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In vitro comparative analysis of human dental stem cells from a single donor and its neuronal differentiation potential evaluated by electrophysiology

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ABSTRACT

Aims: The aim of this study was to find out a mesenchymal stem cells (MSCs) source from human dental tissues of the same donor (follicle, papilla and pulp), which exhibits higher neurogenic differentiation potential in vitro.
Main methods: MSCs were isolated from dental tissues (follicle, papilla and pulp) by digestion method. All MSCs were analyzed for pluripotent makers by western blot, cell surface markers by flow cytometry, adipo- and osteocytes markers by RT-qPCR. The neuronal differentiated MSCs were characterized for neuronal specific markers by RT-qPCR and immunofluorescence. Functional neuronal properties were analyzed by electrophysiology and synaptic markers expression.
Key findings: All MSCs expressed pluripotent markers (Oct4, Sox2 and Nanog) and were found positive for mesenymal markers (CD44, CD90, CD105) while negative for hematopoietic markers (CD34 and CD45). Furthermore, MSCs were successfully differentiated into adipocytes, osteocytes and trans-differentiated into neuronal cells. Among them, dental pulp derived MSCs exhibits higher neurogenic differentiation potential, in term of expression of neuronal specific markers at both gene and protein level, and having higher Na^+ and K^+ current with the expression of synaptic markers.
Significance: The three types of dental MSCs from a single donor broadly possessed similar cellular properties and can differentiate into neuronal cells; however, pulp derived MSCs showed higher neurogenic potential than the follicle and papilla, suggesting their use in future stem cells therapy for the treatment of neurodegenerative disorders.

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1. Introduction

From last decade, cell based therapies come up as a novel therapeutic option for the treatment of wide variety of neurological disorders i.e. Parkinson's disease, Alzheimer's disease, spinal cord injury [1,2]. Neural stem cells (NSCs) are considered as the most ideal source to be applied in stem cell based therapies; however, NSCs are difficult to harvest from adult origins, so therefore, other stem cells sources are needed to explore [3].

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent stem cells having multilineage differentiation potential and self-renewal capabilities [4]. MSCs had been derived from various human and animal sources. MSCs derived from human dental tissues keep a promising role in the future regenerative medicine because of their ease of collection, and their capacity to undergo self-renewal and multilineage differentiation [1,5]-[7]. As the dental MSCs are isolated from extracted tooth, a medical waste in the routine dental procedures, which increase its importance in cell-based therapies without any ethical issues. To date, five different types of stem cells have been isolated from human dental tissues [8]. Postnatal dental pulp stem cells (DPSCs) were the first human dental stem cells isolated by Gronthos et al. in 2000 [5]. After this first isolation of human dental stem cells, stem cells from human exfoliated deciduous teeth (SHED) [6], human periodontal ligament stem cells (PDLSCs) [9], dental follicle precursor cells (DFPCs) [10] and from the apical papilla (SCAP) [11] were successfully isolated and characterized. Stem cells

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isolated from all dental sources exhibits fibroblast like morphology, express cells surface markers and differentiate into mesodermal lineages i.e. osteocytes [12–14] adipocytes (Miura et al., 2003; Jeon et al., 2011) and chondrocytes (Park et al., 2012) as well as neural cells [6,15], muscle cells [6] and hepatocytes [7,16,17]. The properties of MSCs derived from dental tissues were found similar to those of MSCs derived from bone marrow (BM-MSCs) and skin [14,18]. However, as the dental stem cells have a neural crest origin, they have higher neurogenic capacities than other MSCs [19]. It is considered that stem cells derived from dental tissues have analogous properties to that of neural crest [5]. Previously, human dental stem cells were successfully differentiated into neuron like cells, both in vitro and in vivo [1,5,6,15,20]; however, till now the neuronal differentiation abilities of different dental stem cells are not completely deciphered.

In this study we isolated and characterized human dental MSCs derived from follicle, papilla and pulp tissues of the same donor tooth after impacted third molar extraction. Furthermore, we evaluated their in vitro neurogenic capacity by providing suitable induction conditions and assessed their behavior to functionally active neuron like cells with electrophysiological studies.

2. Materials & methods

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and media from Gibco (Life Technologies, USA), unless otherwise specified.

2.1. Isolation and initial culture of MSCs from human dental follicle, papilla and pulp

MSCs derived from human dental follicle, papilla and pulp were isolated and cultured from a single tooth sample as described previously [14,17,44]. Briefly, human third molar were collected from adult male ($n = 7$, aged 14–18 years) at Dental hospital of Gyeonsang National University under approved medical guidelines (GNUH IRB-2012-09-004), after obtaining the informed consents from patients. Samples were rinsed with Dulbecco's phosphate buffer saline (DPBS) containing 1% penicillin-streptomycin (10,000 IU and 10,000 μg/ml, respectively; Pen-Strep). Dental follicle was separated from the tooth surface and papilla was plucked from the apical part of the tooth by using sterile scalpel. Dental pulp tissue was separated from the pulp chamber of dental crown following fracture with bone forceps. The tissues were chopped into pieces and then digested in DPBS supplemented with 1 mg/ml collagenase type I in a 37 °C incubator with frequent gentle agitation for 40 min. After digestion, the cell suspensions were filtered through a 100 and 40 μm nylon cell strainer (BD Falcon, Bedford, MA, USA) in order to harvest single cell suspension, and further digestion was prevented by adding Advanced Dulbecco's modified eagle's media (ADMEM) supplemented with 10% fetal bovine serum (FBS). The suspensions were centrifuged at 500 ×*g* for 5 min. Supernatants were discarded and the pellets were reconstituted in ADMEM supplemented with 10% FBS (10% ADMEM). Total of 1×10^5 cells were initially seeded into 10 cm culture dishes containing 10% ADMEM. Culture dishes were kept at 37 °C in a humidified incubator containing 5% Co_2 in air. Upon confluence, cells were dissociated with 0.25% (*w*/*v*) trypsin EDTA solution and sub-cultured till passage 3. Cells from passaged 3 were used for further characterization and analysis.

2.2. Analysis of pluripotent markers by western blotting

For western blot analysis of pluripotent markers (Oct4, Sox2 and Nanog), protein lysate was prepared from third passage of all cells (Follicle, Papilla and Pulp) using RIPA buffer (PIERCE, Rockford, IL, USA) containing protease inhibitor and further quantified using BCA protein assay kit (PIERCE). Each protein sample (20 μg) were separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for 3 h at 100 V and transferred to polyvinylidene difluoride membrane (PVDF, Biorad, Hercules, CA, USA) for 2 h at 200 V. Furthermore, membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (1×-TBS) for 1 h at room temperature, followed by washing in 0.1% TBST. The membranes were incubated with primary antibodies of goat anti-Oct4 (1:200, 43–50 kDa, Santa Cruiz, State, USA), mouse anti-Nanog (1:200, 35 kDa, Santa Cruiz), rabbit anti-Sox2 (1:200, 34 kDa, Santa cruiz) and rabbit anti-β-actin (1:1000, Cell signaling, MA, USA) for overnight at 4 °C. After three times washing with 0.1% TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:1000, Santa Cruiz), rabbit anti-goat (1:1000, Santa Cruiz) and goat anti-rabbit (1:1000, Santa Cruiz) secondary antibodies for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Supersignal® West Pico chemilluminescent substrate, PEIRCE, Rockford, IL, USA). The membranes were then exposed to X-ray film in dark and bands were analyzed.

2.3. Cell surface antigen profiling by flow cytometry

Before performing other experiments, cells derived from human dental follicle, papilla and pulp were characterized for the presence of mesenchyme markers (CD13, CD29, CD 44, CD73, CD 90, CD105) and absence of hematopoietic marker CD 34 and CD 45 using flow cytometry (BD FACSClibur, Becton Dickinson, San Jose, CA, USA) as previously described by [17]. Briefly, cells at 80% confluence were fixed with 3.7% formaldehyde for 30 min, followed by incubation with fluorescence isothiocyanate (FITC) conjugated mouse Anti-CD34, CD44, CD45 and CD90. For analysis of CD 105 (mouse monoclonal, Santa Cruiz Biotechnology Inc., CA, USA), CD13 (goat polyclonal, Santa Cruiz), CD29 (mouse monoclonal, BD Bioscience 561795) and CD73 (mouse monoclonal, BD Bioscience 550256) expression, cells were treated with primary antibody for 1 h at 4 °C. The cells were then washed with DPBS and treated with secondary antibody FITC conjugated goat anti mouse IgG (BD, Pharmingen™), donkey anti-goat (Santa Cruiz) for 1 h at 4 °C in dark. Total of 10,000 FITC labeled cells were measured by flow cytometer using CellQuest software (BD, Beckson Dickinson). The standard was established by isotype match control.

