치의학석사 학위논문

인간 과잉치 치수 유래 다분화능세포의 체외 분화에 나노구조표면이 미치는 영향

Effects of nanostructured surfaces on in vitro differentiation of multi-potent pulp cells derived from human supernumerary tooth

2013년 2월

서울대학교 치의학대학원

치의학과

조환성

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지도교수 : 노 상 호

이 논문을 치의학석사학위논문으로 제출함

2013년 2월

서울대학교 치의학대학원

치의학과

조 환 성

조환성의 석사학위 논문을 인준함
2012년 11월

위원장 _____(인)

부위원장 _____(인)

위원 _____(인)

Abstract

Effects of nanostructured surfaces on in vitro differentiation of multi-potent pulp cells derived from human supernumerary tooth

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Human dental pulp stem cells (hDPSCs) are multipotentmesenchymal stem cells that have several differentiation potentials. The tissue differentiated from these cells can provide insights for regenerative therapeutics and tissue engineering. The mesiodens is the most frequent form in the supernumerary tooth and dental pulp stem cells from mesiodens can be differentiated into several lineages similar to that from normal deciduous tooth. Recently, it has shown that the influence of nanostructured substrate to differentiation was dramatic positively. In this study, we examined the osteogenic and adipogenic differentiation of

dental pulp cells from mesiodens,which contains hDPSCs, cultured on nanostructured substrate in the presence of each induction media. The enhancement of osteogenic and adipogenicdifferentiation on nanostructured substratewas discovered. In particular, the contribution of nanostructured substrate to the elevation of adipogenic differentiation induction was remarkable. In conclusion, specific surface nanotopography can enhance adipogenic and osteogenic differentiation in vitro, and it may contribute the development of hard tissue regeneration technology.

Key words : supernumerary teeth, human dental pulp cell, osteogenic differentiation, adipogenic differentiation, nanostructured surfaces

Student ID : 2009-22733

Contents

Introduction

During tooth development, the interactions between ectodermic and mesenchymal components from the cranial neural crest are important for differentiation of dental hard tissues. Recently, dental pulp stem cells (DPSCs) can be extracted from human dental pulp and exhibited various differentiation potential into not only odontoblasts but also adipocytes, neurons, chondrocytes, myocytes, osteoblasts.[1)~4)] It has also been reported that they are considered for the therapeutic tool of stem cells because of high efficiency, low morbidity and extensive differentiation ability.[5)]

Dental pulp stem cells (DPSCs) can be primarily derived from the pulp tissues of various teeth types. However, It is not simple to obtain the human dental pulp stem cells (hDPSCs) from healthy deciduous teeth or permanent teeth. By the recent research, dental pulp tissue from a supernumerary tooth, considered to be a usually discarded tissue, also expressed stem cells and differentiation markers, which suggested their stem cell origin and differentiation capability similar to deciduous tooth.[6)] The mesiodens is the most frequent form in the supernumerary tooth and may give rise to eruptive disturbance or root resorption for adjacent teeth. Therefore, the extraction of mesiodens is strongly recommended and dental pulp tissue from mesiodens is very useful as multipotent stem cell resources.

Osteoblasts differentiated from multipotent stem cells are able to shed

light on patients needed to be provided bone regeneration therapy and have a critical role on development of biocompatible osteogenic material.

Differentiated Adipocytes can also contribute to the basis for future alternative bioenergy industry and the underlying mechanism of adipogenic differentiation provides a clue for solving the obesity problem. Recently, it has shown that the ossification process derived from DPSCs has applied for clinical application in bone reconstruction. [7)-9)]

Generally, cells in their natural environment interact with other cells and surrounding extracellular matrix, soluble factors in nano-size scale for adhesion, immune response, differentiation and so on. By the way, Harrison in 1911 first demonstrated the ability of the substratum to influence cell orientation, migration and cytoskeletal organization.[10)] Since then, development of the several nano-fabrication techniques leads to various studies for strong biological effects of nanotopography on a several cell types. [11)] Although the substratum nanotopography to influence cell proliferation, migration, adhesion has been diversely studied, the effect of nanostructures on differentiation of dental pulp cells has rarely been understood.

