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Original Article

Isolation and *in vitro* Characterization of Mesenchymal Stem Cells Derived from the Pulp Tissue of Human Third Molar Tooth

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Abstract

Background: It is still controversial that the stem cells isolated from human dental pulp meets the criteria for mesenchymal stem cells (MSCs). The aim of the present study was to examine whether or not they are MSCs, or are distinct stem cells population residing in tooth pulp.

Methods: Adherent fibroblastic cells in the culture of pulp tissue from human third molars were propagated through several successive subcultures. Passaged-3 cells with a tendency to differentiate into odontoblastic cells were used to examine the key properties of MSCs including typical tripotent differentiation potential into bone, cartilage and adipose cell lineages and the expression of typical surface antigens. Moreover, they were examined for growth capacity in culture.

Results: Dental pulp stem cells successfully progressed towards differentiation among three skeletal cell lineages. More than 90% of the cell population exhibited the expression of surface antigens known to be found on mesenchymal lineages such as CD105, CD90, CD44, and CD73, while only less than 2% expressed endothelial-hematopoietic epitopes including CD56, CD11b, CD34, CD31, CD33, and CD45. The cells exhibited a relatively high proliferation capacity with population doubling time of about 21.9 hours.

Conclusion: The dental pulp stem cells are of MSC population, and may be considered suitable for use in regenerative medicine, owing to their relatively rapid rate of in vitro propagation.

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Keywords ● Dental pulp ● odontoblast ● mesenchymal stem cells ● proliferation

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Introduction

Mesenchymal stem cells (MSCs) are a promising tool for cell-based treatment of variety of tissue defects due to their capacity of extensive replication and potential of multilineage differentiation. These cells were first isolated and described from bone marrow as a colonogenic fibroblastic cell population capable of producing deposits of bone and cartilage-like tissues in culture.[1] Due to the disadvantages associated with marrow MSCs including the burden of marrow harvest, the limited number of MSCs in marrow tissue and *in vitro* ageing problems

of the cells, many attempts have been made in the search for other sources of MSCs.[2-4] In this regards, several tissues including adipose, cord blood, peripheral blood, amniotic fluid, cartilage and bone have been shown to contain MSCs.[5-11]

Dental pulp is another exciting tissue that may contain MSCs. This tissue has been the subject of a number of investigations attempting to isolate and characterize stem cell populations. The evidences indicating the existence of stem cell population in pulp tissue originate from the observations made on the repair process occurring in dentin following carious lesions. The cells responsible for reparative dentin formation are thought to arise from the precursor cells, residing somewhere within the pulp tissue.[12,13] In this regard, a study by Tsukamoto and colleagues showed that fibroblastic cells from pulp of deciduous and supernumery teeth were able to produce dentin like nodules at culture.[14] Similar results were reported by Couble et al in an explant culture of pulp tissue in a medium containing glycerol phosphate.[15]

Definitive evidences for the existence of stem cells in pulp tissue was provided by Gronthos et al, who culture-expanded fibroblastic cell population from pulp tissue, and examined their differentiating potential both *in vitro* and *in vivo*. According to their reports, the cells tended to differentiate into bone and neural cell lineages *in vitro*, and produced dentin-producing cells *in vivo*.[16] Subsequent investigations indicated the bone differentiating potentials of the dental pulp stem cells as well.[17,18] Zhang et al reported to succeed in differentiating dental pulp-derived stem cells into more cell lineages than those that had been reported before.[19]

According to the Stem Cell Committee of International Society for Cell Therapy, MSCs are cells capable of growing as adherent cells, differentiating among bone, cartilage and adipose cells, and expressing some certain surface markers including CD105, CD73 and CD90, but nothematopoietic-endothelial epitopes such as CD45, CD34, CD11b and CD19.[20] The studies that have so far been carried out regarding pulp stem cells have not made it clear whether or not the fibroblastic cells from pulp tissue, which are capable of differentiating into odontoblast-like cells, meet these criteria.

