Decellularized Matrix Derived from Neural Differentiation of Embryonic Stem Cells Enhances the Neurogenic Potential of Dental Follicle Stem Cells

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Abstract

Introduction: Dental follicle stem cells (DFSCs) possess neurogenic potential because they originate from the embryonic neural crest. This study investigated whether neural differentiation of DFSCs can be enhanced by culture on decellularized matrix substrata (NSC-DECM) derived from neurogenesis of human embryonic stem cells (hESCs). **Methods:** The hESCs were differentiated into neural stem cells (NSCs), and NSC-DECM was extracted from confluent monolayers of NSCs through treatment with deionized water. DFSCs seeded on NSC-DECM, Geltrex, and tissue culture polystyrene (TCPS) were subjected to neural induction during a period of 21 days. Expression of early/intermediate (Musashi1, PAX6, NSE, and βIII-tubulin) and mature/late (NGN2, NeuN, NFM, and MASH1) neural markers by DFSCs was analyzed at the 7-, 14-, and 21-day time points with quantitative real-time polymerase chain reaction. Immunocytochemistry for detection of βIII-tubulin, PAX6, and NGN2 expression by DFSCs on day 7 of neural induction was also carried out. **Results:** Quantitative RT-PCR showed that expression of PAX6, Musashi1, βIII-tubulin, NSE, NGN2, and NFM by DFSCs was enhanced on NSC-DECM versus either the Geltrex or TCPS groups. Immunocytochemistry showed that DFSCs in the NSC-DECM group displayed more intense staining for βIII-tubulin, PAX6, and NGN2 expression, together with more neurite outgrowths and elongated morphology, as compared with either Geltrex or TCPS. **Conclusions:** DECM derived from neurogenesis of hESCs can enhance the neurogenic potential of DFSCs. *(J Endod 2017;43:409–416)*

Key Words

Cell fate, dentistry, lineage, neurogenic, regeneration, tissue engineering

Significance

Neural differentiation is one challenge for achieving natural-resembling regenerated pulp tissue. This study used neural stem cell decellularized matrix to enhance the neural differentiation of dental follicle stem cells.

The human dental follicle (dental sac) surrounding the unerupted tooth is of ectomesenchymal origin and gives rise to periodontal tissue during development (1). Because of its embryonic neural crest origin, there exists a resident stem/progenitor cell subpopulation within the human dental follicle with neurogenic potential, which are commonly referred to as dental follicle stem cells (DFSCs) (2). Previously, Li et al (3) demonstrated that transplanted DFSCs could repair spinal cord injury within a rat model when combined with an aligned poly-caprolactone/polyactic-glycolic acid electrospun scaffold. Nevertheless, this study was based on transplantation of undifferentiated DFSCs, and there is a strong likelihood that some degree of neural induction of these cells *in vitro* before transplantation *in vivo* could further enhance their regenerative potential (4).

To date, there have only been a handful of studies (5–7) that have reported the neural induction of DFSCs *in vitro*. Within the natural physiological environment *in vivo*, stem cells, neural progenitors, and mature neurons are enveloped by a highly complex extracellular matrix (ECM) that has crucial roles in modulating neurogenesis (8). Because the suboptimal two-dimensional *in vitro* culture medium is very different from the three-dimensional microenvironment of the stem cell niche *in vivo*, this could compromise the neurogenic differentiation pathway of DFSCs. This limitation may be overcome by using a bioactive ECM substrata to promote the neurogenesis of DFSCs *in vitro*.