Lately, it was reported that the culture on nanostructured surfaces (fabricated by UV-assisted capillary force lithography) with induction media synergistically enhanced osteogenic differentiation of human mesenchymal stem cells. This study suggested that the nanotopographic surfaces can influence to regulate stem cell differentiation in the presence of inductive biochemical signals. [12)]

In this study, we investigated the osteogenic and adipogenic differentiation of human dental pulp cells from mesiodenson nanostructured surfaces in the presence of each induction media. To confirm the degree of differentiation, Alizarin Red S and Red oil O were each used for staining of differentiated cells and the each osteoblast /adipocyte-specific marker genes expression were analyzed for 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 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. The extracted teeth were cut around the cemento-enamel junction using cutting disk to expose the pulp tissue as previously described. [13]. 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 cells were cultured in MEM-Eagle containing 10% (v/v) fetal bovine serum (FBS; Life technologies, NY, USA) and 100IU/ml penicillin-100 μg/ml streptomycin (Life technologies).

The cells extracted from mesiodens were seeded onto plastic tissue culture plate and incubated for 3 days at 37° C in humidified atmosphere containing 5% CO_2. Available dental pulp cells were selected on the basis of their ability to adhere onto the culture plate. The culture medium was refreshed once every 3 days to allow further growth. The adherent cells grown to more than 70% confluency were named as passage zero (P0)

cells. Later, passages were defined accordingly. For passaging, the cells were washed with Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS) (Life technologies) and detached by incubating with trypsin-EDTA solution (0.25% (w/v), Life technologies) for 5min at 37° C. Growth medium was added to inactivate trypsin. The cells were counted and then cultured in 75 cm^2 plates (BD Labware Europe) at a density of 10^6 cells/plate. Growth medium was replaced every 3 days over a period of 10-14days. At passage 3, the cells were stored in Liquid Nitrogen in MEM-Eagle containing 10% (v/v) demethylsulfoxide. For appropriate differentiation, the cells were re-suspended in growth medium as mentioned above.

Scaffold fabrication

Electrospinning was used to construct nano-fiber scaffolds from Poly L-lactic acid (PLLA) (MW = 157,000), or PLLA supplemented with single-walled carbon nanotubes (SWCNT) or multi-walled carbon nanotubes (MWCNT) (Palasmachem). PLLA (3.5% w/v) was dissolving in chloroform/N,N-dimethylformamide (8.5:1.5, v/v). For carbon nanotubes (CNT)-containing PLLA solutions, CNT were first well dispersed in chloroform and then combined with DMF and PLLA in the same proportions stated above. The final concentrations of CNT in solution were equivalent to 3% of the PLLA mass. Solutions were ultrasonicated and then stirred overnight to ensure homogeneity. The polymer solution, plus or minus CNT, was ejected, via a syringe pump,

through a blunted 21-gauge needle. A potential of 25 kV was applied between the needle and the collector. The nanofiber jet was collected on a stainless steel cylinder rotating at 2,400 rpm 15 cm from the spinneret tip.

Scaffold surface modification

The surfaces of some replica scaffolds were plasma treated to render them hydrophilic. Plasma surface treatment was performed using a low frequency plasma generator set on 40 kHz. (Diener Electronics GmbH +Co. KG, Germany). Oxygen (O_2) was introduced into the reaction chamber with the glow discharge ignited for 3min to modify the scaffold surfaces.

Human dental pulp cells cultured on the nanostructured pattern surface

The 250nm width ridge/groove nanopatterned surfaces were immersed in a 0.1% gelatin solution for 12hrs and rinsed with PBS. Human dental pulp cells were plated on the nanopattern surfaces in DMEM medium containing 10% FBS,then attached cells were cultured in each induction media for 3, 7 and 14 days.[14)]

Osteogenic differentiation of human dental pulp cells

For osteogenic differentiation, cells (P5~P7; 3,000 cells/cm^2) were seeded onto collagen-type I precoated coverslips in 6-well plates. The differentiation medium (MEM-Eagle supplemented with 0.1 μM

dexamethasone, 0.05 μM ascorbate-2-phosphate (Wako Chemicals, Richmond, VA, USA), 10 mM β-glycerophosphate, 100U/ml penicillin-100mg/ml streptomycin and 10% FBS) was replaced once per two days. After 3, 7 and 14days, osteogenic differentiation was assessed by osteogenic marker genes expression (osteocalcin and Runx2) and alizarin red staining. For Alizarin Red staining, the cells were washed for 5mins in ice-cold 70% ethanol. The cells were stained with alizarin red solution (2%, pH 4.2) for 30s. The stained cells were dehydrated in pure acetone, washed inacetone-xylene (1:1) solution, cleared with xylene. [13)]

