International Journal of Oral Biology, Vol. 38, No. 4 December 30 2013, p. 161-167

http://dx.doi.org/10.11620/IJOB.2013.38.4.161

International Journal of Oral Biology

Effects of nanoscale ridge/groovepattern arrayed surface on *in vitro* differentiation of multi-potent pulp cells derived from human supernumerary teeth

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(received September 28, 2013; revised October 25, 2013; accepted December 10, 2013)

Human dental pulp stem cells (DPSCs) are multi-potent mesenchymal stem cells that have several differentiation potentials. An understanding of thetissues that differentiate from these cells can provide insights for future regenerative therapeutics and tissue engineering strategies. The mesiodens is the most frequent form of supernumerary tooth from which DPSCs can differentiate into several lineages similar to cells from normal deciduous teeth. Recently, it has been shown that nanoscale structures can affect stem cell differentiation. In our presentstudy, we investigated the effects of a 250-nm nanoscale ridge/groove pattern array on the osteogenic and adipogenic differentiation of dental pulp cells from mesiodenscontaining human DPSCs. To this end, the expression of lineage specific markers after differentiation induction was analyzed by lineage specific staining and RT-PCR. The nanoscale pattern arrayed surface showed apositive effect on the adipogenic differentiation of DPSCs. There was no difference between nanoscale pattern arrayed surface and conventional surface groups onosteogenic differentiation. In conclusion, the nanoscale ridge/groove pattern arrayed surface can be used to enhance the adipogenic differentiation of DPSCs derived from mesiodens. This finding provides an improved understanding of the effects of topography on cell differentiation as well as the potential use of supernumerary tooth in regenerative dental medicine.

Key words: Supernumerary tooth, Dental pulp stem cell, Osteogenic differentiation, Adipogenic differentiation, Nanoscale ridge/groove pattern array

Introduction

Mensenchymal stem cells (MSCs) can be obtained from various tissues such as bone marrow, adipose tissue, skin, cartilage, umbilical cord and placenta [1-5]. It has recently been confirmed that dental pulp stem cells (DPSCs) also belong to MSCs and can differentiate into multiple lineages including odontoblasts, osteoblast, adipocytes, neurons, chondrocytes and myocytes [5-8]. They also share similar gene expression patternswith bone marrow stem cellswhich are criteria of MSCs.

DPSCs can only be recovered from the dental tissue and this makes it difficult to study DPSCs from healthy permanent teeth. Recent study demontrated that the properties of DPSCs from deciduous teeth are similar to normal permanent teeth [9]. The characteristics of DPSCs from supernumerary teeth

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are also similar to deciduous teeth [10]. A supernumerary tooth is an additional tooth to the normal series and can be found in all regions of the dental arch. In general, supernumerary teeth are removed for esthetic or functional reasons and discarded. However, it can be a benefit to recover and utilize DPSCs from those supernumerary teeth as a valuable resource for stem cell therapies because the stem cells can be collected non-invasively from the tooth.

Stem cell differentiation is one of the vigorous research fields as well as the maintenance of stemness and propagation of the cell. In general, specific chemicals are commonly used for stem cell differentiation [10, 11]. However, exogenous chemicals can cause unknown toxicities *in vivo*. Hence chemical-free stem cell differentiation can be an alternative way to overcome this problem. It has been reported that the cell morphology is regulated by adhesion molecules, and alteration of its morphology is closely related with cell migration, function, polarity and differentiation [12-15]. This suggests that factors modifying cell shapes may be important to determine the cell fate. Different from chemical approaches [10, 11], there are few studies on the differentiation induction of DPSCs by physical stimulation.

In this study, we investigated the osteogenic and adipogenic differentiation of human DPCs from mesiodens on 250-nm nanoscale ridge/groove pattern arrayed surface in the presence of each induction media. To confirm the degree of differentiation, Alizarin red solution and Oil red O staining were each used for staining of differentiated cells. In the nanoscale pattern arrayed surface group, expression of osteoblast-and adipocyte-specific marker genes were also analyzed by RT-PCR.

Materials and methods

Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated in the text.