The objectives of the present study were three folds: 1- to isolate and culture expand the stem cells from dental pulps of human third molars, and to differentiate them among odontoblastic cell lineages in vitro, 2- to find out whether or not the cells capable of producing odontoblastic cells are of MSC population described elsewhere (i.e. the cells with differentiating capacity into bone, cartilage and adipose cell lineages and with characteristic surface antigen profile that was mentioned above), and 3- to investigate some growth kinetics of dental pulp-derived stem cells.

Materials and Methods

Pulp Tissue and Cell Culture

Five human third molars were used to harvest the pulp tissue for experimentation. The teeth were obtained from young adult patients (20-25 years old) at the dental clinic of Shahid Beheshti University of Medical Sciences using the guidelines approved by the Ethic Committee of Royan Institute. The specimens were transferred to Adult Stem Cell Lab of the Royan Institute for stem cell isolation. To reveal the pulp chamber, tooth surfaces was cut around the root-enamel boundary using dental fissure burs. Pulp tissue were then gently collected from the chambers, and subjected to enzymatic digestion using an enzyme solution consisting of 3 mg/ml collagenase type I and 4 mg/ml dipase (both from Sigma, Germany) for 30 minutes at 37°C. Three ml of Dulbecco's Modified Eagle Medium (DMEM, Gibco, Germany) were added to the digest followed by supplementation with 15% Fetal Bovine Serum (FBS, Gibco, Germany) and centrifugation at 1200 rpm for 5 minutes. The pellet was then suspended in fresh proliferation medium consisting of DMEM supplemented with 15% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (both antibiotics from Sigma, Germany) plated in 6-well culture plates at 1000 cells/well. The plates were then incubated in an atmosphere of 5% CO_2 and a temperature of 37°C. Three days after the culture initiation, the medium was removed, the cells were washed with phosphate Buffer Solution (PBS), and fresh medium was added. The culture medium was changed every three days until the cultures confluence was achieved. Afterwards, the cultures were trypsinized, and subcultured at a ratio of 1:3 (passage 1). Two additional subcultures were performed to multiply the cells, which were then used in the following experiments.

Flow Cytometric Analysis of Cell Surface Epitopes

Flow cytometric analysis was used to determine the surface phenotypic profile of the isolated cells. For this purpose, about 10^6 cells

of passaged-3 cultures, 5 µl of either Propidium Iodide (PE) or Fluorescein Isothiocyanate (FITC) -conjugated antibody and 5 µl of blocking buffer were placed in 5 ml tubes and incubated at 4°C for 20-25 min in a dark place. Isotopes of Immunoglobulin G2 (IgG2) and Immunoglobulin G1 (IgG1) were used as control groups. The cells were washed with 1 ml washing buffer (PBS supplemented with 1% FBS) and centrifuged at 1200 rpm. The cell pellet was then suspended in 300-500 µl washing buffer and analyzed by flow cytometry (FACS calibur cytometer equipped with 488 nm argon lasers). WinMDI software was used to analyze the flow cytometric results. The cells were stained suing FITC-conjugated CD31, CD33, CD45, CD90, CD105 and PE-conjugated CD11b, CD34, CD44, CD56, CD73 and CD19 antibodies (all from Becton Dickenson).

Odontobalst Differentiation

Stem cells derived from the pulp of human third molars, at passage 3, were cultivated at 10^5 cells in 6-well culture plates in proliferation medium and allowed to reach confluence. Afterwards, the medium was substituted with differentiating medium including DMEM supplemented with 0.5 µM vitamin D3 (Sigma, USA), 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β glycerol phosphate (Sigma, USA).[21] The cultures were then incubated for the next 3 weeks during which the medium was changed twice weekly. At the end of differentiation period, the cultures were analyzed for odontoblast differentiation by either alizarin red staining for mineralization or by RT-PCR for odontoblast specific gene expression.

Differentiation among Mesenchymal Cell Lineages

Osteogenic culture: The passaged-3 dental pulp cells were plated at 10^5 cells in 6-well culture plates in the proliferation medium and allowed to become confluent. The medium was then replaced by differentiating medium composed of DMEM supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, Germany), 10 nM dexamethasone (Sigma, Germany) and 10 mM β glycerol phosphate (Sigma, Germany).[22] The cultures were kept in differentiating medium for 21 days, and the medium was changed twice a week. At the end of this period, osteogenic differentiation was examined by the alizarin red staining for culture mineralization as well as the RT-PCR analysis for bone specific gene expression.