Adipogenic differentiation of human dental pulp cells

For adipogenic differentiation, cells (P5~P7; 2,000 cells/cm^2) were seeded in 6 well plate for 1 day.The differentiation medium (MEM-Eagle supplemented with 2mM L-glutamine, 0.5 μM dexamethasone, 0.5mM isobutylmethylxanthine (IBMX), 50 μM indomethacin, 100U/ml penicillin-100mg/ml streptomycin and 20% FBS) was replaced once per two days. After 3, 7 and 14days, adipogenic differentiation was assessed by adipogenic marker gene expression (PPAR-γ) and Oil Red O staining. For Oil Red O staining, the cells were fixed in 10% formalin for over 1h and stained with fresh Oil Red O solution for 2hrs. [15)]

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 PrimeScriptRTase (Takara Bio, Kyoto, Japan) according to the manufacturer's instruction. In brief, the reverse transcription reaction was carried out in 20㎕ (5X PrimeScript buffer, 2.5㎛M oligodT primer, 0.5mMdNTP, 1unit RNase inhibitor and 10units of Superscript II enzyme) at 42° C. 1㎕ of the single strand cDNA product was used as template for each PCR reaction. Standard PCR conditions were as follows: 10min at 94° C, followed by 30cycles of 30 s of denaturing at 94° C, 30s of annealing at 55° C, and 30s of extension at 72° C..The primer sequences are as follows: osteocalcin;Forward: 5-atg agagccctcacactc ctc-3, Reverse: 5-gcc gtagaagcgccgata ggc-3, Runx 2; Forward: 5-cag accagcagc act cca ta-3, Reverse: 5-ttc aatatggtcgccaaa ca-3, peroxisome proliferator activated receptor-γ (PPAR-γ); Forward: 5-cgt ggccgcagatttgaa ag-3, Reverse: 5-aaa ggagtgggagtggtc tt-3, GAPDH; Forward: 5-ggg ctgctttta act ctg ct-3, Reverse: 5-tgg cag gtttttctagac gg-3

Results

Primary cell culture and differentiation potential of human dental pulp tissue

In the first series of experiment, the heterogenous dental pulp cells extracted from mesiodens were cultured primarily and the mixed cell population was observed (Fig. 1A). After 3 passages, the adhered and expanded clonal cells show fibroblast-like morphology (Fig. 1B). To confirm the differentiation potential of dental pulp cells, the cells were cultured for 28days in the presence of adipogenic and osteogenic induction media. The each cells exhibited round bubble-like adipocyte appearance (Fig. 1C) and osteoblast producing osteoid materials (Fig. 1D).

Adipogenic and osteogenic differentiation of human dental pulp cells on the nanopatteren surface

To elucidate the effects of nanopattern surface for differentiation of dental pulp cells, the nanopatterned surfaces coated in gelatin were prepared (Fig. 2). First, the cells were cultured on non-nanopattern or nanopattern surface in a time-dependent manner with adipogenic induction media. Then, adipogenic differentiation on nanostructured surface compared to non-nanopattern surface, especially at 7 and 14 days, was synergistically enhanced (Fig. 3). To confirm the extent of differentiation for the cells on nanopattern surfaces at 3, 7 and 14 days,

the expression of adipogenic marker gene, PPAR−γ, was analyzed and slightly increased (Fig. 5D−F).

Second, the cells were also cultured with osteogenic induction media as the condition mentioned above. Not similar to the case of adipogenic differentiation, the effects of nanopattern for osteogenic differentiation was insignificant (Fig. 4). The result that expression of osteogenic marker genes, osteocalcin and Runx2 were increased show normal osteogenic differentiation (Fig. 5A−C).