The culture substrata that were used previously for *in vitro* neural induction of DFSCs include bare tissue culture polystyrene (TCPS) (5, 7) and purified proteinaceous macromolecules such as poly-L-lysine, poly-L-ornithine, and laminin (6), all of which are unable to mimic or recapitulate the highly complex ECM environment of stem cells undergoing neurogenesis *in vivo*. It is possible that a more bioactive substrate derived from stem cell neurogenesis itself can provide signaling cues to further enhance the neurogenic potential of DFSCs. On the basis of previous studies (9, 10), decellularized matrix (DECM) extracted from neural stem cells (NSCs) differentiated from pluripotent human embryonic stem cells (hESCs) will be

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investigated as a potential bioactive substratum for enhancing the neurogenesis of DFSCs *in vitro* in comparison with bare TCPS and Geltrex, a commercially available basement membrane–derived cell culture substratum similar to Matrigel (11).

Materials and Methods

Cells, Culture Media, and Other Consumables

The hESCs (WA01 line) were obtained from Wicell Institute (Madison, WI). Human DFSCs (catalog no. FT002 F, lot no. 020609) were purchased from AllCells LLC (Alameda, CA). Unless mentioned otherwise, all cell culture media and supplements were procured from Life Technologies Inc (Carlsbad, CA), all cell and tissue culture lab ware were procured from Becton-Dickinson Inc (Franklin Lakes, NJ), and all chemical reagents were procured from Sigma-Aldrich Inc (St Louis, MO). Cell cultures were carried out within 5% CO_2 incubators set at 37°C.

Expansion Culture and Neural Induction of hESCs

The hESCs were routinely expanded and passaged in 6-well culture plates that were pre-coated with Geltrex by using mTeSR1 medium (catalog no. 05850; Stem Cell Technologies Inc, Vancouver, BC, Canada), as described previously (12). Coating of 6-well culture plates with Geltrex (catalog no. A15696-01; Life Technologies Inc) was carried out by placing 0.5 mL Geltrex in each well for 24 hours before cell seeding.

The protocol used for inducing hESCs into NSCs was similar to that described previously (12). Briefly, induction of hESCs into NSCs was carried out on Geltrex-coated 6-well plates with Neurobasal medium (catalog no. 21103-049; Life Technologies Inc), to which was added 1% (v/v) penicillin-streptomycin antibiotic solution and 2% (v/v) Neural Induction Supplement (catalog no. A16477-01; Life Technologies Inc). NSCs from the second to fourth passage were used for DECM derivation.

Derivation of DCM from NSCs

Adherent DECM on 6-well dishes were derived from NSCs by using a similar protocol to that described previously (13), except that cellular lysis was carried out with autoclaved Milli-Q ultrapure water (EMD Millipore GmbH, Darmstadt, Germany) instead of a mixture of Triton X-100 and ammonium hydroxide. Briefly, confluent cell sheets of NSCs were initially rinsed with phosphate-buffered saline (PBS), followed by treatment with autoclaved Milli-Q ultrapure water for 45 minutes at 37°C to lyse the cells. Then PBS was used to rinse the remaining DECM within the wells, followed by DNAse (100 U/mL) treatment for 1 hour at 37°C and a final rinse in autoclaved Milli-Q ultrapure water before air-drying. The dried adherent DECM on 6-well culture dishes (NSC-DECM) was stored at 4°C before utilization in further experiments. Altogether, 2 separate preparations of DECM were made for this study, all from the same batch of NSCs derived from hESCs.

Assessment of Protein, Collagen, and Glycosaminoglycan Contents of NSC-DECM and the Geltrex Coating

The total protein, collagen, and glycosaminoglycan contents of adherent NSC-DECM and Geltrex coating on the surface of 6-well dishes were assessed by the bicinchoninic acid assay (BCA) (catalog no. 23225; ThermoFisher Scientific Inc, Waltham, MA), hydroxyproline assay (catalog no. MAK008; Sigma-Aldrich Inc), and the Blyscan sulfated glycosaminoglycan assay (catalog no. B1500; Biocolor Ltd, Carrickfergus, County Antrim, UK), respectively, as described previously (13). Each of these assays were performed on 3 separate wells (n = 3) of adherent NSC-DECM and Geltrex coating on 6-well culture dishes.