Isolation of human dental pulp cells (DPCs) and cell culture

To isolate human dental pulp tissue, mesiodens (maxillary central supernumerary teeth) (n=8) were extracted from children at the Department of Pediatric Dentistry in Dental Hospital of Seoul National University according to the guidelines provided by ethics committee (S-D20100005). The extracted teeth were cut around the cemento-enamel junction using cutting disk to expose the pulp tissue as previously described [16]. The pulp tissue was gently separated from the crown and root using sterile endodontic file and digested in 1% (w/v) collagenase type I to generate single-cell suspensions. The digested cells were seeded into 24-well culture dishes with DPSC culture medium supplemented α-MEM containing 10% (v/v) fetal bovine serum (FBS; Life technologies, NY, USA) and 100 IU/ml penicillin-100 μg/ml streptomycin (Life technologies) and then incubated at 37°C in humidified atmosphere containing 5% CO_2. The culture medium was refreshed once every 3 days to allow further growth. The adherent cells grown to more than 70% confluence were named as passage zero (P0) cells. When cells were grown 70% confluence, they were sub-cultured at 1/5 dilution for later passaging. The culture media was replaced every 3 days till growing to proper confluence.

Fabrication of polyurethane acrylate (PUA) mold

Fabrication of PUA mold was performed as previously described [17]. Briefly, the PUA mold for nanoscale patterning was fabricated by curing PUA pre-polymer (311RM, Minuta Technology, Osan, Korea) on silicon master molds prepared by photolithography. The ultraviolet (UV)-curable PUA mold material consists of a functionalized precursor with an acrylate group for cross-linking, a monomeric modulator, a photo-initiator and a radiation-curable releasing agent for surface activity. To fabricate a sheet-type mold, the liquid precursor was drop-dispensed onto a silicon master mold, and then a flexible, transparent polyethylene terephthalate (PET) film was brought into contact with the precursor surface. Subsequently, the mold was exposed to UV light (λ = 200–400 nm) for 20 s through the transparent backplane (dose = 100 mJ/cm^2). After UV curing, the mold was peeled from the master and additionally cured overnight to terminate the remaining active acrylate groups prior to use as a first replica. The resulting PUA mold used in the experiment was a thin sheet with a thickness of ~50 μM.

Fabrication of the nanoscale ridge/groove-patterned surface

Fabrication of the nanoscale ridge/groove-patterned surface PUA micro- and nanoscale ridge/groove pattern

arrays were fabricated on glass coverslips using UV-assisted capillary force lithography. The glass coverslip was rinsed with ethanol in an ultrasonic bath for 30 min, washed in a flow of distilled water and dried in a drying oven. To increase the adhesion between the PUA nanostructures and the glass interface, an adhesion promoter (phosphoric acrylate: isopropyl alcohol, 1.25:10, v/v) was coated onto the glass substrate. A small amount of the PUA precursor (~ 0.1–0.5 ml) was drop-dispensed onto the substrate, and a first-replicated PUA mold (same material but without active acrylate groups) was directly placed onto the surface. The PUA precursor spontaneously moved into the cavity of the mold by means of capillary action and was cured by exposure to UV light (λ = 250–400 nm) for 30 s through the transparent backplane (dose = 100 mJ/cm^2). After curing, the mold was peeled from the substrate using sharp tweezers.

Culture of human DPCs on the nanostructured pattern surface

The 250-nm nanoscale ridge/groove pattern arrays were immersed in a 0.1% gelatin solution for 12 h and rinsed with PBS. Human DPCs (5 to 7 passages; 3,000 cells/cm^2) were plated on the nanoscale pattern surfaces with DPSC culture medium, then attached cells were cultured in each differentiation media for 3, 7 and 14 days [17].

Osteogenic differentiation of human DPCs

To determine the differentiation potential, the basal medium was replaced with an osteogenic mediumconsisting of α-MEM supplemented with 0.1 μM dexamethasone, 0.05 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS. The osteogenic medium was replaced every other day. After 3, 7 and 14 days of induction culture, osteogenic differentiation was assessed by the expression of osteogenic marker genes, Osteocalcin (OC) and Runx2, and Alizarin red staining. For Alizarin red staining, the cells were fixed in 10% formalin for over 1 h, and were stained with alizarin red solution (2%, pH 4.2) for 15 min. The stained cells were dehydrated in pure acetone, washed inacetone-xylene (1:1) solution, cleared with xylene [16].