Adipogenic culture: Dental pulp cells, at passaged-3, were plated at 10^5 cells in 6-well culture plates in the proliferation medium until achieving confluence. The proliferation medium was then replaced with adipogenic differentiating medium consisting of DMEM supplemented with 50 µg/ml ascorbic acid 3-phosphate, 100 nM dexamethasone and 50 µg/ml indomethacine.[22] The cultures were incubated for 21 days during which the medium was changed twice weekly. At the end of the culture period, the differentiation was determined using Oil red staining for lipid droplet as well as RT-PCR analysis for adipose-related gene expression.

Chondrogenesis: For chondrogenic differentiation, 2.5×10^5 passaged-3 cells derived from the pulp of the human third molar teeth were pelleted under 300 g for 5 minutes and provided with differentiating medium which was composed of DMEM supplemented by 10 ng/ml transforming growth factor-ß3 (TGF- ß3, Sigma, Germany), 10 ng/ml bone morphogenetic protein-6 (BMP6, Sigma, Germany), 50mg/ml insulin transferin selenium+ premix (Sigma, Germany), 1.25 mg bovine serum albumin (Sigma, Germany) and 1% FBS (Sigma, Germany).[22] The cultures were incubated at 37°C and 5% CO2 for three weeks with medium changes of twice weekly. To prepare the cultures for the assessment of cartilage differentiation, the pellets were fixed with 10% formalin, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Five-µm-thick sections were then made and stained by toluidine blue. Further examination was done using RT-PCR analysis for cartilage specific gene expression in the culture.

RT-PCR Analysis

Total RNA was isolated from the differentiated cells using the RNX^{TM} (-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). In order to eliminate residual DNA, the samples of the isolated RNAs were treated with 1U/ml of RNase-free DNaseI (EN0521; Fermentas,Opelstrasse 9,Germany) per 1 mg of RNA in the presence of 40 U/ml of ribonuclease inhibitor (E00311; Fermentasm, Germany) and 1×reaction buffer with $MgCl_2$ for 30 min at 37°C. To inactivate the DNaseI, 1 ml of 25mM EDTA was added to the sample which was then incubated at 65°C for 10 min. Standard RT reactions were performed with 2 µg total RNA using oligo (dt) as a primer and a RevertAid^{TM} First Strand cDNA Synthesis Kit (K1622; Fermentas, Germany) according to the manufacturer's instructions. To provide a negative control in the subsequent PCR, for every reaction set, one RNA sample was prepared without RevertAid^{TM}M-MuLV Reverse

Transcriptase (RT- reaction). To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Reaction mixtures for PCR included 2 ml cDNA, 1×PCR buffer (AMS™; CinnaGen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primer (table 1), and 1U Taq DNA polymerase.

Growth Characteristics of the Cells

Colony Forming Unit-Fibroblast (CFU-F) Assay: MSCs are colonogenic cells, and CFU-F assay is routinely used to assess the potential of *in vitro* growth of MSCs. To determine the colonogenic ability of the pulp-derived MSCs, 100 cells of the passaged-3 culture were plated in a 25-cm^2-plastic flask in the DMEM, supplemented with 15% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and were incubated at 37°C and 5% CO2 for a period of 10 days.[22] At the end of the cultivation period the number of the colonies was determined using an inverted light microscope.