Discussion

According to this study, human dental pulp cells extracted from supernumerary teeth exhibited similar cell morphology to healthy deciduous teeth as previously described. [6] Primary dental pulp cells are a heterogeneous population of cells including dental pulp stem cells. [16] Although they still needs to verify as definite dental pulp stem cells, they can be selected and maintained in vitro as dental pulp stem cells by appropriate culture media for dental pulp stem cell. [1] In this study, the cells cultured in dental pulp stem cell media have two distinct features identical to DPSCs as previously known; spindle-shaped fibroblast-like appearance and more rounded epithelial cell-like appearance.[17] They also have differentiation ability similar to DPSCs in the presence of induction media. [1~4] (Fig. 1)

So far, numerous studies have shown that in the presence of each induction media, each differentiated cells can be derived from multipotent stem cell. On the contrary, it was reported that nanopattern structure alone can induce the differentiation of human embryonic stem cells into neuronal lineage without the use of any differentiation-inducing agents. [14]. Therefore, we could expect that the culture on nanostructured surfaces with induction media will represent the additive effect for differentiation into each lineage from dental pulp cells. Recently, You *et al* have shown the synergistically enhanced differentiation in the presence of nanostructured surfaces with induction media as expected.

[12]. In this study, dental pulp cells also exhibited additive differentiation effect on nanostructured surfaces with induction media more than with induction media alone. Particularly, the extent of adipogenic differentiation was dramatic, whereas that of osteogenic differentiation was not. The biological environment of osteoblast on selected nanostructured surface specifically may be inappropriate due to inadequate adherence. Further study is needed for optimization of enhanced differentiation of osteoblast on nanostructred surfaces with various ridge/groove width.

As mentioned above, the osteoblasts and adipocytes differentiated from the multipotent stem cells can provide insight for regenerative medicine such as bone graft, fat injection and others. The more rapid differentiation technology can also facilitate mass production and increase the success rate for patient-customized tissue repair. In addition, it facilitates greatly the elucidation of the underlying mechanism for differentiation and trans-differentiation research.

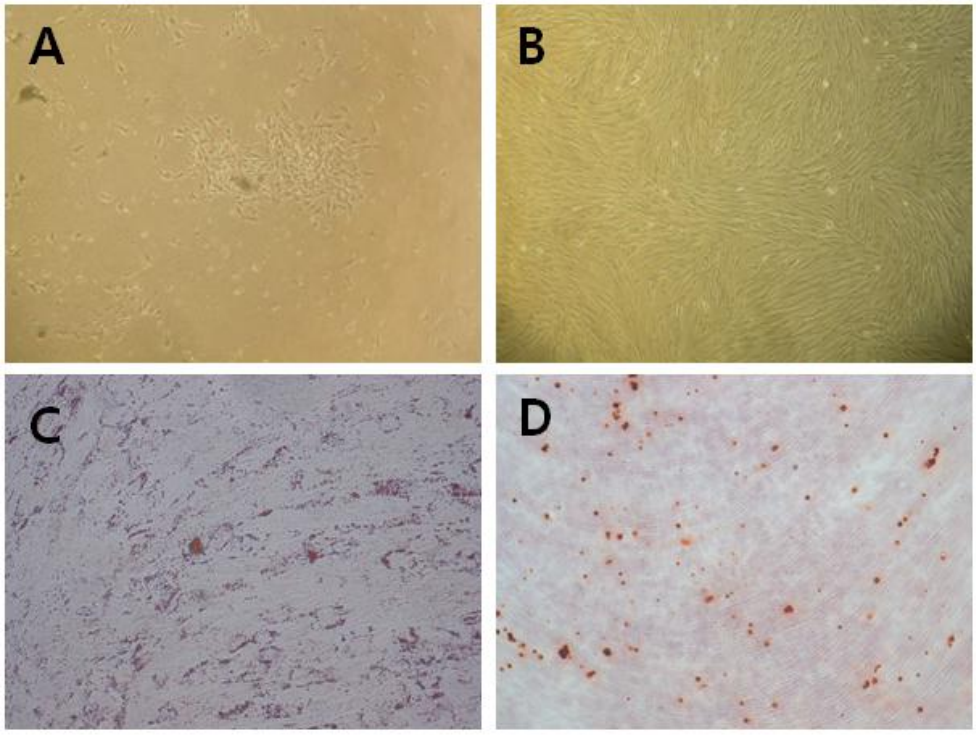

Figure1. Microscopic features and differentiation property of human dental pulp cells. (A) The feature of initial primary culture of dental pulp tissue as passage zero (P0) cells. The cells showed heterogenous population. (B) At passage 3, the feature of dental pulp cells. The cells exhibited spindle-like morphology. (C,D) Adipogenic (C) and osteogenic (D) differentiation of dental pulp cells. At passage 5~7, the cells were cultured for 28days in the presence of each induction media and stained with Oil Red O and Alizarin Red solution, respectively.