Quantitative Real-time Polymerase Chain Reaction Analysis of Pluripotency and Neural Marker Expression

The RNeasy Plus Mini Kit (Qiagen, Valencia, CA) was used to extract the total RNA of cultured cells, which was then reverse-transcribed with SuperScript VILO Master Mix (Life Technologies, Grand Island, NY). The StepOne Real-Time PCR System (Applied Biosystems, Grand Island, NY) and SYBR Select Master Mix (Applied Biosystems) were used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Table 1 lists the primer sequences used for qRT-PCR analysis, which were based on those used in previous studies (14–18). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference control gene. The qRT-PCR analyses used the following amplification parameters: 2 minutes at 50°C, 20 seconds at 95°C, and 40 cycles of 3 seconds at 95°C, followed by 30 seconds at 60°C. The relative cycle threshold (Ct) was computed with the $2^{-\Delta\Delta Ct}$ method and normalized against endogenous GAPDH gene expression. Fold changes in gene expression by DFSCs subjected to neural induction on either NSC-DECM or Geltrex were computed with respect to DFSCs cultured on the TCPS control on day 7. For each gene analyzed by qRT-PCR, there were 3 biological replicates (n = 3) collected from 3 separate wells of cell cultures within 6-well culture dishes.

Immunofluorescence Staining for Detection of Fibronectin and Collagen within NSC-DECM and the Geltrex Coating

NSCs were seeded at a density of 2.0×10^4 cells/cm^2 on glass coverslips inserted within 6-well culture plates and cultured for at least 1 week until confluence was attained before DECM derivation, as described previously. Coverslips were pre-coated with Geltrex in a similar procedure described previously for 6-well culture plates. The coverslips with either NSC-DECM or Geltrex coating were initially blocked for 2 hours in PBS with 10% (v/v) fetal bovine serum. After washing 3 times in PBS, the samples were incubated with primary antibodies against fibronectin (mouse, catalog no. FBN11; ThermoFisher

TABLE 1. Primer Sequences Used for qRT-PCR

Gene		Primer sequences
Pluripotency markers		
OCT4	F	5′-CAAAGCAGAAACCCTCGTGC-3′
	R	5′-TCTCACTCGGTTCTCGATACTG-3′
NANOG	F	5′-TGATTTGTGGGCCTGAAGAAAA-3′
	R	5′-GAGGCATCTCAGCAGAAGACA-3′
SOX2	F	5′-AACCCCAAGATGCACAACTC-3′
	R	5′-GCTTAGCCTCGTCGATGAAC-3′
Early and intermediate neural markers		
PAX6	F	5′-ACCCATTATCCAGATGTGTTTGCCCGAG-3′
	R	5′-ATGGTGAAGCTGGGCATAGGCGGCAG-3′
Musashi1	F	5′-GAGCTTACAGCCATTCCTCTCAC-3′
	R	5′-GCGCTGATGTAACTGCTGACC-3′
βIII-tubulin	F	5′-AGACCTACTGCATCGACAACGAGG-3′
	R	5′-GCTCATGGTGGCCGATACCAGG-3′
NSE	F	5′-GTCCCACGTGTCTTCCACTT-3′
	R	5′-TGGGATCTACAGCCACATGA-3
Mature and late neural markers		
NGN2	F	5′-CGCATCAAGAAGACCCGTAG-3′
	R	5′-GTGAGTGCCCAGATGTAGTTGTG-3′
NeuN	F	5′-GCGGCTACACGTCTCCAACATC-3′
	R	5′-ATCGTCCCATTCAGCTTCTCCC-3′
NFM	F	5′-GTCAAGATGGCTCTGGATATAGAAATC-3′
	R	5′-TACAGTGGCCCAGTGATGCTT-3′
MASH1	F	5′-AAGAGCAACTGGGACCTGAGTCAA-3′
	R	5′-AGCAAGAACTTTCAGCTGTGCGTG-3′

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