Population doubling time (PDT): It is defined as the time by which a given cell population doubles in number. It is indeed an indication of the rate of proliferation of the given cell population in culture. To determine PDT of the isolated cells from dental pulp, passaged-3 cells were plated at 250 cells/well of 12-well culture plate in a DMEM supplemented with 15% FBS and 100 IU/ml penicillin, 100 µg/ml streptomycin and incubated for a period of 10 days at the end of which the cell were lifted and counted with hemocytometer. Population doubling time was determined using the following formula, where N_0 is the initiating cell number (which was 250 cells), N is the harvesting cell number (which was 512800 cells) and C.T is the culture time (which was 10 days).[23]

$$(PDT)=\frac{C.T}{Log\frac{N}{N_0}\times 3.31}$$

Daily examination of cells growth: Cells in culture grow in a specific pattern consisting of lag phase (adaptation phase), log phase (proliferation phase) and plateau. To determine these features for dental pulp MSCs, passaged-3 cells were counted and plated at 250 cells per well in 12-well culture plates and allowed to achieve confluence. During the culture time, the number of cells was determined on a daily basis with a hemocytometer. The growth curve for the culture was plotted using the average values of daily cell numbers.

Results

Cell Culture

Dental pulp cells were observed to grow with colony formation at primary culture. The colonies were mainly composed of fibroblastic cells. A number of small clear cells were also observed on the fibroblastic cells (figure 1A). The cultures tended to achieve confluence in 10 days. Confluent cultures were composed of multiple bundles of fibroblastic cells each running in a particular direction, a feature typical of human marrow-derived MSC culture (figure 1B). Dental pulp-derived fibroblastic cells tended to proliferate at a faster rate than the cells in the primary culture, so that the subcultures reached confluence in about 6 days. They retained fibroblastic morphology at all subcultures.

Flow Cytometry

Flow cytometeric results showed that CD105, CD90, CD44 and CD73 were expressed on the majority (more than 97%) of

Table 1: The list of the primers used in RT-PCR experiments

Gene name	Direction	Sequence	Accession number
DMP1	Forward	GCAGAGTGATGACCCAGAG	NG_008988.1
	Reverse	GCTCGCTTCTGTCATCTTCC	
DSPP	Forward	CCATTCCAGTTCCTCAAAGC	NG_011595.1
	Reverse	CTGCCCACTTAGAGCCATTC	
Aggrecan	Forward	TCAACAACAATGCCCAAGAC	NM_001135.2
	Reverse	AGCGACAAGAAGAGGACACC	
Collagen type II	Forward	GCAGCAAGAGCAAGGAGAAG	NG_008072.1
	Reverse	AACTGTGAGAGGGTGGGATG	
PPAR-Gamma	Forward	CTAAAGAGCCTGCGAAAG	NC_000003.11
	Reverse	TGTCTGTCTCCGTCTTCTTG	
PPAR- Alpha	Forward	TGCTATCATTTGCTGTGGAG	NC_000022.10
	Reverse	ACTCCGTCTTCTTGATGAT	
RunX2	Forward	CAAGTAGCAAGGTTCAACGA	NG_008020.1
	Reverse	CGGTCAGAGAACAAACTAGG	
Osteocalcin	Forward	GGCAGCGAGGTAGTGAAGAG	NC_000001.10
	Reverse	CAGCAGAGCGACACCCTAGAC	
GAPDH	Forward	CTCATTTCCTGGTATGACACC	NC_000012.11
	Reverse	CTTCCTCCTGTGCTCTTGCT	

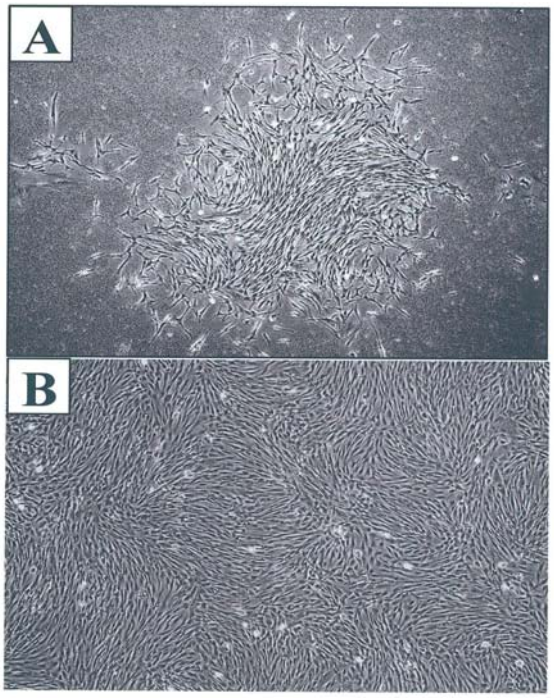

Figure 1: The culture of pulp tissue cells. A) Primary culture before achieving confluence. A large colony consisting of fibroblastic cells can be seen B) The same culture after achieving confluence. This culture is composed of multiple bundles of fibroblastic cells each running in a particular direction.