2.4. In vitro lineage differentiation

Adipogenic and osteogenic differentiation of three types of dental MSCs were performed by using previously described protocols [14,17]. Briefly, MSCs were cultured in 10% ADMEM supplemented with lineage specific factors for 21 days and medium was changed twice a week. Adipogenic medium consisted of 1 μM dexamethasone, 10 μM insulin, 100 μM indomethacin and 500 μM Isobutylmethyl Xanthine (IBMX). For the detection of lipid droplets, differentiated cells were stained by Oil red O solution for 30 min. Osteogenic medium consisted of 50 μM ascorbate-2-phosphate, 10 mM glycerol-2-phosphate and 0.1 μM dexamethasone. Cells were stained with Alizarin red and von Kossa for the detection mineralization and calcium deposition, respectively. The cells cultured in 10% ADMEM were used as negative control.

2.5. Neurogenic differentiation of MSCs from dental follicle, papilla and pulp

Cells culture flasks or wells were coated with Geltrex LDEV (Gibco) for 2 h at 37 °C, and washed with DPBS before cells were added. Neurogenic differentiation potential of MSCs was conducted as described previously by Arthur et al. (2008) [28], with minor modifications. MSCs are taken from passage 3 and were grown in 10% ADMEM in 6 wells and 12 wells coated chamber slides. When cells reached at 70–80%, the ADMEM media was changed with neural basal A media (Invitrogen; Carlsbad, CA, USA) supplemented with 1% Pen-Strep, 20 ng/ml epidermal growth factor (EGF), 30 ng/ml basic fibroblast growth factor (bFGF) and 1× B27 supplement for 3 weeks. Media

was changed twice a week. After 21 days, cells were either fixed with 4% paraformaldehyde (PFA) for immunostaining or prepared for RNA isolation, real time polymerase chain reaction (RT-PCR) and electrophysiological analysis.

2.6. Immunofluorescence staining

After neuronal inductions of MSCs, immunostaining were performed for neuronal specific markers using previously described protocols [14,45]. Briefly, cells were fixed with 3.7% formaldehyde for 40 min and permeabilized with 0.2% Triton X-100 supplemented with 1% BSA. After premeabilization, cells were blocked with 1% BSA (in DPBS) for 1 h and then incubated with primary antibodies (1:100), MAP2 goat polyclonal, β-tubulin III-goat polyclonol, neurofilament M (NF-M)-goat polyclonol and nerve growth factor (NGF)-rabbit polyclonol (Santa Cruiz Biotechnology, Inc.), tau rabbit polyclonal (ab64193, 1:20), synapsin rabbit polyclonal (ab8, 1:500) and synaptophysin rabbit monoclonal (D35E4, 1:100, Cell Signaling) at 4 °C overnight. After primary antibody treatment, cells were washed with DPBS and incubated with FITC or Alexa flour conjugated secondary antibodies (Santa Cruiz Biotechnology, Inc) for 1 h. For nuclear staining, cells were treated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature (rt). Finally cells were observed under fluorescence microscope (Leica, Wetzlar, Germany).

2.7. Real time quantitative PCR (RT-qPCR) analysis

Total RNA was isolated from control and induced (Adipogenic, Osteogenic, Neurogenic) MSCs using RNeasy mini kit (Qiagen, CA, USA) and quantified with spectrophotometer (NanoDrop 1000, Thermo Scientific). Complementary DNA (cDNA) was synthesized from total purified RNA (2 μg) using Omniscript reverse transcription kit (Qiagen) with 10 μM OligodT primer at 37 °C for 1 h. cDNA samples were diluted to a uniform concentration of 50 ng/μl.

RT-qPCR reaction was performed using Rotor Gene Q (Qiagen) with 50 ng of cDNA quantified with Rotor-Gene™ 2× SYBR® Green mix (Qiagen) supplemented with 10 μM of specific primers set (Table 1). RT-qPCR reaction was performed with initial denaturation at 95 °C for 10 min; followed by 40 cycles of PCR at 95 °C for 10 s, 60 °C for 6 s and 72 °C for 6 s. The melting curves, amplification curves and cycle threshold values (C_t) were determined by using Rotor-Gene Q series software (Qiagen). All samples were run in triplicates and YHWAZ was used as an internal control.

2.8. Electrophysiology

After 3 weeks of neuronal induction, cells were trypsinized and replated onto glass coverslips at concentration of 2×10^4 cells/ml in neuronal differentiation media and incubated for 48 h. Electrophysiology of differentiated and non-differentiated MSCs were conducted using previously described protocol [34], with minor modifications. To record K^+ and Na^+ currents, whole cell patch clamping was performed at rt using a patch clamp amplifier (Axopatch 200, Axon instruments, Union City, CA, USA). The bath solution is consisted of 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, finally pH is adjusted to 7.4. For measuring K^+ current, the pipette resistance ranges between 2 and 4 MΩ when filled with pipette solution comprised of (in mM): KCl, 150; $MgCl_2$, 1; EGTA, 5 and HEPES, 10 while for Na^+ currents the pipette solution consists of (in mM): CsCl, 140; KATP, 2; $MgCl_2$, 4; EGTA, 10; HEPES, 10 and tetraethylammonium (TEA), 1 (pH 7.3). K^+ and Na^+ currents were arouse in both control and differentiated cells by voltage steps (between −120 and +60 mV for K^+ currents, −40 and +50 mV for Na^+ currents) from a holding potential of −80 mV. K^+ channel was blocked by adding TEA to the bath solution while recording Na^+ currents and Na^+ channel was blocked by adding lidocaine (100 μM) and bupivacaine (100 μM) in the bath solution while recording K^+ currents. The recorded currents were filtered at 2 kHz and analyze using the Digidata 1322 interface (Axon Instruments). Whole cell current were analyzed with the pCLAMP (version 10.4, Axon Instruments) and Origin® (Microcal Software, Inc., Northampton, MA, USA) programs.

2.9. Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's test using SPSS 21.0 (SPSS Inc., Chicago, Il, USA). Data was expressed as means ± SE. Differences were considered significant at $p < 0.05$.

Table 1
List of primers used in RT-qPCR.

Gene	Primer sequence (5′-3′)	Product size (bp)	Annealing Tm (°C)	Accession no/reference
FABP4	F: TGAGATTTCCTTCATACTGG R: TGGTTGATTTTCCATCCCAT	128	60	NM_001442.2
LPL	F: AGACACAGCTGAGGACACTT R: GCACCCAACTCTCATACATT	137	60	NM_000237.2
PPAR	F: TTGCTGTCATTATTCTCAGT R: GAGGACTCAGGGTGGTTCAG	124	60	AB565476.1
BMP2	F: TAGACCTGTATCGCAGGCAC R: GGTTGTTTTCCCACTCGTTT	149	60	NM_001200.2
OSTEONECTIN	F: GTGCAGAGGAAACCGAAGAG R: AAGTGGCAGGAAGAGTCGAA	202	60	J03040.1
RUNX2	F: CCTTGGGAAAAATTCAAGCA R: AACACATGACCCAGTGCAAA	181	56	NM_001015051
β3-tubulin	F: AGTGTGAAAACTGCGACTGC R: ATACTCCTCACGCACCTTGC	110	60	U47634.1
NF-H	F: CAGCCAAGGTGAACACAGAC R: GCTGCTGAATGGCTTCCT	189	60	NM_021076.3
MAP2	F: TGGCATTGACCTCCCTAAAGAG R: TTGCTTCCGTTGGCATTTCG	80	60	NM_002374
NF-M	F: GACGGCGCTGAAGGAAATC R: CTTGGCGGAGCGGATGGCCT	142	60	NM_005382.2
YWHAZ	F:ACGAAGCTGAAGCAGGAGAAG R:TTTGTGGGACAGCATGGATG	111	60	BC108281.1

3. Results

3.1. Characterization of MSCs from dental follicle, papilla and pulp

3.1.1. Morphological evaluation, expression of cell surface and pluripotent markers

Three donor samples, from which we successfully isolated MSCs (follicle, papilla and pulp) were considered for further studies. After 6 days of initial seeding, adherent fibroblast like spindle colonies were observed from all the tissues which become homogeneous at passage 3 upon sub-culturing (Fig. 1A). The expression of pluripotent transcription factors (Oct4, Sox2 and Nanog) were found positive in all types of MSCs by western blotting (Fig. 1B, C). The selected cell surface markers were analyzed by flow cytometry. All MSCs were positive for mesenchymal markers (CD44, CD90, CD105) as well as dental stem cells specific markers (CD13, CD29, CD73) and were found negative for hematopoietic stem cell markers (CD34, CD45) (Fig. 1D, E).