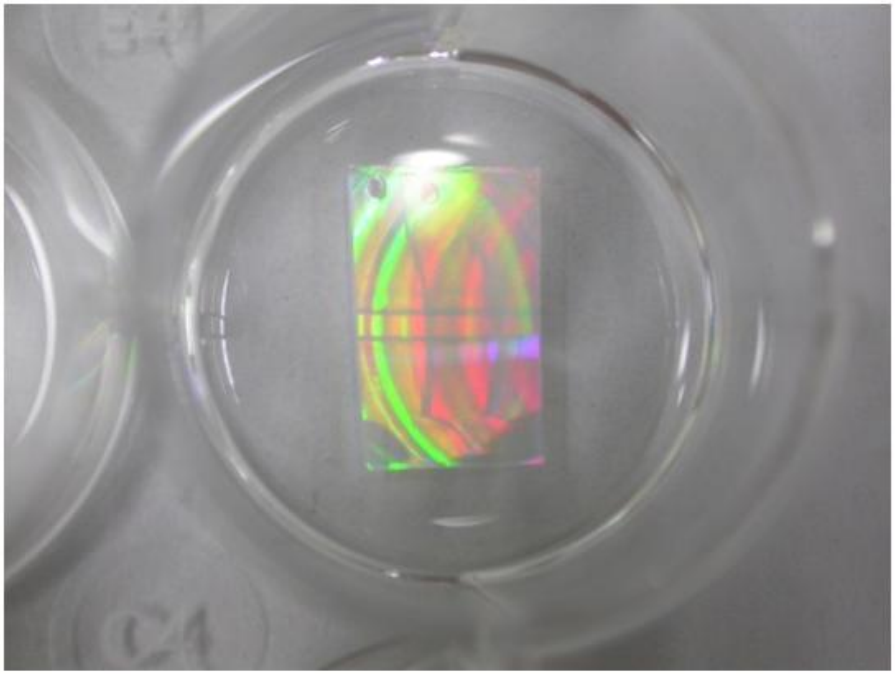

Figure2. Setting of nanostructured pattern surface. Human dental pulp cells were plated on the nanopatterned surfaces immersed in gelatin.

