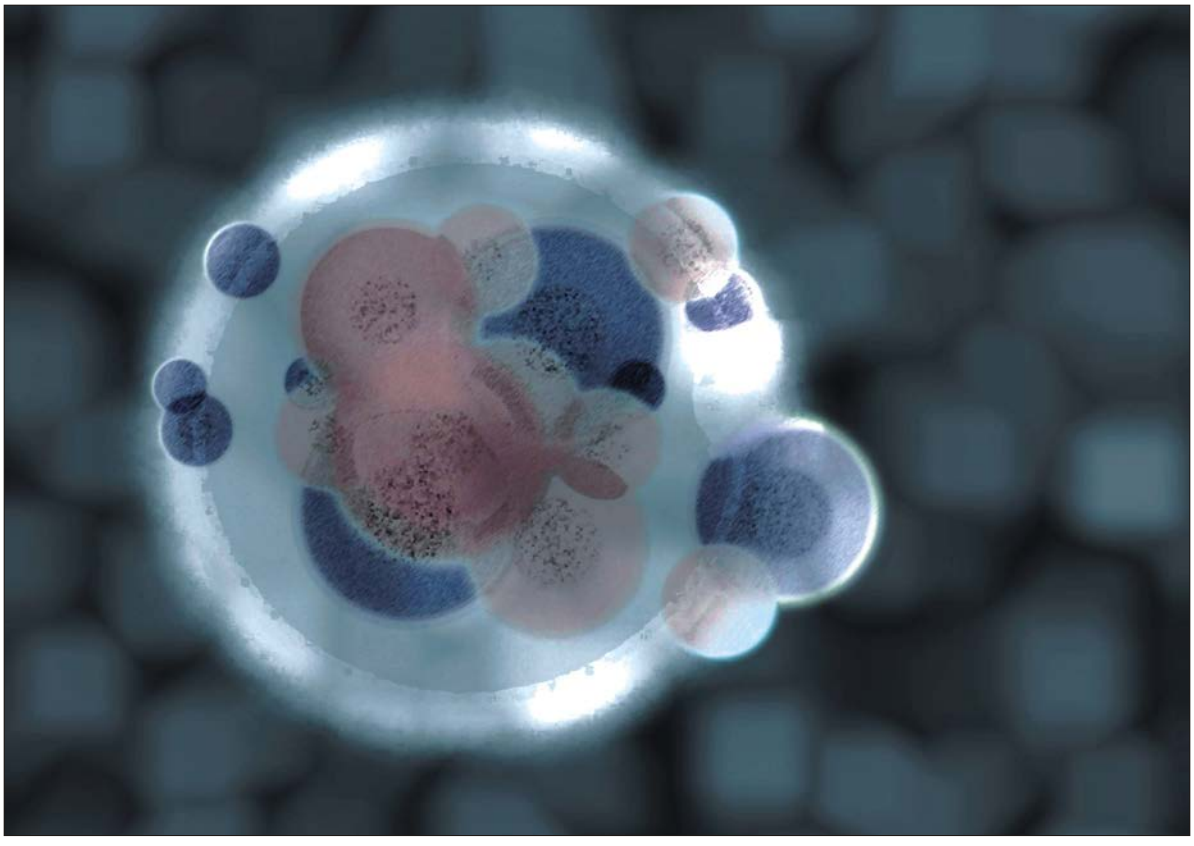

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Isolation and Characterization of Dental Pulp Stem Cells from Murine Incisors

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Abstract: Studies have proven that dental pulp contains cells with stem cell activity. Although mice are widely used as a model organism, murine dental pulp stem cells (mDPSCs) are still poorly characterized. In this study, we aim to provide a modified and cost-effective method for isolating mDPSCs and to characterize the isolated cells using molecular markers. Mice incisors' dental pulps were digested in collagenase 1A and the cell suspensions cultured in α-MEM supplemented with 20% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin. The morphology of the cells was observed and passage 4 cells were subjected to molecular characterization using RT-PCR. We found that the freshly isolated dental pulp cells through enzymatic dissociation method showed a heterogeneous cell morphology which became more homogeneous following cell passage. Semi-quantitative RT-PCR showed analysis that the murine incisors' DPSCs are $Cd105^{+}$, $Cd13^{+}$, $Cd29^{+}$, $Cd146^{+}$, $Cd166^{+}$, $Cd90^{low}$, $Cd73^{low}$, $Cd105^{-}$ and $Cd34^{-}$. Based on these findings, we concluded that dental pulp stem cells from murine incisor could be easily isolated through the method described and the incisor's dental pulp could be a source of mesenchymal stem cells. Further studies are required to explore the differentiation potential of the isolated mDPSC *in vitro*.

