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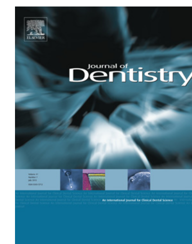

Immortalization and characterization of human dental mesenchymal cells

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ARTICLE INFO

Article history:
Received 11 November 2014
Received in revised form
7 February 2015
Accepted 8 February 2015

Keywords:
Dental mesenchymal cell
Immortalization
SV40 large T antigen
Tooth-specific marker

ABSTRACT

Objectives: Due to the rarity of human embryonic samples and limited proliferating capability of primary human dental mesenchymal cells, it is valuable to create an immortalized human dental mesenchymal cell line for studying dental mesenchymal cell differentiation and signalling pathways during detinogenesis in humans.

Methods: In this study, dental mesenchymal cells from human molar tooth germs at 19-week gestation were isolated and immortalized with pSV40. Single cell colonies were then selected by 96-well plate dilution. The immortalized cell line was characterized using immmunofluorescent microscopy, RT-PCR and Western blot for the expression of SV40 large T antigen and five genes specific for the mesenchymal stage during tooth development. The differentiation and mineralization activities of the immortalized and primary cells were compared using adipogenic and calcifying induction.

Results: The immortalized dental mesenchymal cell line displayed a higher proliferation rate, expressed several tooth-specific markers including Msx1, Pax9, Lhx6, Barx1, and Runx2, and maintained the ability to differentiate and form mineralized nodules.

Conclusions: Our results demonstrated that the immortalized human mesenchymal cell line retained the characteristics similar to primary human dental mesenchymal cells and can be used for studying the mechanisms of human dental mesenchymal cell differentiation and signalling pathways involved in human odontogenesis.

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1. Introduction

During mouse tooth development, the morphological signal initially appears in the dental epithelium, and then shifts to the mesenchyme. The constant reciprocal interplay of inductive signals between the epithelium and mesenchyme gives rise to the tooth formation.[1,2] In dental mesenchymal cells, the signalling pathways such as WNT (wingless-type MMTV integration site), BMP (bone morphogenetic protein), FGF (fibroblast growth factors) and SHH (sonic hegdehog) pathways are involved in its interaction with epithelial cells.[3] Over the past 20 years, studies using transgenic mouse model have proved that these signalling pathways are critical for tooth development regulation but the exact mechanisms on how these pathways exert their regulatory roles in human dental mesenchymal cells are still unclear. This is probably due to several difficulties in studying human dental mesenchymal

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http://dx.doi.org/10.1016/j.jdent.2015.02.008
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cells. First, the human samples for these cells are rare. Second, the primary mesenchymal cells isolated have limited proliferative abilities and undergo replicative senescence. Third, the difference between individuals greatly reduces the reproducibility of the primary cells in experiments. Therefore, in order to overcome these hurdles for investigating signalling pathways in dental mesenchymal cells for human tooth development, it is necessary to establish an immortalized human dental mesenchymal cell line.

Several murine immortalized mesenchymal cell lines have been established from mouse dental mesenchymal cells and displayed similar characteristics of the primary cells.[4–7] Although human and mouse tooth have demonstrated similar developmental processes that include lamina, bud, cap, and bell stages, there are still many discrepancies between them.[8,9] For example, the expression patterns of some regulatory genes exhibit distinct between human and mouse in tooth development.[9–11] Human has two sets of teeth (the deciduous teeth and permanent teeth), whereas mouse has only one. The mouse cell line may not reflect the signalling pathways in human and is not the ideal tool for human tooth development study. In addition, several immortalized human dental cell lines have already been established from mature tooth cells and proved useful tools in research.[12–15] However, they may not be suitable for studying early events during human tooth development as the conditions in mature and mesenchymal tooth cells are very different. In the present report, we employed simian virus 40 large T-antigen (SV40 T-Ag) and G418 to create an immortalized human dental mesenchymal cell line from developing tooth. The immortalized cells demonstrated infinite proliferative ability and maintained the phenotypic and genotypic characteristics of the primary cells *in vitro*.

2. Materials and methods

2.1. *Ethics statement*

All experimental procedures involving the usage animals were approved by the Animals' Ethics Committee at Fujian Normal University. The aborted foetuses were provided by the Hospital for Woman and Children Health of Fujian Province. Written informed consents were obtained from participants for the use of their medically aborted embryos for scientific research. The obtainment and application of human embryos in this study was permitted by the Ethical Committee of Fujian Normal University.

2.2. *Isolation of primary human dental mesenchymal cells*

Mandibular molar tooth germs of 19-week human embryo were obtained from medical aborted foetuses with the patients' approval at Southeast of the Women's Hospital (Fuzhou, Fujian, China). The tooth germs were washed with phosphate buffered saline (PBS) and digested in a solution containing 3 mg/ml collagenase type I and 4 mg/ml of dispase II for 30 min at 37 °C. After digestion, the mixture was filtered using a cell strainer (Corning) and the cells were maintained in minimum essential medium eagle-alpha modification (α-MEM) containing 10% foetal bovine serum (FBS) plus penicillin (100 Unit/ml) and streptomycin (100 μg/ml) and cultured at 37 °C in a humidified atmosphere of air with 5% CO_2. The culture media was replaced after 24 h and the cells were left to grow for 14 days before splitting. In further application, the cells were split at 1:4 ratio when reaching confluence.

2.3. *Establishment of immortalized human dental mesenchymal cells*

Primary human dental mesenchymal cells cultured after 3 PDLs were transfected with pSV3-neo, a plasmid containing coding sequences of SV40 T-Ag and a neomycin (G418)-resistance gene by liposome-mediated method.[4] After transfection, cells were maintained in the same culture media supplemented with 650 μg/ml G418. After selection, a serial dilution of the resistant cells at the 8th passage was added into a 96-well plate to create single colonies. Eight mono-clones were selected and grown continuously in culture media containing 650 μg/ml G418. Seven out of the eight clones became senescent after 35 population doublings (PDLs) and cannot expand for over 50 PDLs. Finally, the only clone that successfully survived over 50 PDLs was expanded into a cell line (ihEDMC4, immortalized human embryonic dental mesenchymal cells) and cultured for over 99 PDLs. The cell line ihEDMC4 cultured after 50 PDLs and primary human dental mesenchymal cells cultured after 3 PDLs were used for the characterization studies in this work. Morphology of primary and ihEDMC4 cells was observed using light inverted microscope IX51 (Olympus, Tokyo, Japan). Cell number and viability was measured using the Countstar automated cell counter (IC 1000, Shanghai Ruiyu Biotech Co., Ltd., China).

2.4. *Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA of primary and ihEDMC4 cells were obtained with TRIzol reagent (Invitrogen, San Diego, CA, USA) and cDNA was synthesized from 2 μg of the total RNA using Reverse Transcription System (TaKaRa, Dalian, China). The primer sequences used in this work are shown in Table 1. In a typical experiment, the PCR mixture was first heated to 95 °C for 5 min, then entering 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. After the cycles, the mixture was left at 72 °C for 7 min and kept at 4 °C. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

2.5. *Western blot assay*

Total cellular proteins were extracted using lysis buffer (6.25 M urea, 100 mM DTT, 20 mM Hepes pH 8.0, 25 mM NaCl, 0.05% Triton 100) containing protease inhibitor (Roche Applied Science, Mannheim, Germany). The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) buffer (50 mM Tris–Cl, pH 7.5. 150 mM NaCl) containing 0.05% Tween 20 (TBS-T) at room temperature for 1 h and incubated overnight at 4 °C with

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