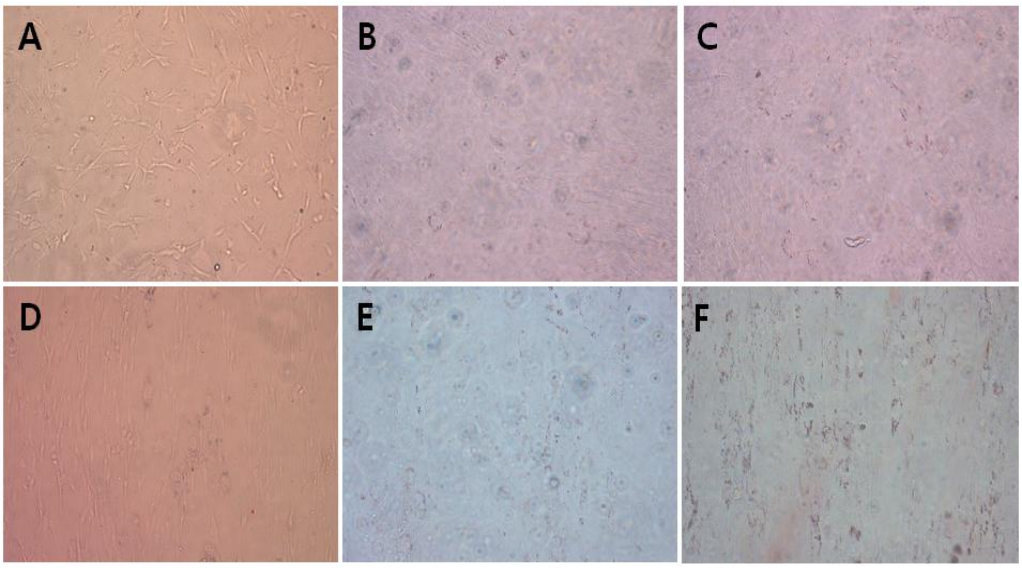

Figure3. Synergistically enhanced adipogenic differentiation of human dental pulp cells on the nanopatteren surface in a time-dependent manner. (A,B,C) Dental pulp cells were cultured on non-nanopattern surface for 3 (A), 7 (B) and 14 (C) days in the presence of adipogenic induction media and stained with Oil Red O (D,E,F) Dental pulp cells were cultured on nanostructured surface for 3 (D), 7 (E) and 14 (F) days in the presence of adipogenic induction media and stained with Oil Red O. Enhanced staining pattern was shown that on nanostructured surface compared to the differentiation on non-nanopattern surface, especially at Day 14.

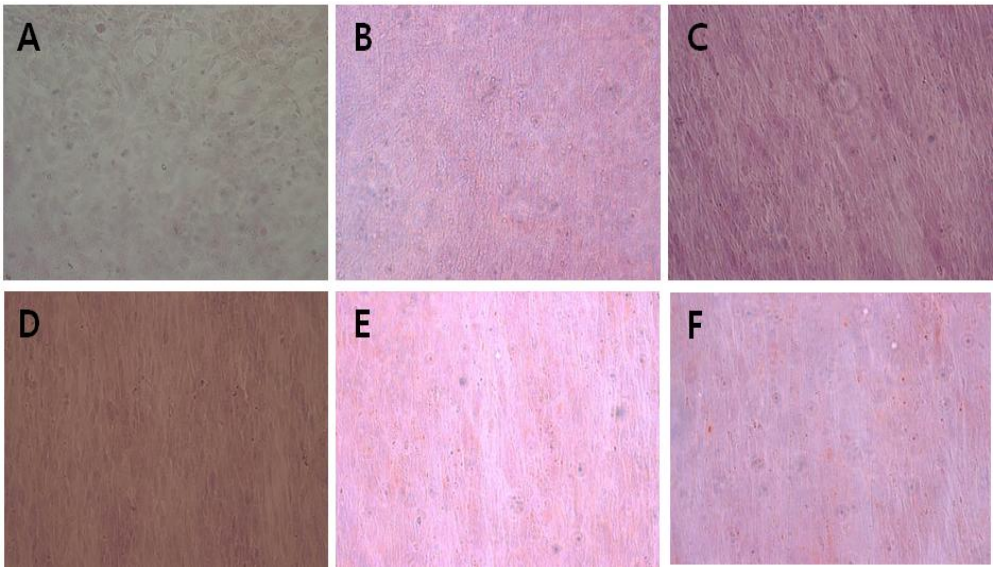

Figure4. Increased osteogenic differentiation of human dental pulp cells on the nanopatteren surface in a time-dependent manner. (A,B,C) Dental pulp cells were cultured on non-nanopattern surface for 3 (A), 7 (B) and 14 (C) days in the presence of osteogenic induction media and stained with Alizarin Red (D,E,F) Dental pulp cells were cultured on nanostructured surface for 3 (D), 7 (E) and 14 (F) days in the presence of adipogenic induction media and stained with Alizarin Red. Slightly increased staining pattern was generally shown for that on nanostructured surface compared to the differentiation on non-nanopattern surface.

