidelines approved by the IRB of the Dankook Dental Hospital. For human exfoliated deciduous teeth (SHED) culture, tooth was fractured and pulp tissue was separated from the crown and root. To primary cultures of periodontal ligament cells and gingival fibroblast, the periodontal ligament tissue and the spinous layer of gingival were collected, respectively. The pieces of tissue were digested in a solution of 3 mg/mL collagenase type I (Sigma Aldrich, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma Aldrich, St. Louis, MO, USA) for 1 hour at 37°C. Cell suspension was obtained by passing through a 70- μ m strainer, and incubated in α -MEM containing 20% fetal bovine serum (Hyclone, GE Healthcare, Buckinghamshire, UK) and antibiotics at 37°C in 5% CO₂. For differentiation and mineralization, cells were incubated in α -MEM containing 10% fetal bovine serum, 5 mM β -glycerophosphate,

500 nM dexamethasone, and 100 μ M ascorbic acid for 15 days [33,34].

Alizarin red staining

After osteogenic differentiation, cells on 6-well culture plate were fixed with 70% ethanol for 5 minutes at room temperatures, and then allowed to dry. For staining, cells were treated with 2% alizarin red S (pH 4.5), and washed thoroughly. For staining of mineral deposits on cells cultured in microfluidic device, 70% ethanol was treated in reservoirs for 5 min at room temperature, and then 2% alizarin red S (pH 4.5) was injected into the device by using syringes. Washing processes were performed by using an automated micro pump system. For quantification of alizarin red staining, 10% (v/v) acetic acid 800 μ L added to each well, and incubated during 30 min in room temperature with shaking. Then, cells scraped from plate using cell scraper (SPL Lifesciences, Korea). After, 10% (v/v) acetic acid was moved to 1.5 mL microcentrifuge tube and vortexing for 30 s. Supernatant was overlaid with 500 μ L mineral oil (Sigma Aldrich, St. Louis, MO, USA) and heated in 85°C for 10 min. After heating, 1.5 mL microcentrifuge tube moved to ice for 5 min. And then supernatant was centrifuged at 20000 g for 15 min, supernatant excluded mineral oil 500 μ L was transferred to a new tube. Supernatant of 500 μ L added 200 μ L 10% ammonium hydroxide, each 150 μ L aliquoted to 96 well-plates. Absorbance was measured at 405 nm. Statistical analyses on three readings were carried out using Student's t-test, and *p* values of less than 0.05 were considered significant. t-test; ****p*<0.001, *****p*<0.0001. Data are expressed as the mean (n=3) with error bars representing 0.1 SEM and t-test was analyzed using Graph-Pad Prism 6 program (La Jolla, CA, USA).

Quantitative real-time PCR

The expression levels of alkaline phosphatase (ALP), osteocalcin, and osteonectin were analyzed by quantitative real-time polymerase chain reaction (PCR) (qPCR). Briefly, total cellular RNA was isolated from cells, and used for complementary DNA synthesis by using ReverTra Ace[®] qPCR RT kit (Toyobo Corporation, Osaka, Japan). The qPCR was performed by using iTaq[™] Universal SYBR[®] Green Supermix (BioRad, Hercules, CA, USA) system. Primers used are listed in Table 1. The cycling parameters of qPCR were followed; 1 cycle for 1 min at 95°C, 40 cycles for 15 sec at 95°C and 1 min at 60°C. After PCR, a dissociation curve was constructed in the range of 65°C to 95°C. The GAPDH was used as an internal control to normalize the variability in target gene expression. Relative quantity (Δ Cq) for each gene is calculated with this formula: Δ Cq = $2^{[Cq(Min) - Cq(Sample)]}$. Cq (Min), average Cq for sample with the lowest average Cq for GOI; Cq (Sample), average Cq for sample; GOI, Gene

of Interest. Normalized expression ($\Delta\Delta Cq$) is the relative quantity of each gene normalized to the quantities of GAPDH gene. The calculation for normalized expression is described in the following formula: $\Delta\Delta Cq = \Delta Cq_{(Sample)} / \Delta Cq_{(Ref)}$. Statistical analyses on three readings were carried out using Student's t-test, and p values of less than 0.05 were considered significant. t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are expressed as the mean ($n=3$) with error bars representing 0.1 SEM and t-test was analyzed using GraphPad Prism 6 program.