3.1.2. Lineage differentiation capacity

To confirm in vitro differentiation capacity, MSCs from follicle, papilla and pulp were subjected into adipo- and osteogenesis. All MSCs were successfully differentiated into adipocytes and osteocytes and were stained for cytochemical changes. The formation of intracellular lipid droplet in adipocytes was confirmed by Oil red O staining (Fig. 2A) and the expression of adipocyte related genes i.e. fatty acid binding protein (*FABP*), lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor (*PPARγ*) were found significantly ($p < 0.05$) higher in differentiated cells as compared to the control by RT-qPCR (Fig. 2B). Osteogenesis was evidenced by the formation of mineralized nodules and calcium deposits, and confirmed by staining with Alizarin Red and von Kossa (Fig. 2A). Osteogenic specific markers i.e. osteonectin (*ON*), runt-related transcription factor 2 (*RUNX2*) and bone morphogenic protein 2 (*BMP2*) were analyzed by RT-qPCR and showed significantly higher ($p < 0.05$) expression as compared to the undifferentiated cells (Fig. 2B).

3.1.3. Neurogenic differentiation of MSCs from dental follicle, papilla and pulp

To evaluate the neurogenic potential of follicle, papilla and pulp derived MSCs, cells from passage 3 were induced in neurogenic media. In all MSCs, after 21 days of neuronal treatment, changes in cellular morphology were observed along with long axons and branch dendrites (Fig. 3A). However, the pulp derived MSCs exhibits more morphological changes towards neurons as compare to follicle and papilla (Fig. 3A-III). Analysis of neuronal specific markers by immunostaining revealed that neuronal cells morphological changes were sustained with the expression of MAP2, β-tubulin, neurofilament medium (NF-M) and nerve growth factor (NG-F) in all three types of MSCs (Fig. 3B). Integrated optical density (IOD) histograms represented the expression of proteins in differentiated MSCs (Fig. 3C). Furthermore, expression of neuronal specific markers i.e. *MAP2* (4.74 ± 0.28, 3.64 ± 0.35, 6.30 ± 0.30) *β-tubulin* (5.60 ± 0.25, 4.38 ± 0.32, 7.55 ± 0.32) *NF-H* (4.51 ± 0.29, 3.95 ± 0.14, 6.41 ± 0.65) and *NF-M* (3.27 ± 0.26, 3.05 ± 0.42, 4.03 ± 0.66) were evaluated by RT-qPCR in follicle, papilla and pulp derived MSCs respectively, which shows significantly ($p < 0.05$) higher expression in differentiated (DF) MSCs as compare to non-differentiated (NDF) (Fig. 4).

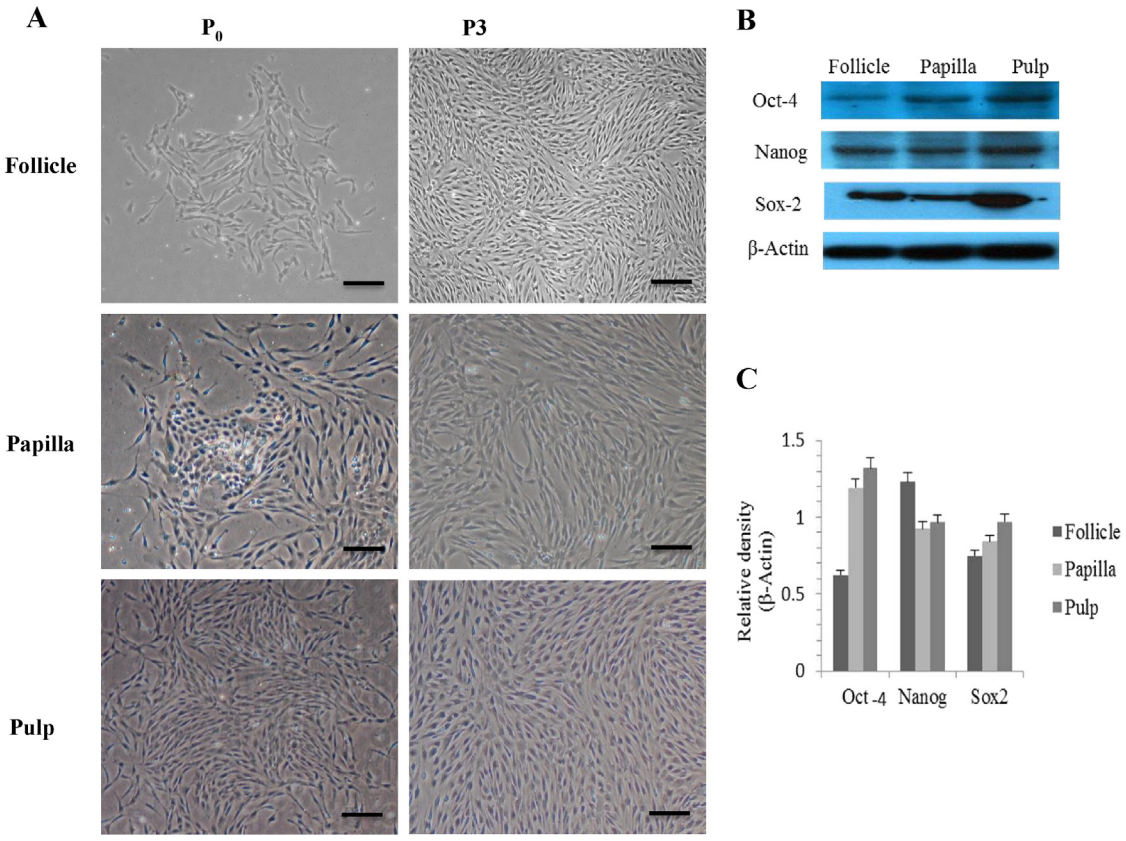
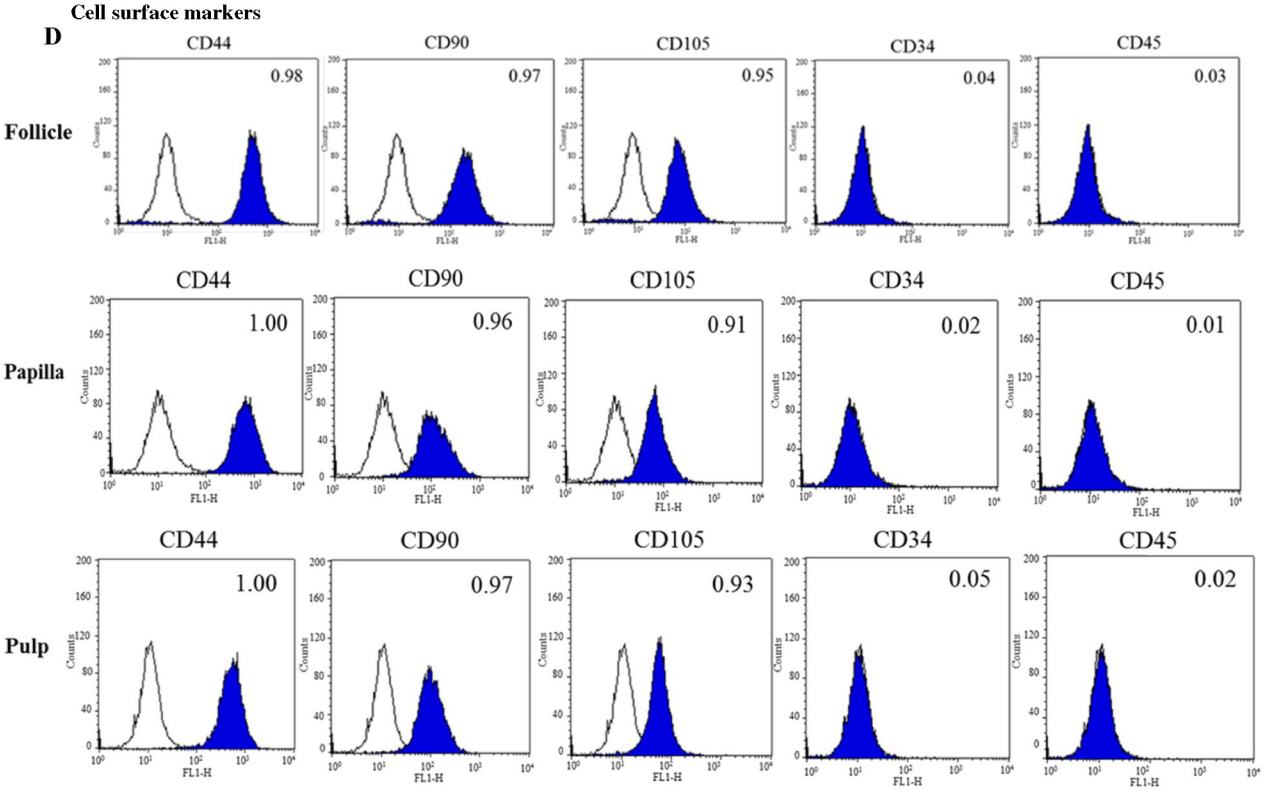