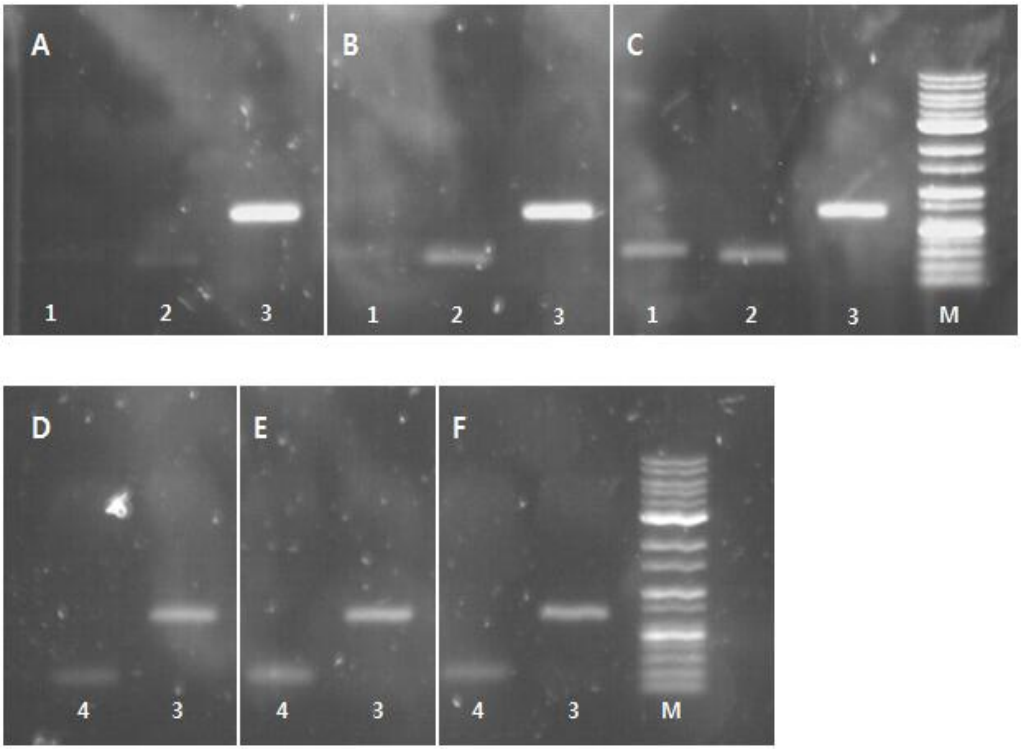

Figure5. Marker genes expression for osteogenic and adipogenic differentiated human dental pulp cells on the nanopatteren surface in a time-dependent manner. (A,B,C) Dental pulp cells were cultured on nanostructured surface for 3 (A), 7 (B) and 14 (C) days in the presence of osteogenic induction media and marker genes(osteocalcin andRunx2) expression was analyzed using RT-PCR (D,E,F) Dental pulp cells were cultured on nanostructured surface for 3 (D), 7 (E) and 14 (F) days in the presence of adipogenic induction media and marker gene (PPAR-γ) expression was analyzed using RT-PCR.Each numbers represent the following genes. 1: osteocalcin, 2: Runx2, 3:GAPDH 4: PPAR-γ

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국문초록

인간 과잉치 치수 유래 다분화능 세포의 체외 분화에 나노구조표면이 미치는 영향

조환성

서울대학교 치의학대학원

치의학과

인간 치수 줄기세포는 다양한 분화능력을 가지는 다능성 간엽 줄기세포이다. 이 세포들로부터 유래된 분화 조직은 재생의학이나 조직공학의 발전을 위한 실마리를 제공할 수 있다. 정중과잉치는 과잉치중에 가장 빈도 높은 형태로써, 정중과잉치에서 추출한 치수 줄기 세포들은 정상적인 유치에서 추출된 치수 줄기세포와 유사하게 다양한 세포로 분화할 수 있다. 최근, 나노구조로 이루어진 기질이 세포 분화를 촉진한다는 연구 결과가 있다.

따라서, 이번 연구에서는 정중과잉치로부터 추출한 치수 조직세포를 골모세포나 지방세포로 분화할 수 있는 유도 배지하에서 나노구조로 된 기질이 어떠한 역할을 하는지 알아보고자 하였다. 기대했던 대로 각각의 분화 유도 배지에서 나노패턴의 기질은 분화를 촉진하는 역할을 하였다. 특히, 지방세포분화를 유도하는데 있어 나노패턴의 영향은 더욱더 크게 기여했다. 결론적으로, 특이적인 나노패턴구조는 골모세포나 지방세포를 분화할 수 있는 환경하에서 그 분화 정도를 촉진한다는 것을 보여주었다.

주요어 : 과잉치, 인간 치수세포, 골모세포 분화, 지방세포 분화, 나노구조표면
학 번 : 2009-22733