Adipogenic differentiation of human DPCs

To determine differentiation potential, the basal medium was replaced with an adipogenic medium consisting α-MEM supplemented with 2 mM L-glutamine, 0.5 μM dexamethasone, 0.5 mM isobutylmethylxantine, 50 μM indomethacin, 100 U/ml penicillin, 100 mg/ml streptomycin and 20% FBS. The adipogenic medium was replaced every other day. After 3,7 and 14 days, adipogenic differentiation was assessed by the expression of adipogenic marker gene, peroxisome proliferator activated receptor-γ (PPAR- γ), and Oil Red O staining. For Oil Red O staining, the cells were fixed in 10% formalin for over 1 h and stained with fresh Oil Red O solution for 2 h [18].

RNA isolation and expression analysis

Total RNA was extracted from the differentiated cells using RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using oligodT priming and PrimeScript RTase(Takara Bio, Kyoto, Japan) according to the manufacturer's instruction. In brief, the reverse transcription reaction was carried out in 20 μl (5X PrimeScript buffer, 2.5 μM oligodT primer, 0.5 mM dNTP, 1 unit RNase inhibitor and 10 units of Superscript II enzyme) at 42°C. Oneμl of the single strand cDNA product was used as template for each PCR reaction. Standard PCR conditions were as follows: 10 min at 94°C, followed by 30 cycles of 30 s of denaturing at 94°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C. The primer sequences are as follows: OC; Forward: 5-ATGAGAGCCCTCAC ACTCCTC-3, Reverse: 5-GCCGTAGAAGCGCCGATAG GC-3, Runx2; Forward: 5-CAGACCAGCAGCACTCCATA -3, Reverse: 5-TTCAATATGGTCGCCAAACA-3, PPAR-γ; Forward: 5-CGTGGCCGCAGATTTGAAAG-3, Reverse: 5-AAAGGA GTGGGAGTGGTCTT-3, GAPDH; Forward: 5-GGGCTG CTTTTAACTCTGCT-3, Reverse: 5-TGGCAGG TTTTTCT AGACGG-3.

Results

Primary cell culture and differentiation potential of human DPCs

In the first series of experiments, the heterogeneous DPCs obtained from mesiodens were cultured primarily and the mixed cell populations were observed (Fig. 1A). After 3 passages, the adhered and expanded homogenous population of the cells showed fibroblast-like morphology

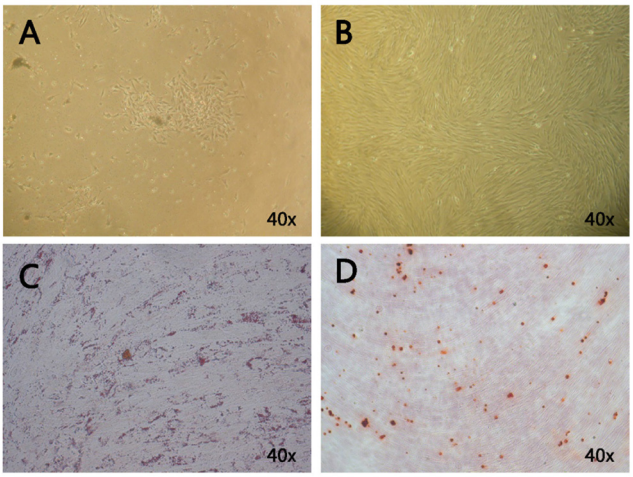

Fig. 1. Morphology and differentiation properties of dental pulp cells (DPCs). (A) Primary human DPCs derived from pulp tissue of a supernumerary tooth. (B) *In vitro* cultured DPCs at passage 3. (C) Differentiated DPCs stained with Oil red O after 28 days of adipogenic induction. (D) Differentiated DPCs stained with Alizarin red after 28 days of osteogenic induction.