Fig. 1. Characterization of MSCs from dental follicle, papilla and pulp. (A) Formation of adherent, fibroblast like cell morphology at passage 0 and formation of homogenous colonies at passage 3. Scale bar = 50 μm. (B) MSCs from follicle, papilla and pulp, showing positive expression of Oct4 (43–50 kDa), Sox2 (34 kDa) and Nanog (35 kDa) at protein level. β-actin (42 kDa) was used as an internal control. (C) Relative density of pluripotent markers to β-actin. (D) Cell surface markers analysis of MSCs from follicle, papilla and pulp. All MSCs showed positive expression of CD44, CD90 and CD105, while negative expression of CD34 and CD45. (E) Dental stem cells specific cell surface markers. MSCs from follicle, papilla and pulp showed positive expressions of CD13, CD29 and CD73. The blank histograms represent an antibody isotype control and filled shaded histogram indicates MSC-specific antibodies.



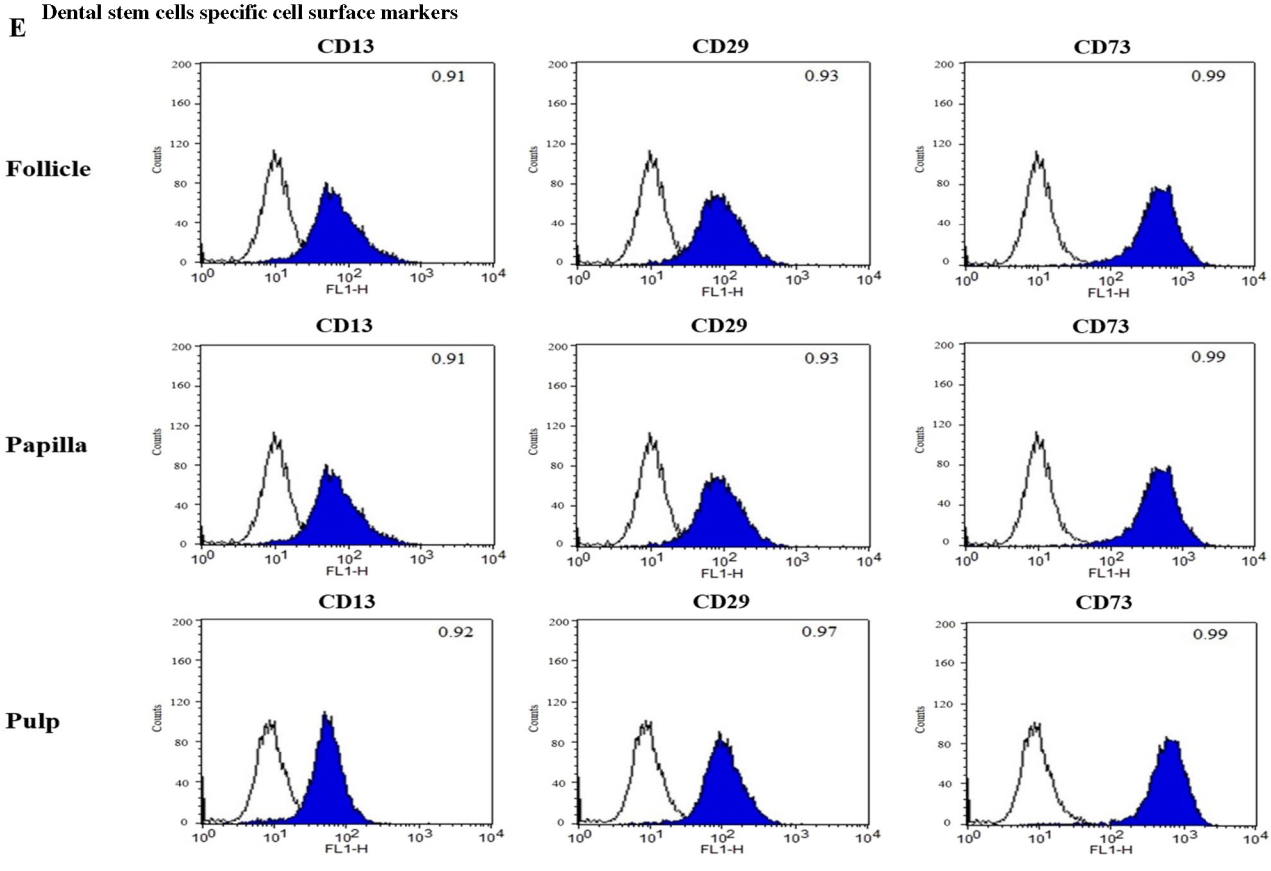


Fig. 1 (*continued*).