RESULTS

Fabrication of the microfluidic cell culture device

The microfluidic cell culture device was fabricated using a soft lithographic technique [35–38] to investigate mineralization of SHED by indirectly culturing various oral cells under a mimicked cell microenvironment. This microfluidic culture system was designed for continuous perfusion culture and forming a chemical gradient. A schematic of this device is shown in Figure 1. The device has gradient generating parts containing two reservoirs with a micropump and a 32-cell culture chamber array consisting of eight-cell culture chambers as a group for exposure to different reagent concentrations with four replications. Each

Table 1. Primers used for the quantitative real-time-polymerase chain reaction analysis

Gene name	Sequences
GAPDH	Forward 5' GTA TGA CAA CAG CCT CAA GAT 3' Reverse 5' CCT TCC ACG ATA CCA AAG TT 3'
ALP	Forward 5' CTT GAC CTC CTC GGA AGA CAC TC 3' Reverse 5' CGC CCA CCA CCT TGT AGC C 3'
Osteocalcin	Forward 5' TGA GTC CTG AGC AGC AG 3' Reverse 5' TCT CTT CAC TAC CTC GCT 3'
Osteonectin	Forward 5' CTG TTG CCT GTC TCT AAA CC 3' Reverse 5' CAC CAT CAT CAA ATT CTC CT 3'

ALP: alkaline phosphatase

of 32 culture micro chambers is 100 μm high, 720 μm wide, and 1000 μm long. The media reservoir is 4 mm high and has a diameter of 8 mm. Each cell culture chamber was separated with micro valves to avoid mixing reagents during non-flow conditions (Fig. 1A). The microfluidic cell culture device consisted of two polydimethylsiloxane (PDMS; Dow Corning, Midland, MI, USA) layers. A thick pneumatic layer was fabricated by filling a 4-mm thick master mold with PDMS. This thickness was required to connect the pneumatic Tygon tubing (0.01" ID $\times$ 0.03" OD; Tygon, Akron, OH, USA) into a micro channel. A 150- μm thick fluidic membrane layer was fabricated by pouring PDMS into a master mold and spinning at 800 rpm for 30 seconds. The PDMS-coated master mold was kept on a horizontally balance before curing. The gradient generator and micro chamber channels were 100 μm high and the channel for controlling the micropump and micro-valve array was 150 μm high. A thick pneumatic layer and thin fluidic membrane were aligned and adhered to each other after exposure to the 100 W oxygen plasma for 40 seconds under 150 mTorr pressure. Methanol was added to the space between two layers prior to aligning as lubricant. The PDMS device was then bonded onto a slide glass with oxygen plasma treatment. To prevent irreversible bonding between the PDMS micropump and micro valve, negative pressure was applied onto the pneumatic layer with connecting pneumatic Tygon tubing during bonding the PDMS device on slide glass. The connection ports for micro-valves, micropump, and inlet ports were punched using a 19-gauge tapped needle. The reservoirs were punched with an 8-mm diameter biopsy punch prior to bonding onto the slide glass. The device contained 8 $\times$ 4 micro chamber arrays. Each chamber had separate micro-valves for refreshing media and supplements. A concentration gradient was maintained in the micro chamber by perfusing media and supplements. The micropump maintained a linear concentration gradient and minimized dead volume of media and supplements. Culture media and supplements were introduced automatically at a 1.7-

Figure 1. Schematic diagram of the microfluidic device for co-culture. (A) The microfluidic device was composed of two functional units, including multiple cell culture chamber arrays and media reservoirs. (B) Photograph of the microfluidic co-culture device. Red and blue dyes were used in reservoirs 1 and 2, respectively, to detect a gradient.

$\mu\text{L}/\text{min}$ flow rate and a 12-nL stroke volume by controlling the opening/closing of the micro-valves. Reservoirs 1 and 2 were filled with red and blue inks, respectively, to detect the mixing of media from each reservoir using the micropump. The two inks were mixed and a color gradient was generated in the form of a tree (Fig. 1B).

Verification of cell culture and mineralization assay using the device

The device was UV sterilized by lights, and the surface of microchamber was coated with fibronectin so cells could attach prior to cell culture. SHED were introduced through inlet valve located on the left side of the culture chambers, and cultured to 30–40% confluency. Mineralization media containing additives was introduced into the microchambers to verify whether mineralization could be induced normally in cells cultured in the device. Reservoirs 1 and 2 were filled with media containing 100% of the additives and media without additives, respectively, to create the additive concentration gradient (Fig. 2A). A 0–100% concentration gradient of mineralization additives was created in each micro culture chamber. Cells were incubated under the continuous perfusion culture conditions for 15 days without any change in cell viability (Fig. 2B, a). The number of cells decreased according to increase in concentration of the mineralization additives, indicating that cell proliferation was inhibited during differentiation (Fig. 2B, from panel 8 to panel 1 in a). Next, we set up a direct alizarin-red assay to detect mineralization under microfluidic conditions. After 15 days of differentiation, all media in the microchambers and reservoirs was removed with the pump, and staining and washing solutions were introduced into the microchambers. As expected, cells cultured in media containing 100% of the additives were highly mineralized (Fig. 2B, panel 1 in b), and alizarin-red staining decreased according to the decrease in concentration of mineralization additives (Fig. 2B, from panel 1 to panel 8 in b), suggesting that the mineralization detection assay could be successfully performed directly in this microfluidic device.