Key words: Murine incisor, dental pulp, stem cell, semi-quantitative RT-PCR

INTRODUCTION

The subject of stem cells has caught the interest of many researchers due to its promising future in regenerative medicine application. Since embryonic stem cells face huge bouts of ethical issues, attempts are made to look for better alternatives of stem cells. Stem cells of dental sources have been isolated from different part of the tooth such as dental papilla, dental follicle, periodontal ligament and apical papilla (Bakopoulou *et al*., 2011; Tziafas and Kodonas, 2010). Teeth are developed from the same lineage of cranial neural crest ectomesenchyme, yet the gene expression profiles and differentiation potency of stem cells of different tooth sources are not the same (Bakopoulou *et al.,* 2011; Morsczeck *et al*., 2010; Tziafas and Kodonas, 2010; Volponi *et al*., 2010; Lee and Lee, 2010). Thus, the dental stem cells from different sources need to be characterized in an effort to search for cells with good proliferative ability and wide differentiation potential.

Murine incisors and rabbit molars are well known for its ability for continuous eruption throughout life. In such cases, stem cells inside the heterogeneous population of dental cells are thought to be involved in the process of continuous growth by differentiating into odontoblasts that produce dentin (Mao and Prockop, 2012). Other than that, Tecles *et al.* (2005) has shown that the stem cells are also involved in reparative mechanism in which the cells would be activated in response to pulpal injury.

Human DPSC are actively being characterized as scientists focused on the application of DPSC in cell therapy. A lot of research involving dental pulp also targets big-sized organisms (Snyder *et al*., 2011; Kaneko *et al*., 2013; Iohara *et al*., 2006). Thus, although mice is being used as a model organism for a lot of studies primarily due to its small size, low cost of maintenance and easy handling and short generation time-which is only about 10 weeks after being born, there are still lack of data on mice dental pulp stem cells. This might be primarily due to the limited amount of dental pulp tissues in small organisms like mice. Therefore, in this study, we aim to

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isolate and characterize the stem cells isolated from mice dental pulp. A more cost-effective method of characterizing the stem cells was also used by combining touchdown PCR and semi-quantitative RT-PCR.

MATERIALS AND METHODS

Subjects, isolation and culture of dental pulp stem cells: The mandibles were dissected from of 6-8 weeks ICR strain mice and the incisor was separated from the surrounding alveolar bone and tissues with the help of stereomicroscope (Leica EZ4, Germany). The dental pulp was gently removed from interior of the incisor and subjected to digestion with 0.8 mg mL^{-1} (928 CDU) collagenase 1A (Sigma, USA) in Phosphate Buffered Saline (PBS) (Sigma, USA) at 37°C for 1 h with shaking. The digested tissues were then homogenized by repetitive pipetting to release the cells from the tissue clumps. The cells were washed with 1X PBS and cultured in complete media (α-Minimum Essential Medium (α-MEM) (Invitrogen, USA) supplemented with 20% (v/v) Fetal Bovine Serum (FBS) (Biowest, South America) and 1% (v/v) penicillin-streptomycin (Biowest, South America). The first medium change was done 24 h after culture, followed by every three days. The cells were passaged when it reaches ~80% confluence. The cells were detached by incubation with 0.25% (v/v) trypsin-EDTA (Biowest, South America) for five minutes and sub-cultivated at a ratio of 1:2.