(Fig. 1B). To confirm the differentiation potential of DPCs, the cells were seeded onto the gelatin-coated culture dish and cultured for 28 days with adipogenic or osteogenic induction medium. Each the differentiated cell groups exhibited round bubble-like adipocyte appearance (Fig. 1C) and osteoblast producing osteoid materials (Fig. 1D).

Adipogenic and osteogenic differentiation of human DPCs on 250-nm nanoscale ridge/groove pattern arrayed surface

To elucidate the effects of 250-nm nanoscale ridge/groove pattern arrayed surfaceon differentiation of DPCs, the nanoscale pattern arrayed surface were coated with gelatin (Fig. 2A) and DPCs were seeded onto both the nanoscale pattern arrayed surface and the gelatinized surface of the conventional culture dish. Two days after seeding, the morphology of attached cells from both experimental groups was compared. The cells on nanoscale pattern group showed linear arrangement shape (Fig. 2B), while the cells on conventional surface placed irregularly (Fig. 2C).

To investigate whether nanotopography would influence differentiation, DPCs were cultured on nanoscale pattern arrayed surface or conventional gelatinized surface withadipogenic or osteogenic induction media. In order tocompare the level of adipogenic differentiation, Oil red O staining was conducted to measure accumulated intracellular lipid droplets on 3, 7 and 14 day of culture. The cells on nanoscale pattern arrayed surface showed remarkable increase in Oil red O staining on Day 14 (Fig. 3). Next, Alizarin red staining was conducted to compare the level of osteogenic differentiation. Different from adipogenic induction result, the Alizarin red staining result showed that nanoscale pattern arrayed surface does not improve osteogenic differentiation induction by Day 14. Conventional surface tended to have more red stained (presumptive osteogenic) cells on Day 14 (Fig. 4). In the gene expression analysis by RT-PCR, PPAR-γ, an adipogenic marker gene, tended to express stronger in

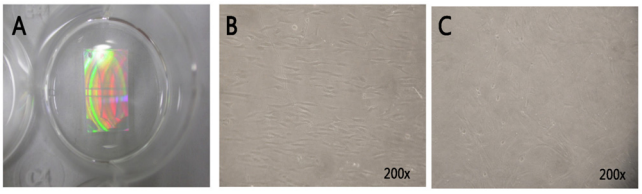

Fig. 2. The 250-nm nanoscaleridge/groove pattern arrayed surface and dental pulp cells (DPCs) cultured on the surface. (A) Gelatin coated nanoscale ridge/groove pattern array (rectangular sheet in the middle of dish well of a 24-well dish; 1.55 cm inner diameter). (B) DPCs cultured on nanoscale pattern arrayed surface showing linear arrangement shape. (C) DPCs cultured on gelatinized conventional dish surface showing irregular distribution.

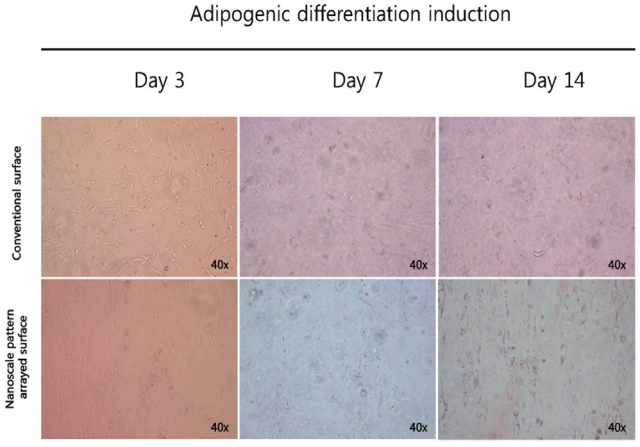

Fig. 3. Adipogenic differentiation of dental pulp cells (DPCs) cultured on nanoscale ridge/groove pattern arrayed surface. DPCs were plated on either nanoscale pattern arrayed or conventional surface and were pre-cultured in dental pulp stem cell culture medium for 24 h. Then the medium was replaced with adipogenic medium and the cells were cultured for additional 3, 7 and 14 days. The differentiated cells were stained with Oil red O. The cells on nanoscale pattern arrayed surface showed remarkable increase in Oil red O staining on Day 14.

nanoscale pattern arrayed surfacethan in conventional surface (Fig. 5), whereas osteogenic marker genes, Runx2 and OC, expressed relatively earlier and stronger in conventional surfacethan those in nanoscale pattern arrayed surface (Fig. 6).