Mineralization of SHED under indirect hGF and hPDLSC co-culture

We investigated the effect of indirect co-culture on mineralization of SHED in various concentrations of additives. A total of 1×10^5 early passage hGFs and hPDLSCs were cultured in α -MEM supplemented with 10% fetal bovine serum. Three-days after the last media change, the culture media was collected and used as co-culture media. SHED (6×10^5 cells/mL) were injected into the 32 micro chamber arrays through the inlet valve using a 1-mL syringe. Twenty cells were captured uniformly in each micro cell culture chamber by closing the micro valves.

Figure 2. Culture and mineralization of stem cells from human exfoliated deciduous teeth (SHED) using the microfluidic co-culture device system. (A) Cell culture and mineralization assay in the microfluidic system. Reservoir 1 was filled with media supplemented with 100% mineralization additives (5 mM β -glycerophosphate, 500 nM dexamethasone, and 100 μM ascorbic acid), and reservoir 2 was filled with media without additives (0%). (B) Magnified photograph of a column in the cell chamber array shown as a square in (A). Mineralization of SHED as the concentration of additives was decreased from 100% to 0%. a: microscopic phenotypes of SHED cultured in microfluidic cell chambers, b: alizarin red-S staining to detect mineralization.

Cells that were stagnant in the reservoirs were removed with a pipet. The captured SHED attached and proliferated on the fibronectin-coated glass surface. Reservoirs 1 and 2 were filled with fresh media and media cultured with hPDLSCs or hGFs for co-culture, respectively (Fig. 3A). During injection into the micro-chamber array, the media in chambers mixed through the winding gradient generator. To verify whether SHED min-

Figure 3. Mineralization efficiency of stem cells from human exfoliated deciduous teeth (SHED) under treatment with mineralization additives and media cultured with human gingival fibroblasts (hGFs) and periodontal ligament stem cells (hPDLSCs). (A) Media composition used in indirect co-culture. Reservoirs 1 and 2 were filled with media cultured with cells and fresh media, respectively. Three devices were prepared for three different concentrations of mineralization additives. Device I: 100% additives, Device II: 40% additives, Device III: no additives (0%). (B) Mineralization of SHED co-cultured with media cultured with hGFs. A concentration gradient (0–100%) of the media cultured with hGFs was prepared in the microfluidic cell chambers. One column from each of the three devices was selected, and mineralization efficiencies were compared. a: microscopic phenotypes of SHED cultured in microfluidic cell chambers, b: alizarin red-S staining to detect mineralization. Scale bars=200 μ m. (Continued to the next page)

Figure 3. (Continued from the previous page) (C) Mineralization of stem cells from human exfoliated deciduous teeth (SHED) co-cultured with media cultured with human periodontal ligament stem cells (hPDLSCs). A concentration gradient (0–100%) of media cultured with hPDLSCs was prepared in microfluidic cell chambers. a: microscopic phenotypes of SHED cultured in the microfluidic cell chambers, b: alizarin red-S staining to detect mineralization. Scale bars=200 μ m.

eralization could be induced by co-culture with hGFs or hPDLSCs, the concentration gradient of the media cultured with hPDLSCs or hGFs was created at fixed concentrations (100%, 40%, or 0%) of additives in separate individual devices a, b, and c, respectively. Cells were incubated under continuous perfusion culture conditions for 14 days, and mineralization was analyzed by alizarin red-S staining in the microfluidic device. Mineralization of SHED did not increase with the concentration gradient of media cultured with hGFs and hPDLSCs (Fig. 3, from bottom to top in panels b in B and C). As expected SHED, mineralization efficiency increased remarkably according to the concentration of additives (Fig. 3, from left to right in panels b in B and C). To confirm whether mineralization efficiency under microfluidic conditions was consistent with that under normal scale cell culture conditions, we performed an indirect cell culture of SHED treated with cultured media in a 6-well cell culture plate. When SHED were grown in media cultured

with hGFs and hPDLSCs (Fig. 4A, b and c), their mineralization efficiencies were not different from those of cells grown in fresh media (Fig. 4A, a). Regardless of indirect co-culture, mineralization of SHED decreased as the additive concentration decreased (Fig. 4B, panels 1–3).