Semi-quantitative reverse transcriptase PCR: Total RNA was isolated from passage 4 dental pulp stem cells using Trizol (Invitrogen, USA). Two-step reverse transcriptase PCR (RT-PCR) was executed in which 1 μg of total RNA was used to generate the first strand cDNAs using RevertAid First Strand cDNA Synthesis Kit (ThermoScientific, USA). Second strand synthesis was conducted by means of touchdown PCR (Korbie and Mattick, 2008) using 0.25 μL of oligo-dT primed cDNAs, 0.125 units GoTaq, 1X GoTaq reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTP and 1 μM of sense and antisense primers each. Amplification was performed in a total of 40 cycles in Mastercycler Gradient PCR machine (Eppendorf, USA). The first phase of the touchdown PCR was carried out in 17 cycles (denaturation at 95°C for 1 min, annealing at the highest T_m of the primer pairs+8°C with decrement of 0.5°C per cycle and elongation at 72°C for 40 sec). This was followed by 23 cycles of amplification at the T_m. The T_m was calculated according to nearest neighbour formula using OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). The touchdown PCR program was started with initial denaturation step at 95°C for 2 min and terminated with 5 min final elongation step at 72 °C. The primers used for the characterization of dental pulp stem cells were as shown in Table 1. PCR products were separated by electrophoresis on a 1.7% (w/v) agarose in 1XTAE buffer. The gel was stained using EtBr and the image visualized under UV transillumination (AlphaInnotech, USA). Band intensities were quantified using ImageJ 1.46 (http://rsb.info.nih.gov/ij).

Table 1: Gene-specific primer sequences used in touchdown PCR

Gene	Primer	Sequence (5'-3')
Gapdh	Sense	CAACGGCACAGTCAAGG
	Antisense	AAGGTGGAAGAGTGGGAGT
Cd13	Sense	GCAAGTTACGCCCCTCTTCT
	Antisense	GTTTGTCCGCTTCGTTCACC
Cd29	Sense	GCGTGGTTGCTGGAATTGTT
	Antisense	AAAGGCGTCGGAGTTACAGG
Cd73	Sense	AGGGAGTGGGTAAGG
	Antisense	GGAGTCGCACAGGAG
Cd105	Sense	AGGCTGAAGACACTGACGACCAT
	Antisense	CTTGGCTGTTCGGCTCTGGATG
Cd146	Sense	GGACCTTGAGTTTGAGTGG
	Antisense	CAGTGGTTTGGCTGGAGT
Cd166	Sense	AACATGGCGGCTTCAACG
	Antisense	GACGACACCAGCAACGAG
Cd90	Sense	ACCAAGGATGAGGGCGACTA
	Antisense	ACAGGCACAGTCCAACTTCC

RESULTS AND DISCUSSION

Isolation of dental pulp stem cells: Mice's maxilla and mandible both consist of three pair of molars and one pair of incisor. The incisor of mice is deeply embedded inside the alveolar bone and the dental pulp is almost the same length as the incisor (Fig. 1a). Figure 1b shows an intact, freshly isolated dental pulp with large blood vessels in both the coronal and radicular pulp. Apical part of the pulp was removed to minimize the contamination with epithelial stem cells which are known to give rise to transient amplifying cells that propagate, migrate anteriorly and differentiate into ameloblasts that produce enamel matrix in mice incisors (Mao and Prockop, 2012).

Two methods usually used in Dental Pulp Stem Cell (DPSC) isolation were the outgrowth method and enzyme dissociation method (Yildirim, 2013). The outgrowth method involved submerging the explant inside complete growth medium and allowing the cells to migrate out of the tissues. Although this method might be able to prevent cell damage resulting from the action of enzyme, the time taken to obtain the cell are however, significantly longer (Huang *et al*., 2006). It had also been shown that the time taken for the cells to become confluence is shorter for the enzyme dissociation method which is one to two weeks as compared to three weeks for the outgrowth method (Huang *et al*., 2006). In this study, the isolated dental pulp was digested with only collagenase