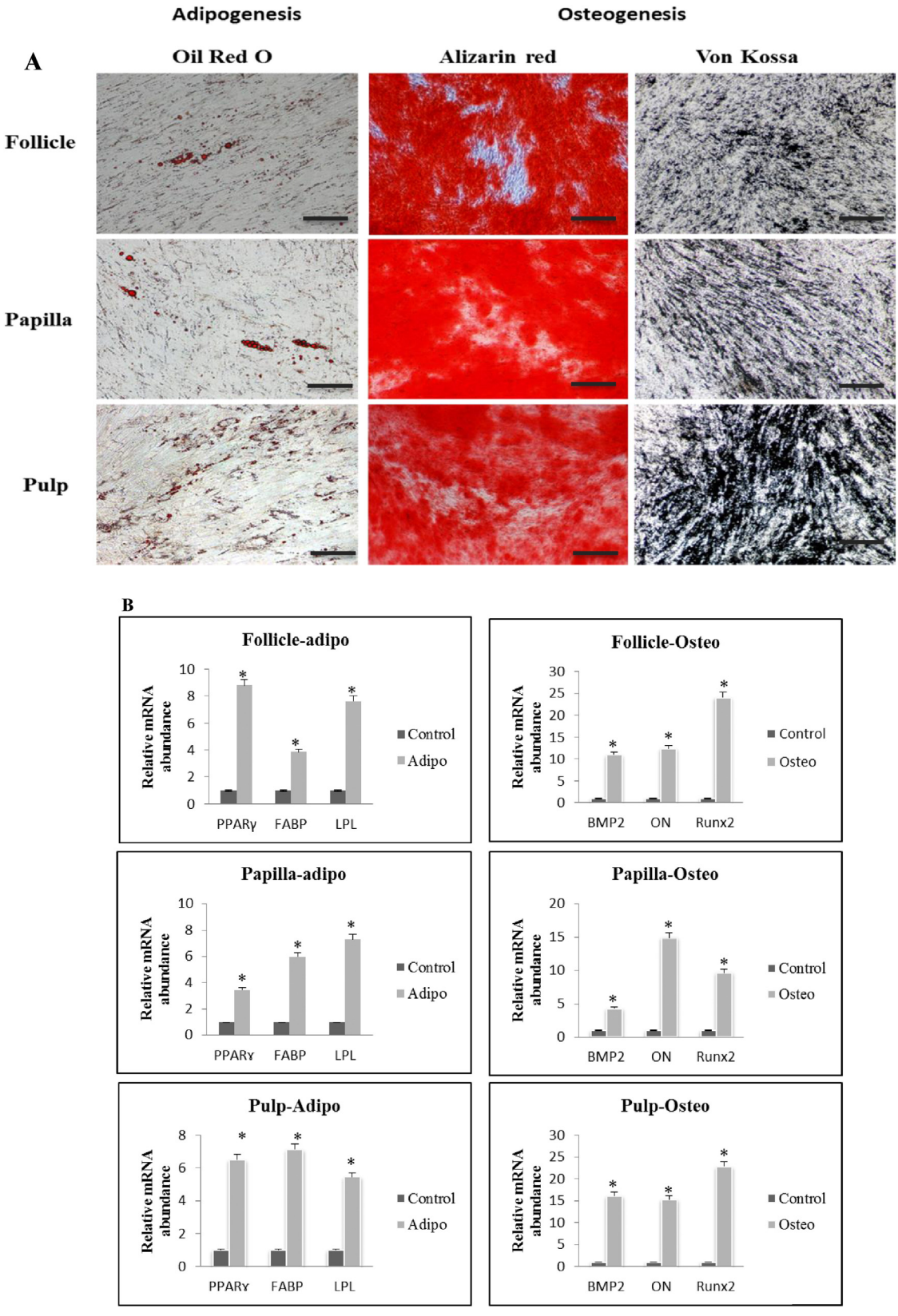


Fig. 2. In vitro lineage differentiation potential of MSCs from dental follicle, papilla and pulp (A) Adipogenesis of MSCs was detected by Oil red O staining of lipid droplets. Osteogenesis was demonstrated by Alizarin red and Von Kossa staining of mineralized nodules and calcium deposition. Scale bar = 100 μm. (B) RT-qPCR expression analysis of lineage specific markers in MSCs differentiated into adipocytes and osteocytes. YWHAZ was used as an internal control. (* represent significant differences ($p < 0.05$) in control and differentiated cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

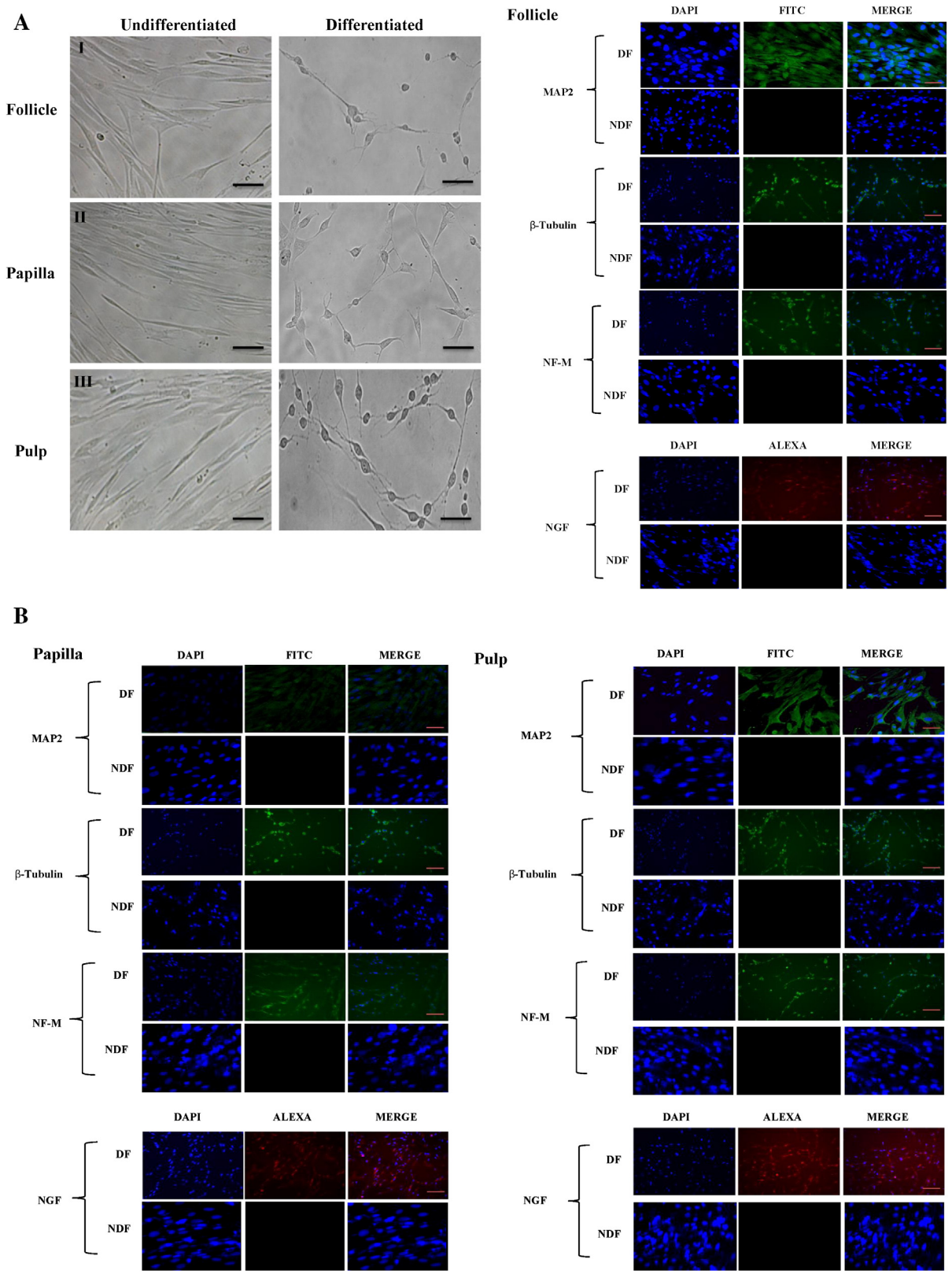


Fig. 3. In vitro trans-differentiation of MSCs of follicle, papilla and pulp into neuron like cells. (A) After 3 weeks of induction with neural conditioned media, differentiated cells showed typical neuron like morphology with dendrites and elongated axons as compared to the undifferentiated cells (I) Follicle, (II) Papilla (III) Pulp. Scale bar = 100 μm. (B) Immunofluorescence analysis showed positive expression of neuronal specific markers MAP2, β-tubulin, NF-M and NGF in neuronal differentiated and undifferentiated MSCs of Follicle, Papilla and Pulp. (C) Integrated density histograms (IOD) of immunofluorescence images, showing expression levels of specific antibodies (Letters a, b, and c indicates significant differences ($p < 0.05$) in expression of proteins among three groups). DAPI indicates the nucleus, and merge indicated the positive expression of neuronal specific proteins. Scale bar = 100 μm.