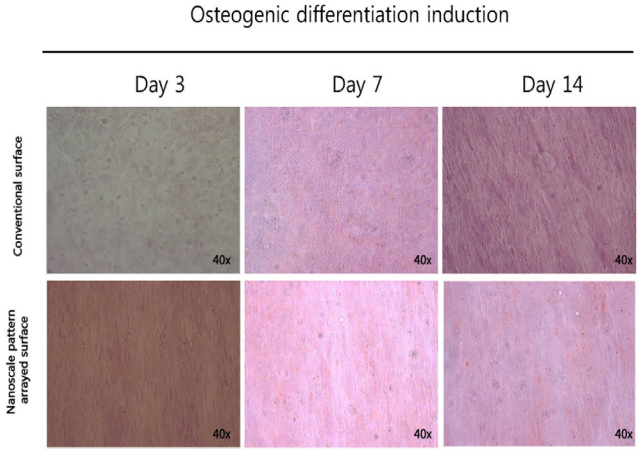

Fig. 4. Osteogenic differentiation of dental pulp cells (DPCs) cultured on nanoscale ridge/groove pattern arrayed surface. DPCs were plated on either nanoscale pattern arrayed or conventional surface and pre-cultured in a dental pulp stem cell culture medium for 24 h.Then the medium was replaced with osteogenic medium and the cells were cultured for additional 3, 7 and 14 days. The differentiated cells were stained with Alizarin red. Conventional surface tended to have more red stained (presumptive osteogenic) cells on Day 14.

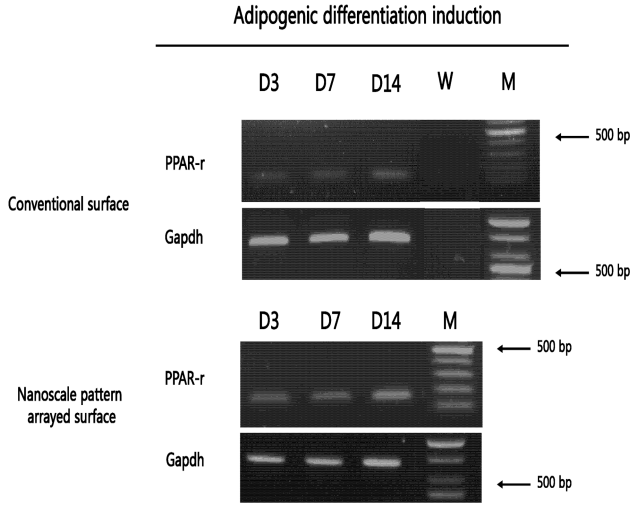

Fig. 5. Expression of adipogeniclineage specific genesin differentiated dental pulp cells (DPCs) cultured on nanoscale ridge/ groove pattern arrayed surface. Expression of an adipogenic lineage gene, PPAR-ɣ, on Day 3, 7 and 14.W: water as negative control. M: 1kb size marker.

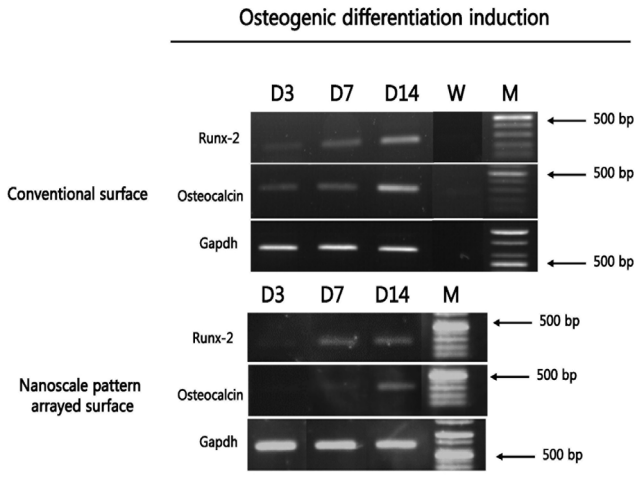

Fig. 6. Expression of osteogenic lineage specific genes in differentiated dental pulp cells (DPCs) cultured on nanoscale ridge/ groove pattern arrayed surface. Expression of osteogenic lineage genes, Runx2 and Osteocalcin, on Day 3, 7 and 14.W: water as negative control. M: 1kb size marker.