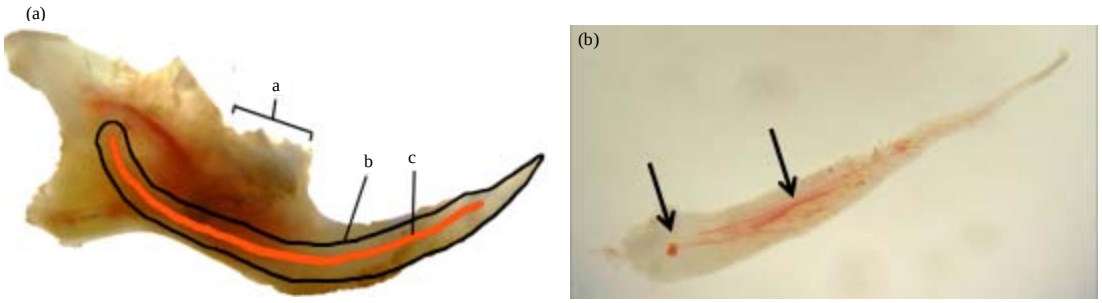

Fig. 1(a-b): Dental pulp (a) Dissected murine's mandible. a: Molars, b: Incisor, c: Location of dental pulp, (b) Extracted incisor's dental pulp showing the highly vascularized structure

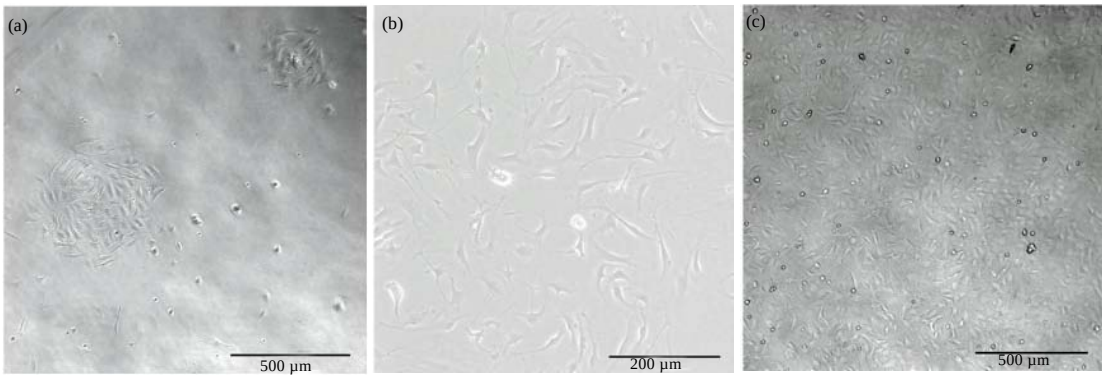

Fig. 2(a-c): Dental pulp cells (DPCs) (a) DPCs colony formation in 1 week culture (passage 0) (scale bar: 500 µm), (b) Subconfluent DPCs (passage 0) (scale bar: 200 µm) and (c) Confluent DPCs (passage 4) cell (scale bar: 500 µm)

as opposed to a combination of collagenase/dispase (Waddington *et al*., 2008) or collagenase/trypsin (Balic *et al*., 2010). Collagenase type I was chosen as the sole enzyme to be used since collagen type I is present predominantly in dental pulp and it is the major organic component which make out the pulp (Yildirim, 2013). Collagenase functions by hydrolysing the X-Gly bond in the sequence R-Pro-X-Gly-Pro which is present almost exclusively in collagen (Yahyouche *et al*., 2011). This makes collagenase an enzyme with a high specificity on collagen.

Cell clusters known as the colony-forming units could be observed following the Dental Pulp Cells (DPCs) isolated through enzyme dissociation method as previously described (Fig. 2a) (Souza *et al*., 2010). A heterogeneous cell shapes such as spindle-like, endothelial-like or epithelial-like cells could be seen in freshly isolated cultures as the enzymatic dissociation technique would digest the tissues and release all sort of cells (Fig. 2b). This cell shapes however, would become more homogenous following subculture as the cells were grown in mesenchymal cell selection media, showing only fibroblastic-like cells (Fig. 2c) (Huang *et al*., 2006).