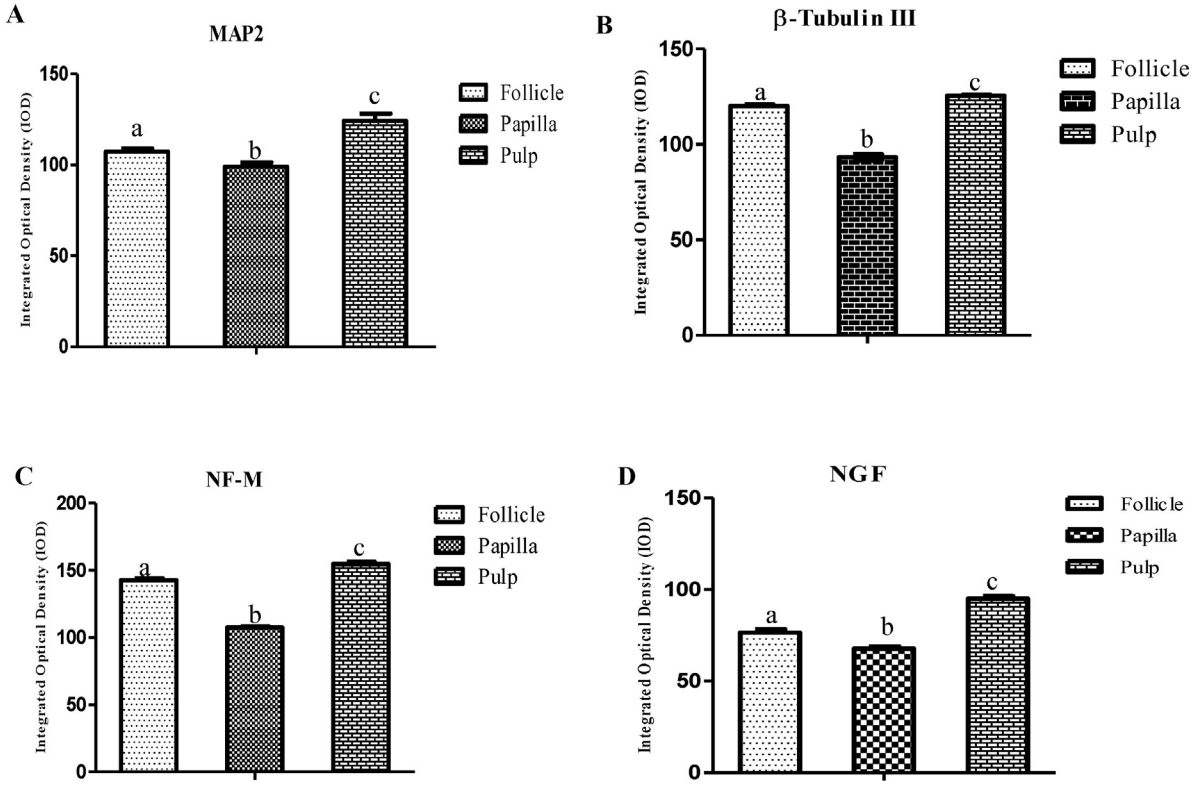


Fig. 3 (*continued*).

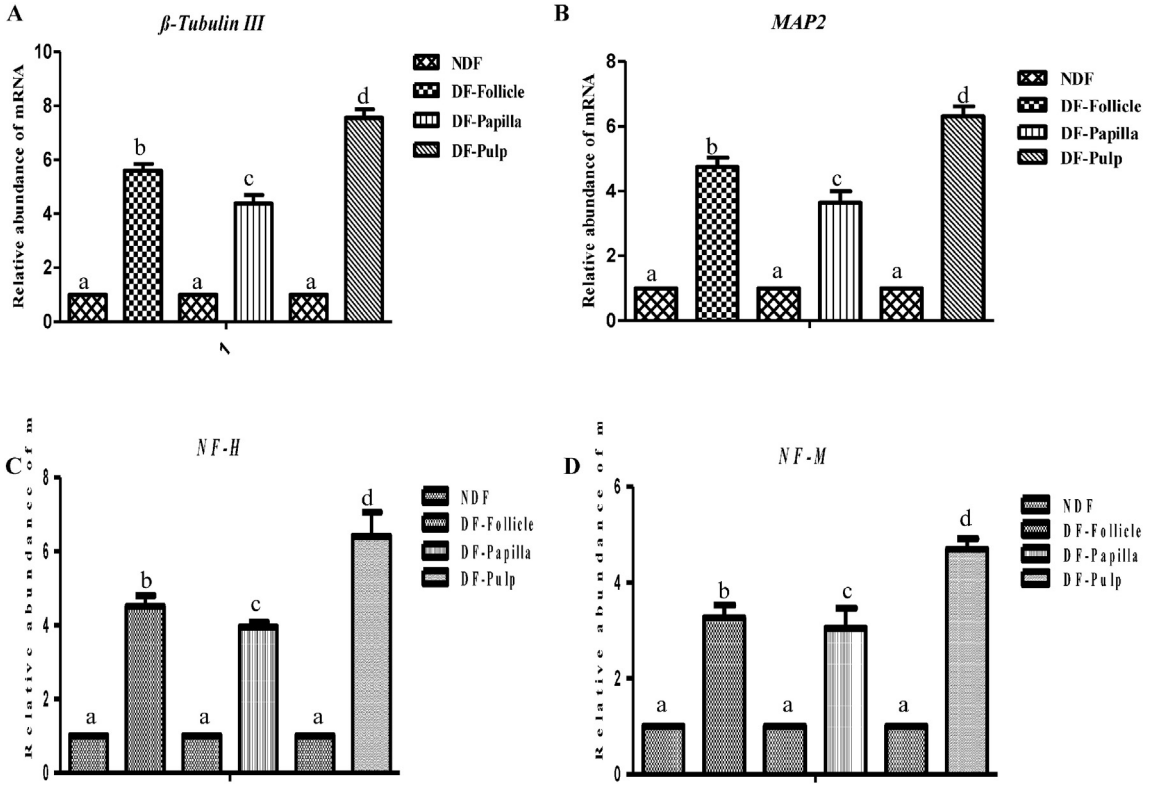


Fig. 4. RT-qPCR analysis of neuronal specific markers (A) *MAP2* (B) *β-tubulin* (C) *NF-H* and (D) *NF-M*, showed significantly higher expression in differentiated cells (DF) as compared to undifferentiated control (NDF) among the three groups (Follicle, Papilla and Pulp). (Letters a, b, and c indicates significant differences ($p < 0.05$) in expression of mRNA between the DF and NDF cells).

3.1.4. Functional analysis of neuronal cells by electrophysiology and synaptic responses

The functional expression of Na^+ and K^+ current were compared between NDF and DF papilla, follicle and pulp derived MSCs. Na^+ currents were recorded in the DF-pulp derived MSCs; however, no Na^+ current was observed in the NDF pulp derived MSCs (Fig. 5A). The Na^+ current was compared among papilla, follicle and pulp-derived MSCs. As shown in Fig. 5B, neurogenic cells derived from pulp showed high expression of Na^+ current as compared to those from papilla and follicle. The number of patches showing Na^+ channel was displayed as a percentage value. The % of patch showing Na^+ currents was 6.7% (2/30 patches), 24.0% (12/50 patches) and 50.0% (14/28 patches) in papilla-, follicle- and pulp-derived neurogenic cells, respectively. The Na^+ current were completely inhibited by treatment with Na^+ channel blockers i.e. 100 μM bupivacaine and 100 μM lidocaine (94.6 ± 2.7% by bupivacaine, 94.2 ± 3. 5% by lidocaine, Fig. 5C and D). The presence of K^+ currents was investigated using K^+-based pipette solution in papilla-, follicle- and pulp-DF cells. The K^+ currents showed significant difference between non-differentiated and differentiated into neurogenic cells, in particular pulp-derived stem cells (927 ± 179 pA in NDF vs 1404 ± 224 pA in DF). The currents showed weak voltage-dependence and inward rectification. In addition, the current was completely inhibited (92 ± 2.3%) by using K^+ channel blocker i.e. TEA (3 mM) (Fig. 5E and F). The pulp derived neuronal cells were further evaluated for the expression of synaptic markers i.e. synaptophysin and synapsin along with axonal specific marker (tau) (Fig. 6).

4. Discussion

Dental tissues are more specialized tissues that do not undergo continuous remodeling like other tissues; therefore, dental tissues derived MSCs are more specific and committed in their differentiation efficiency as compared to other MSCs [21]. The present study tried to endeavor a forthright comparison of neuronal differentiation among MSCs derived