the isolated dental pulp cells, but CD56, CD11b, CD34, CD31, CD33and CD45 were expressed in a very small percentages (less than 1-2%) of them. CD19 was expressed on about 13% of the studied cells (figure 2).

Odontoblast Differentiation

The dental pulp cells cultivated in odontoblast-inducing conditions were seen to undergo some morphologic reorganization including the formation of cell aggregates at some area of culture plate. This area appeared to be a foci of differentiation in which mineralized matrix deposited by the differentiating cells was positively stained with alizarin red (figure 3A). According to RT-PCR analysis the odontoblast-specific mRNAs including Dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) were largely produced in differentiating cells (Figure 3B). All such evidences were indicative of successful odotoblast differentiation of the dental pulp cells in the culture conditions.

Differentiation among Mesencymal Cell Lineages

In some areas of osteoinductive cultures, nodule-like structures were observed. Following the alizarin red staining, red mineralizing areas were formed (figure 4A).

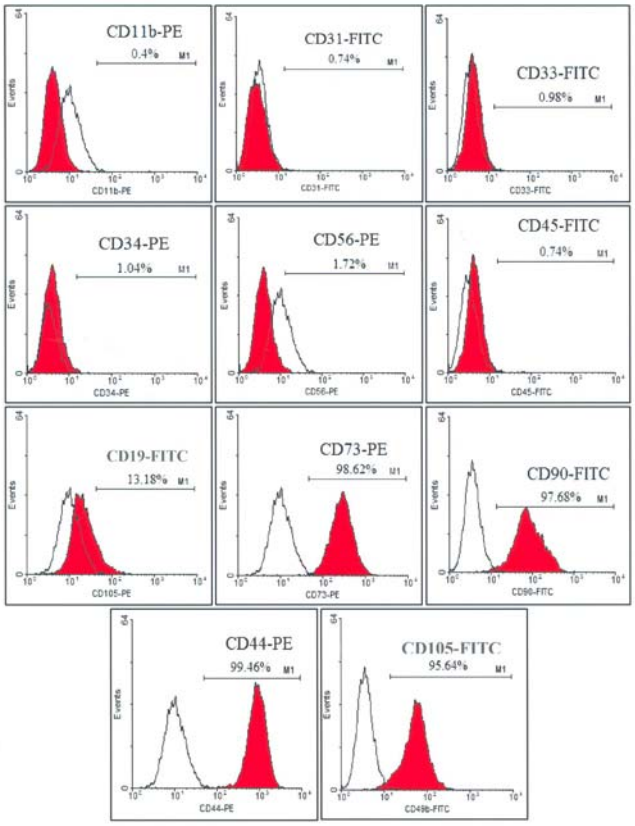

Figure 2: The flow cytometry analysis of the dental pulp passaged-3 cells. The majority of the cells are positive to CD105, CD 90, CD 44, and CD 73. Endothelial-hematopoietic markers including CD 56, CD11b, CD 34, CD 31, and CD 33 are expressed on very small percentages of the cells. CD 19 are expressed on about 13% of the studied cells.