Discussion

The objective of this study was to investigate the effect of the 250-nm nanoscale pattern arrayed surface on adipogenic and osteogenic differentiation of DPCs obtained from the supernumerary tooth which contain human DPSC population.

It has been reported that the characteristics of human DPSCs from supernumerary teeth is similar to the characteristics of healthy deciduous or permanent teeth [9, 10]. This suggests that human DPSC from supernumerary teethis a potentialstem cell source for therapeutic purpose. In this study, the DPCs after primary culture of dental pulp tissue showa heterogeneous population (Fig. 1A). During passaging, however, the population of DPCs in culture with DPSC culture medium became homogeneous (Fig. 1B) and presented two distinct features identical to DPSCs; spindle-shaped fibroblast-like appearance and more rounded epithelial cell-like appearance [19]. In addition, they also had similar differentiation abilities to DPSCs in the presence of either adipogenic or osteogenic induction media (Fig. 1C, D). The results of our study corresponded well with those found in the previous experiments of DPSCs by other research groups [5-8, 10, 20].

Since exogenous chemicals can cause unknown toxicities *in vivo,* chemical-free stem cell differentiation can be an alternative way to overcome this problem. However,

different from chemical-induced differentiation [10, 11], few studies reported about differentiation induction of DPSCs by modifying physical condition of culture. The present study used nanoscale pattern arrayed surfaceto improve stem cell differentiation. In this study, the cells on the nanoscale pattern arrayed surface showed linear arrangement while the cell on the conventional surface located irregularly (Fig. 2A, B).With the intention to determine the effect of nanoscale pattern arrayed surface on either adipogenic or osteogenic differentiation of DPCs, lineage specific staining were performed. After adipogenic differentiation induction, more Oil red O positive cells were observed in nanoscale pattern arrayed surface group than the cells in conventional surface one (Fig. 3). This suggests that nanoscale pattern arrayed surface enhanced adipogenic differentiation of DPCs. On the other hand, there were no differences between two the different surface groupsafter osteogenic differentiation induction (Fig. 4).

In general, adipogenic cells appear to be spherical shapes whereas osteogenic cells show a more polygonal appearance and form nodules [21]. In the present study, while adipogenic differentiation was increased, osteogenic differentiation of DPCs was not improved by the physical environment of nanoscale pattern arrayed surface. Since the nanoscale pattern arrayed surface induces linear arrangement of the cells in culture, this may be one of the causes to suppress osteogenic differentiation of DPCs. It has been known that adipogenesis and osteogenesis are strongly related, and increased adipocyte negatively regulate osteogenesis [22, 23]. Therefore, the physical environment favoring adipogenesis could possibly act conversely for osteogenesis in the present study. Gene expression analysis also partly support this result since PPAR-γ, an adipogenic marker gene, expressed relatively stronger in nanoscale pattern arrayed surface, whereas theexpression of osteogenic marker genes such as Runx2 and OC showed opposite results (Fig. 5, 6).

In conclusion, we demonstrated the effect of nanoscale ridge/groove pattern arrayed surface on adipogenic differentiation of DPCs. The physical stimulation by nanoscale pattern arrayed surface enhanced adipogenic differentiation of human DPCs derived from mesiodens a form of supernumerary tooth. The results above provide an improved understanding of the effects of topography on cell differentiation as well as the potential use of supernumerary tooth in the field of regenerative medicine or dentistry.

Acknowledgements

This study was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2006-2004042) and the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture, Food and Rural Affairs (MAFRA; 111160-04), Republic of Korea.

Conflicts of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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