Osteogenic cytodifferentiation of SHED by indirect co-culture with hGFs and hPDLSCs

Although mineralization efficiency of SHED in media supplemented with additives was not affected by indirect co-culture in the microfluidic device and normal culture system, it could be affected a change in osteogenic cytodifferentiation capability caused by culturing without mineralization additives. To investigate osteogenic cytodifferentiation under indirect co-culture conditions, SHED were co-cultured in media cultured with hGFs and hPDLSCs without additives. The cells were harvested after 7 days, and the expression of osteogenic

Figure 4. Mineralization of stem cells from human exfoliated deciduous teeth (SHED) treated with media cultured with human gingival fibroblasts (hGFs) and periodontal ligament stem cells (hPDLSCs) under macro-culture conditions. (A) SHED were cultured and induced for mineralization in media cultured with hGFs and hPDLSCs supplemented with various concentrations (100%, 40%, and 0%) of mineralization additives in 6-well plates. Mineralization was analyzed after 15 days by alizarin red-S staining. a: without adding media cultured with cells, b: with media cultured with hGFs, c: with media cultured with hPDLSCs. (B) Quantification of Alizarin red S staining. Statistical analyses on three readings were carried out using Student's t-test, and p values of less than 0.05 were considered significant. t-test; *** p <0.001, **** p <0.0001. Data are expressed as the mean (n =3) with error bars representing SEM.

Figure 5. Osteogenic cytodifferentiation of stem cells from human exfoliated deciduous teeth (SHED) in media cultured with human gingival fibroblasts (hGFs) and periodontal ligament stem cells (hPDLSCs). SHED were cultured in media cultured with hGFs and hPDLSCs without additives for 7 days, and used for RNA preparation. As a control, SHED were cultured in a-MEM media with 10% FBS. Transcriptional expression of osteogenic markers was analyzed by quantitative real-time-polymerase chain reaction analysis. a: expression of alkaline phosphatase, b: expression of osteocalcin, c: expression of osteonectin. Statistical analyses on three readings were carried out using Student's t-test, and p values of less than 0.05 were considered significant. t-test; * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001. Data are expressed as mentioned in Materials and Methods.

markers was analyzed by quantitative real-time PCR. Osteogenic cytodifferentiation of SHED was not affected by indirect co-culture with hPDLSCs, and of ALP, osteocalcin, and osteonectin mRNA expression did not increase (Fig. 5, bars for hPDLSC media). However, when SHED were co-cultured with hGFs, osteogenesis was inhibited, and transcriptional expression of the osteogenic markers decreased in two-fold (Fig. 5,

bars for hGF media).

DISCUSSION

In this study, we constructed a microfluidic device system for investigation of indirect co-culture. A microfluidic device is a kind of chip systems, on which various laboratory works are

performed in micro scale. This system has several advantages; small amount of cells and reagents can be used in the whole experiment and the results can be obtained from various conditions quickly and easily. In addition, this device enables to be applied for the high throughput drug screening, which constructs a linear gradient of drug in micro scale.

Tooth develops from communication between neural-crest mesenchyme and oral epithelium [39,40]. SHED are capable of responding to tooth-inducing signals from cells derived from adult human gingival tissue or periodontal ligament tissue to form tooth and to differentiate into hard tissue following *in vitro* culture. To examine the oral cell effect on pulp differentiation, SHEDs were grown in the cultured media of hGFs and hPDLSCs under the indirect co-culture condition. SHED was cultured under the concentration gradients of mineralization additives and the hGFs/hPDLSCs cultured media, which were set up by microfluidic pump system. According to use this system, we are able to estimate the proper concentrations of hGFs and hPDLSCs cultured media and a differentiation additives for differentiation. Expectedly, as the concentration of mineralization additives is increased, the mineralization in SHED is inhibited in the microfluidic device, suggesting that osteoblast differentiation occur in this system properly (Fig. 2B). However, when the osteogenic differentiation of SHED was induced in hGFs- or hPDLSCs-cultured media, an increment in mineralization was not detected in the microfluidic device (Fig. 3B and C). It has been reported that gingival fibroblasts stimulate bone resorption and cell proliferation of osteoblast [41,42]. Based on the molecular analysis of gene expression in normal condition, osteogenic cytodifferentiation of SHED was not much changed or inhibited under the treatment with hPDLSCs- or hGFs-cultured media, respectively (Fig. 5), suggested that an additional adequate system need for investigation of a change down to the minutest details, although this microfluidic device is useful for high throughput treatment of additives and cytokines. For all this limitation, the microfluidic device developed in this study contained an important advantage: microfluidic assay for mineralization. The alizarin red staining was performed directly after cell culture in this device and all the steps of staining and washing were able to be operated automatically.

Finally, we designed a microfluidic co-culture platform in this study, and this device will be useful for culturing cells, flushing of media, and mineralization assay in a single device. This device might be useful for examine whether secretory factors from various oral cells offer an osteo/dentinogenic niche for pulp stem cells and vice versa.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

For the human dental tissue primary culture, adult third molars and baby supernumerary teeth were collected under guidelines approved by the Institutional Review Board of Dankook Dental Hospital (IRB no. H-1506-006-001).

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