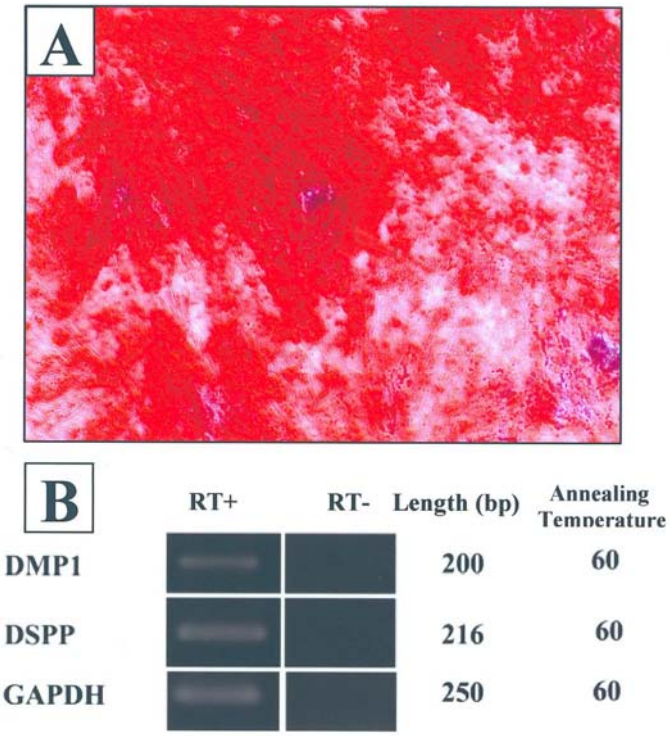

Figure 3: Odontoblast differentiation of the dental pulp stem cells. A) Alizarin red staining indicated the deposition of mineralized matrix among the differentiated cells. B) RT-PCR analysis revealed the expression of odontoblast-specific genes by the pulp cells plated under the differentiation condition.

Differentiation was further demonstrated by RT-PCR detection of the osteocytic markers including osteocalcin and Runx2 mRNA in the cultures (figure 4D).

The potential of adipogenic differentiation in dental pulp MSCs appeared to be relatively weak, since a few cells per microscopic field were observed to be differentiated into adipocyte in differentiation culture. The lipid droplets developed at adipogenic cultures stained red with Oil red staining (figure 4B). According to RT-PCR results, adipose related-genes such as Peroxisome proliferators activatedreceptor-alpha (PPAR-alpha) and Peroxisome proliferators activatedreceptor- gamma (PPAR-gamma) were successfully expressed in the cultures (figure 4D).

In the micro mass culture system for chondrocyte differentiation, metachromatic matrix was detected by toluidine blue staining (figure 4C). Moreover RT-PCR results indicated the expression of cartilage-specific-genes including aggrecan and collagen II by the differentiating cells (figure 4D).

Colonogenic Assay

Dental pulp MSCs were produced an average of 57.4 colonies per each 100 cells per culture flask.

Population Doubling Time

The PDT for dental pulp MSCs was about 21.89 hours.

Growth Curve

The curve plotted for dental pulp MSCs revealed that the cell population had a lag phase of about 3 days, after which they multiplied at a relatively rapid rate and reached plateau on the day 9 of culture period (figure 5).

Discussion

In the present study a population of fibroblastic cells with differentiation potential into odontoblast cells from human third molar was isolated and culture expanded as had been done previously.[12-17] Moreover, the study examined whether or not the isolated cells belonged to mesenchymal stem cells described elsewhere. The findings indicate that the cells were succeeded in passing all the examinations related to MSCs including expressing some mesenchymal lineage surface epitopes, not expressing endothelial and hematopoietic markers, growing as adherent cells, and differentiating into bone, cartilage and adipose cells *in vitro*. All these properties are criteria that indicate the mesenchymal stem cell nature of the fibroblastic cells