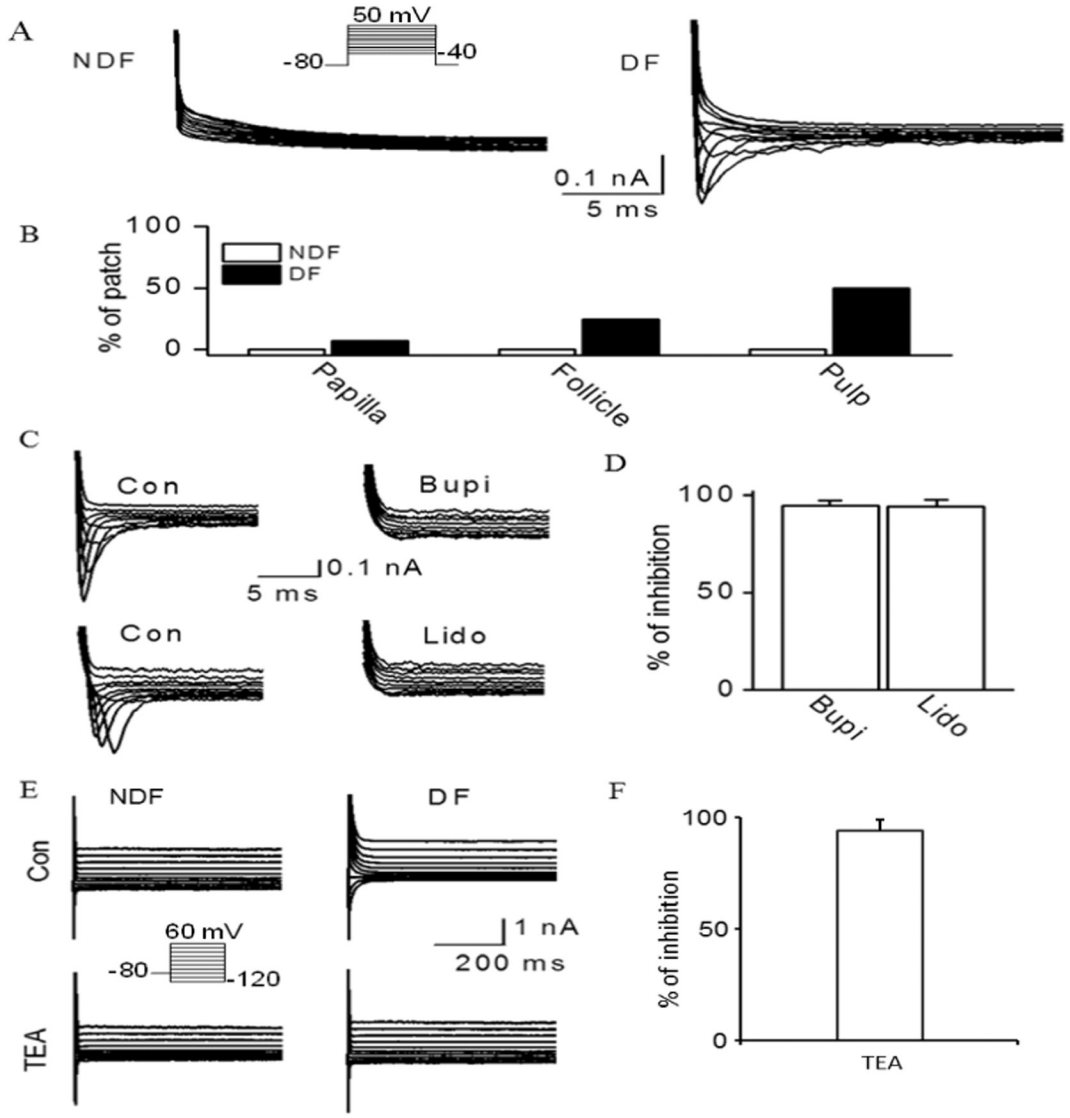


Fig. 5. Recording Na^+ and K^+ currents in neuron-like cells differentiated from human dental stem cells. (A) Whole-cell Na^+ current recorded in neuronal cells differentiated from human dental pulp stem cells. Cell membrane potential was held at −80 mV, and voltage steps from −40 to +60 mV were applied at 10-mV intervals for 100-ms durations. (B) A bar graph showing the expression of Na^+ current in neuronal cells differentiated from papilla, follicle, and pulp stem cells. (C and D) Complete blockage of the currents by Na^+ channel blockers, Bupi (100 μμ) and Lido (100 μμ). Bupi and Lido represent bupivacaine and lidocaine, respectively. Each bar represents the mean ± SE. (E) Comparison of K^+ currents between dental pulp non-differentiated and differentiated cells. Voltage steps from −120 to +60 mV were applied for 600-ms durations in the absence and presence of 3 mM TEA. Pipette and bath solutions contained 150 and 5 mM K^+, respectively. (F) Effect of TEA on K^+ currents of DF dental pulp stem cells. Each bar represents the mean ± SE.

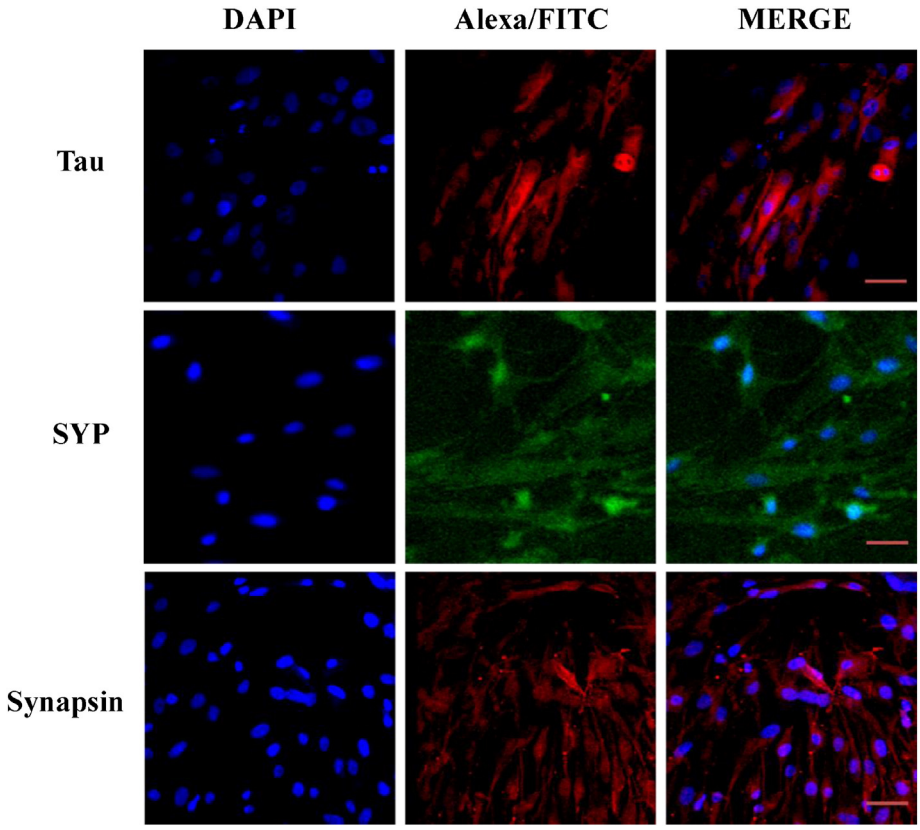


Fig. 6. Analysis of axonic specific marker (tau) and synaptic markers, synapsin and synaptophysin in differentiated pulp derived MSCs. DAPI indicates the nucleus, and merge indicated the positive expression of specific proteins. Scale bare = 100 μm.

from dental follicle, papilla and pulp isolated from a single donor tooth. The three types of MSCs were analyzed for the expression of cell surface and pluripotent markers, in vitro differentiation ability into mesodermal lineages (adipocytes, osteocytes) and ectoderm (neuronal cells). Moreover, their neuronal differentiation capacity were evaluated by analyzing neuronal specific marker at protein and mRNA level and functionally assessed by measuring Na^+ and K^+ current through electrophysiology while expression of synaptic markers by immunofluorescence.

MSCs exhibit plastic adherence, express specific cell surface markers and can differentiate into mesodermal lineages (adipocytes, chondrocytes and osteocytes) in vitro [22]. In the present study, all MSCs exhibited the plastic adherence property after initial seeding and became homogenous cells when further expanded. All the three types of MSCs were found positive for MSCs specific cell surface markers (CD44, CD90, CD105) and dental stem cells markers (CD13, CD29, CD73), while found negative for hematopoietic markers (CD34, CD45). This expression pattern of cell surface markers is consistent with the previous reports [5–6,17] confirming the MSCs like feature of our dental stem cells. The pluripotent or stem cells markers, Oct4, Sox2 and Nanog play a key regulatory role in the self-renewal, pluripotency and cellular differentiation of embryonic stem cells [23]. The similar regulatory role of these transcription factors has been reported in adult MSCs isolated from different sources including dental MSCs [18,24–25]. In our findings, MSCs from dental follicle, papilla and pulp expressed these markers at protein level, which is in accordance to the previous reports. The expressions of these markers deciphered the stemness ability of MSCs and endorsed the presence of highly primitive cells in dental tissues.

MSCs from follicle, papilla and pulp successfully differentiated into adipocytes and expressed adipocytes specific markers i.e. *FABP*, *PPARγ* and *LPL* which are in accordance to the previous reports [17,26,27]. Similarly all the three types of MSCs exquisitely differentiated into osteocytes upon exposure to osteogenic induction media. Differentiated cells were found having mineralized matrix which were confirmed by Alizarin red and von Kossa staining. Furthermore, the gene expression pattern of osteogenic related markers *ON*, *RUNX2* and *BMP2* were up-regulated in differentiated osteoblasts as compared to the normal control cells. Alizarin red and von Kossa staining along with higher expression of osteogenic markers, revealed higher osteogenic potency of follicle and pulp than papilla, and these finding are consistent with the previous studies [3,17].