Molecular characterization of dental pulp stem cells: Semi-quantitative RT-PCR was used to demonstrate the level of expression of mesenchymal stem cells surface markers in the isolated DPSCs at passage 4. The gene expression levels of each marker were represented by the intensity of the amplicons (Fig. 2a-c). In order to ensure accurate quantification of the expressed markers, the intensity density was normalized to an endogenously expressed reference gene *Gapdh*. The DPSCs were shown to express high levels of mesenchymal stem cell markers *Cd29*, *Cd105*, *Cd146*, *Cd166* and significantly low levels of *Cd73* and *Cd90* ($p<0.01$) in relative to housekeeping gene *Gapdh*. The cells are also negative for the expression of *Cd34* and *Cd150*, ruling out contamination with cells of hematopoietic origin (Fig. 3a, b). These findings were in concordance with the

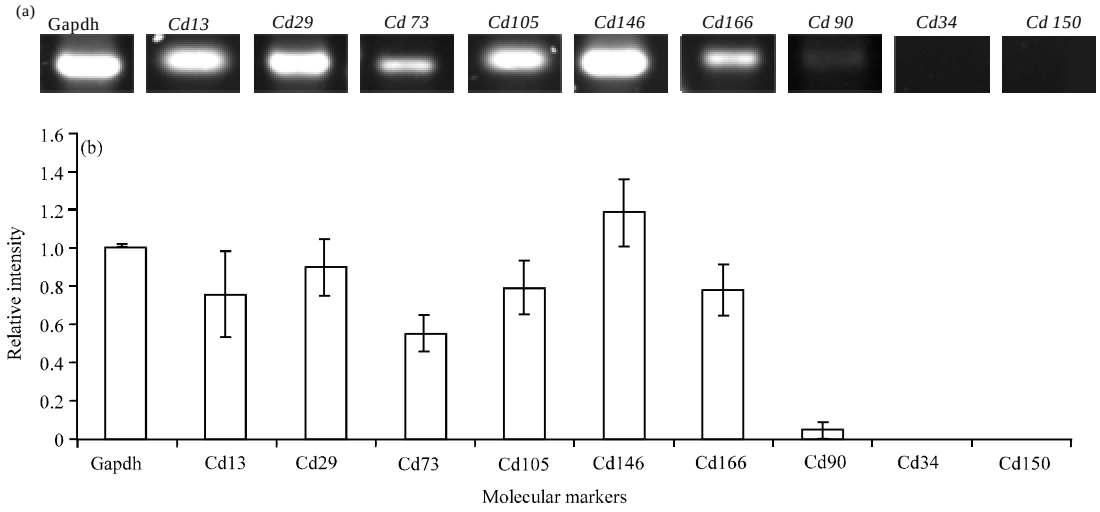

Fig. 3(a-b): Gene expression profiles for dental pulp stem cell from murine incisor using semi-quantitative RT-PCR. (a) Agarose gel electrophoresis of PCR product for the different primers used. The intensity of the PCR product confers directly with the expression of the genes. The amplified genes are *Gapdh* (717 bp), *Cd13* (311 bp), *Cd29* (821 bp), *Cd73* (765 bp), *Cd105* (356 bp), *Cd146* (479 bp), *Cd166* (630 bp), *Cd90* (620 bp), *Cd34* (769 bp), *Cd150* (658 bp), (b) Semi-quantitative RT-PCR showing different levels of mRNA transcribed for mesenchymal stem cell markers in relative to housekeeping genes *Gapdh* as analysed by ImageJ. Values were expressed as Mean±SD (n = 3) and were normalized using *Gapdh* (* : significant ($p<0.01$) in relative to *Gapdh*)

DPSCs isolated from rat (Kaneko *et al*., 2013), swine (Sonoyama *et al*., 2006), human (Atari *et al*., 2011) and primate (Snyder *et al*., 2011).

CONCLUSION

Murine incisor dental pulp stem cells with fibroblastic-like characteristics have been successfully isolated from murine incisors through digestion with a single enzyme (Collagenase 1A). These cells are determined to be $Cd13^{+}$, $Cd29^{+}$, $Cd105^{+}$, $Cd146^{+}$, $Cd166^{+}$, $Cd73^{low}$, $Cd90^{low}$, $Cd34^{-}$ and $Cd150^{-}$ through semi-quantitative RT-PCR. Further studies are required in order to explore the differentiation potential of the isolated dental pulp stem cells *in vitro*.

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