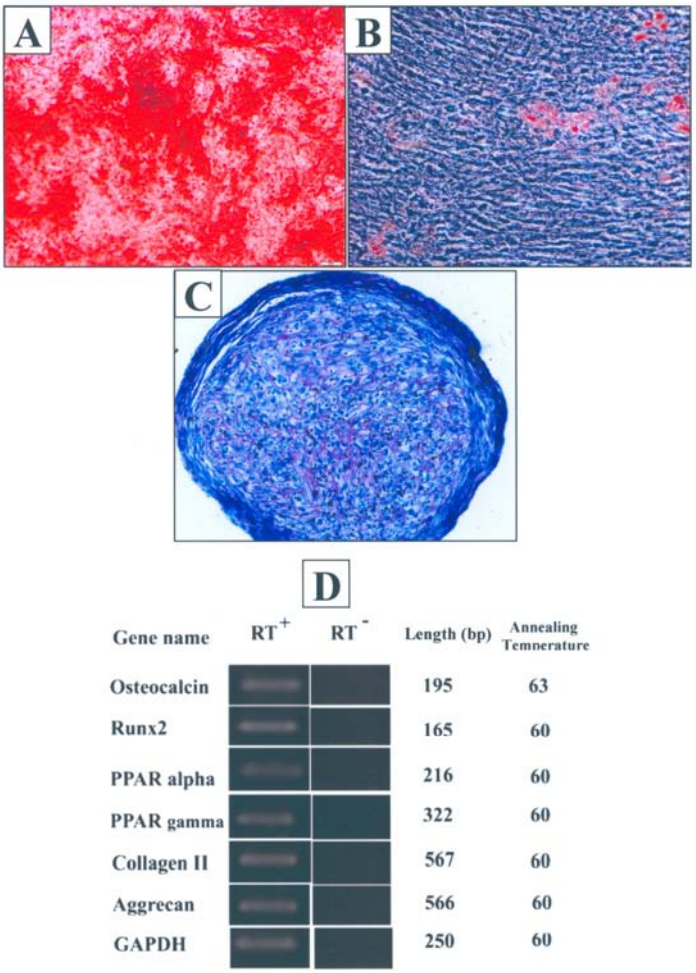

Figure 4: Differentiation of human dental pulp stem cells among skeletal cell lineages. A- Alizarin red staining for osteogenic differentiation indicated the mineralized bone matrix deposition among the cells, B- Lipid droplets within the adipogenic cells stained red by Oil red staining, C- Following toloidine blue staining, methachromatic matrix (a purple color) was appeared in the section prepared from chondrogenic micro mass culture of the pulp stem cells. D- RT-PCR analysis indicated the expression of osteogenic (osteocalcin and Runx2), adipogenic (peroxisome proliferators activated receptor-alpha (PPAR alpha) and PPAR gamma) and chondrogenic (aggrecan and coll II) genes at relevant cultures.

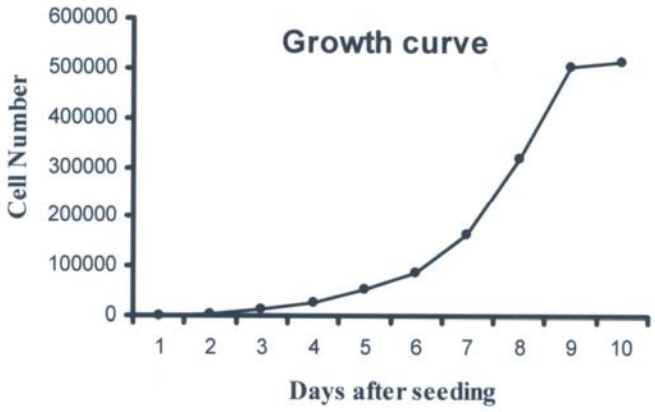

Figure 5: A representative growth curve plotted after daily determination of the dental pulp stem cells in vitro proliferation.

derived from human third molar.[20] In a previous report, Zhang and colleagues demonstrated the tripotent differentiation potential of the human dental pulp stem cells, but they failed to show the surface antigenic profile of the isolated cells.[19] Moreover, Yalvac et al studied the

antigenic profile of isolated pulp stem cells, and demonstrated that the cells could differentiate into bone and adipose tissues, but not into cartilage.[24]

Mesenchymal stem cells have so far been isolated and characterized from a multitude of sources including, cord blood, peripheral blood, amniotic fluid, cartilage, bone and adipose tissue. Each of these sources and the cells isolated from them have some advantages and disadvantages that have been mentioned in the related literature.[5-11] Mesenchymal stem cells from dental pulp have the advantage of being derived from a tissue that is routinely discarded, if not being used. These cells are of utmost importance in regenerative medicine involving dentin. It should be noted that tooth is a complex tissue made from two cell types: odontoblast, which are responsible for dentin production, and ameloblast, which are responsible for enamel deposition. Therefore, creating a whole tooth would be a highly complex process requiring multiple cell types, but partial repair of a tooth would be much more feasible. Dentin-producing MSCs can be used for this purpose in future regenerative medicine.