Dental derived MSCs have the potency to trans-differentiate into ectodermal and endodermal lineages. Trans-differentiation is basically the transformation of cells from one lineage to another lineage, acquiring morphological features, express specific set of markers and also function of that specific cell type (lineage). In this study we investigated the trans-differentiation capacity of dental MSCs into ectodermal lineage (neurons) which has been previously conducted [3,28–29]. As dental mesenchyme was termed as 'ectomesenchyme' due its neural crest origin [21], we hypothesized that these ectomesenchyme-derived MSCs will possess higher neuronal differentiation ability than other MSCs sources. Furthermore, we were curious to know that which dental tissues derived MSCs can differentiate into functionally active neurons. To evaluate the neuronal differentiation ability, we selected three dental tissues (follicle, papilla and pulp) which we can easily isolate from a single donor. To date many protocols were published about the neuronal differentiation of MSCs [30–34]; however, each of them having shortcomings and questions has been raised about the reliability of differentiated neurons [32,34–35]. In 2008, Arthur et al. [28] reported the differentiation of dental pulp stem cells into functionally active neuron both in vitro and in vivo. We followed the same protocol with minor modifications, and measured the neuronal differentiation capacity of follicle, papilla and pulp derived MSCs by looking at morphological

changes, expression of neuronal specific markers (gene and protein level) and finally evaluated the coexistence of voltage-gated sodium and potassium channels.

After neuronal differentiation the cells became polarized with the formation of a single long axon and multiple dendrites, which is the first criterion for neuronal assessment [3]. We further observed that the number of differentiated cells were more in pulp than the follicle and papilla; however, the non-differentiated cells which were maintained in the normal media exhibited elongated fibroblast like morphology. Expression of neuronal specific marker was also reported in the undifferentiated DPSCs, which might be of its neural crest origin. It has been reported that naïve DPSCs express nestin, vimentin, neurofilament, S100 and β-tubulin in undifferentiated state [13,36]. In 2011, Karbanova et al. [37] demonstrated that expression of neural markers in undifferentiated DPSCs is dependent on serum amount in the media and found that 2% serum containing media results an increase in nestin expression as compared to 10% of serum concentration. For normal growth of our dental stem cells, we used growth media supplemented with 10% serum and found nestin expression only in the undifferentiated DPSCs (data not shown).

In this study we found that after in vitro neural induction, all dental MSCs expressed neuronal markers i.e. MAP2, β-tubulin III, NF-M and NF-H. MAP2 is a member of microtubule associated protein family and mostly involve in the stabilization of dendrites [28]. We found positive expression of MAP2 in all differentiated cells which gradually increase from papilla to pulp. Furthermore, we also found the positive expression of β-tubulin III at mRNA and protein level, which is an intermediate neuronal marker and also found in mature neurons. The mature neuronal assessment was performed by looking at the expression of nuerofilaments (NFs) i.e. NF-M and NF-H. NFs are abundant in axon and help in the radial growth of axons during development [38]. Among these NFs, NF-M is an important element of axon cytoskeleton [39], and it express before NF-H during neuronal differentiation [28]. We found that, all dental MSCs which were induce with neuronal induction media, expresses neuronal specific markers at different level compared to the non-differentiated control cells.

These observations suggested the in vitro neuronal differentiational potential of dental MSCs; however, to further substantiate their neuronal differentiation capacity, electrophysiological analysis were performed to check coexistence of voltage-gated ion channels. These gated channels in case of neurons are Ca^+, Na^+ and K^+ channels [40]. Whole cell patch clamping technique that is a gold standard of reporting single ion channel currents [41] was used to record Na^+ and K^+ current in DF and NDF dental MSCs. We found only voltage-gated functional Na^+ channel in DF cells, which were completely absent in NDF cells. The presence of Na^+ channel is important for generation of action potential and it has been considered as an electrophysiological marker for evaluation of fully mature functional neurons [42,43]. This depolarization of neuron plasma membrane is responsible for creating membrane potential or starting a nerve impulse. In our observations, dental pulp MSCs showed higher Na^+ current than the other two cell lines and the current was completely blocked when treated with specific Na^+ channel blockers. It has been deciphered that, after depolarization (Na^+ ions) of neuron plasma membrane, repolarization (K^+ ions) occurs which bring the neurons to resting potential [29]. So we also measured the K^+ current in DF pulp derived MSCs, confirming the coexistence of voltage-gated Na^+ and K^+ channels. The existence of minor outward current (K^+) in NDF pulp derived MSCs, suggesting that DPSCs may have a susceptibility to differentiate into neuronal cell type which in consistent to the previously published report [28]. Furthermore, along with electrophysiological properties, neurons also exhibit synaptic function which is functional interaction between two neurons [46]. There are certain proteins which are either involve in regulation of neurotransmitters or regulation of number of synaptic vesicles across neurons. Synapsin is one of the most specific marker of synapses throughout central and peripheral nervous system. Synaptophysin (SYP) is also a neural synaptic vesicle protein that is expressed in neuroendocrine cells and neoplasms [47]. Dental pulp derived neuronal cells were also found positive for synaptic markers i.e. synapsin and synaptophysin which further reconfirmed its higher neurogenic differentiation potential.

5. Conclusion

Dental derived stem cells exhibited the basic characteristics of MSCs and differentiated into adipocytes and chondrocytes. Because of their neural crest origin, all dental MSCs were successfully trans-differentiated into neuronal cells and showed different level of neuronal specific markers and also displayed functional characteristics of neurons. However, the morphological observation, expression of neuronal markers and electrophysiological properties stands out pulp derived MSCs a prominent source for neuronal differentiation. To our knowledge, this is the first kind of study in which we compare the neuronal differentiation potential of three dental tissues (follicle, papilla and pulp) from a same donor and identify a potent MSCs source for the generation of functional active neurons. This study will provide further insight in the use of dental pulp derived MSCs for the treatment of many neurological disorders.

Authors contributions

Imran Ullah collected the data and wrote the manuscript, Raghavendra Baregundi Subbarao conduct few experiments and revised the manuscript, Eun-Jin Kim performed electrophysiology, Dinesh Bharti performed western blotting, Si-Jung Jang and Ji-Sung Park gave technical assistance, Sharath Belame Shivakumar helped in statistical analysis, Dawon Kang design electrophysiological experiments, June-Ho Byun provide the dental samples, Sung-Lim Lee monitored the work, Bong-Wook Park and Gyu-Jin Rho designed the project, gave financial support, wrote and revised the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors have no competing interest to disclose.

List of abbreviations

MSCs	Mesenchymal stem cells
CD	Cluster of differentiation
NSCs	Neural stem cells
DPSCs	Dental pulp stem cells
SHED	Stem cell in exfoliated deciduous teeth
PDLSCs	Periodontal ligament stem cells
DFPCs	Dental follicle precursor cells
SCAP	Stem cells in apical papilla
FABP	Fatty acid binding protein
LPL	Lipoprotein lipase
PPARγ	Peroxisome proliferator-activated receptor
RT-qPCR	Real time quantitative PCR
ON	Osteonectin
RUNX2	Runt-related transcription factor 2
BMP2	Bone morphogenic protein 2
NF-M	Neurofilament medium
NG-F	Nerve growth factor
IOD	Integrated optical density
DF	Differentiated
NDF	Non-differentiated
TEA	Tetraethylammonium
NFs	Nuerofilaments
DPBS	Dulbecco's phosphate buffer saline

ADMEM	Advanced Dulbecco's modified eagle's media
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride membrane
BSA	Bovine serum albumin
TBS	Tris buffered saline
HRP	Horseradish peroxidase
ECL	Chemiluminescence
FITC	Fluorescence isothiocyanate
IBMX	Isobutylmethyl Xanthine
EGF	Epidermal growth factor
bFGF	Basic fibroblast growth factor
PFA	Paraformaldehyde
DAPI	4′,6-diamidino-2-phenylindole
rt	Room temperature
NaCl	Sodium chloride
KCl	Potassium chloride
CaCl2	Calcium chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EGTA	Ethylene glycol tetraacetic acid
CsCl	Cessium chloride
mM	Milli-mole
mV	Milli-volt
ANOVA	Analysis of variance

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