The present study did suffer from a number of limitations. One limitation was related to the inherent scarcity of pulp tissue in pulp chamber of the human teeth. Due to the small volume of pulp tissue, a number of attempts to establish pulp-derived cell culture were failed. The other limitation was the teeth-induced contamination, which resulted in the contamination of cultures. The contamination could occur either during tooth extraction or transfer to the cell culture lab.

In order to characterize the human third molar pulp stem cells, a number of appropriate growth indices were examined. These include colonogenic ability, PDT and growth curve of the cells. Such indices were helpful in estimating the culture period required by the cells to multiply into adequate number. In MSCs applications, such as the use in tissue engineering or transplant, a huge number of cells are usually required. In such cases, culture expansion of the cells is inevitable, since the cells in the tissue of origin are scarce.

A study by Suchanek and colleagues,[25] reported that PDT for dental pulp stem cells from human third molar was 42.56 hours, while it was 21.89 hours in the present study. Such a discrepancy might have been due to the differences in FBS concentrations in the cell culture medium. Whereas in the current study, the culture medium was supplemented with 15% FBS, the culture medium in the study by Suchanek et al had 10% FBS.[25] In agreement with this speculation, these authors showed that PDT was 55.43 hrs when a concentration of 2% was used.

Previous investigations indicate that the adipose differentiation of the pulp stem cells has been a subject of controversy. While Gronthos and colleagues reported that the fibroblastic cells capable of differentiating into odontoblast like cells *in vivo* and neural cells *in vitro* had no adipogenic potential,[16] some others reported that the dental pulp cells could successfully differentiate into adipose cells.[19,24-26] The findings of the present study indicate that lipid-droplet in stem cells developed at adipogenic conditions. These cells were positively stained by Oil red, and exhibited the expression of adipose specific genes. However, the adipogenic differentiation tended to be far less extensive that a few lipid-containing cells were observed in each microscopic field. Taken together, it seems that the adipogenic differentiation of pulp stem cells was not as strong as their bone or cartilage differentiation. More investigation is required to elucidate the underlying causes and mechanisms of such a disparity.

An important issue in stem cell investigations is the identification and confirmation of the nature of the cells that are isolated and culture-expanded. The best method for such a purpose would be to use specific surface markers of the cell. There is no a specific surface marker for mesenchymal stem cells, however, several non-specific surface antigens have been reported to exist for them.[27-29] To overcome this problem, MSCs Committee of International Society for Cell Therapy has proposed a panel of surface antigens, which their existence must be verified on putative human MSCs before recognizing and presenting them as mesenchymal stem cells. According to the published guidelines of the Society, a panel of endothelial and hematopoietic cell surface markers must not be expressed on MSC surfaces, and a set of cluster of differentiation must be exhibited.[20] The present study did examine such CDs to recognize and confirm the isolated cells as MSCs.

Taken together, the preset study showed that fibroblastic cells isolated from human third molars were able to differentiate among odontoblastic cell lineages. Moreover, it demonstrated that these cells were capable of producing osteocytic, chondrocytic and adipocytic cell lineages in culture. Furthermore, the study showed that the isolated stem cells did exhibit the expression of a number of mesenchymal-related surface antigens, but not the expression of endothelial-hematopoietic-

related markers. Therefore, it might be possible to conclude that the isolated stem cells belong to a family of MSCs that have previously been shown to be isolated from a multitude of tissues. Future quantitative studies such as the comparisons of MSCs from dental pulp with those from other tissues including bone marrow, adipose tissue, umbilical cord blood, amniotic fluid in terms of their differentiation potential into bone, cartilage and adipose cell lineages as well as surface marker profiles and growth kinetics *in vitro* will shed more light on the nature of such stem cells.

Conflict of Interest: None declared

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