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Characterization of gingival tissue explant for odontogenic differentiation



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# Characterization of gingival tissue explant for odontogenic differentiation

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by

Chaehwan Lee

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ABSTRACT OF THE THESIS

Characterization of gingival tissue explant for odontogenic differentiation

by

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Master of Science in Oral Biology

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Professor Mo Kwan Kang, Chair

**Introduction**: Since the detection of Mesenchymal Stem Cells (MSCs) in dental pulp tissue, many laboratories have conducted studies associated with pulp regeneration. Recently we introduced the pulp tissue grafting, a new approach to pulp regeneration, which would diminish the inconveniences of the existing cell-based therapy. The pulp tissue grafting might be able to avoid the cell culture expansion of MSCs, a necessary step for cell-based therapy, and the associated regulatory requirements for pulp regeneration therapy. However, as the pulp tissue grafting necessitates the patient's pulp tissue, it also renders with clinical limitations for clinical

applications. In the present study, we investigated the possibility of using gingival tissue as a source of MSCs for regenerative endodontics treatment.

**Materials and Methods**: We cultured cells from minced gingiva (MG) explant and isolated outgrowing cells. To determine the possible MSCs of these outgrowing cells, the phenotypes of these cells were analyzed, such as replication kinetics, migration capacity, multi-differentiation potential, and immunophenotypes. Also, we examined osteogenic/odontogenic differentiation capabilities of these cells in dentin slices model with two different scaffold, PuraMatrix$^{TM}$ hydrogel and poly-L-lactic acid (PLLA) *in situ*.

**Results**: We found that gingival tissue explants yielded group of cells with robust replication kinetics and multi-differentiation potential to osteogenic/odontogenic and adipogenic differentiation. Inasmuch as these isolated cells demonstrated the chractyeristics of mesenchymal stem cell, we termed them the minced gingival-mesenchymal stem cells (MG-MSCs). The MG-MSCs were able to replicate up to 45 population doublings (PDs) before senescence, while gingival MSCs (GMSCs) prepared from enzyme digestion method as reported previously (25) continued to replicate beyond 60 PDs. Senescence-associated β-galactosidase (SA β-Gal) staining showed that MG-MSCs underwent senescence significantly earlier compared to GMSCs. Although both MG-MSCs and GMSCs were able to differentiate into adipogenic lineage, demonstrating multi-differentiation capacities, only MG-MSCs were able to differentiate to osteo/odontogenic cells. Also MG-MSCs expressed much stronger level of stem markers, e.g., Oct3/4, Sox2, and Nanog, as well as odontogenic differentiation markers, e.g., DMP-1 (Dentin matrix acidic phosphoprotein-1) and DSPP (Dentin Sialophosphoprotein), compared with GMSCs. When MG was seeded on the scaffold (poly-L-lactic acid and PuraMatrix$^{TM}$) in dentin-slice model, we noticed migratory cells from the tissues that had

attached on the dentin surface and expressed DSP differentiation marker, again indicating the presence of MG-MSCs in the minced gingival tissue.

**Conclusion**: Our data indicate that MG transplantation yield highly proliferative MG-MSCs that exhibit the features of MSCs and undergo odontogenic differentiation in contact with dentin surface. Taken together, we believe that MG-MSCs can be utilized for direct tissue transplantation purpose of pulp-dentin regeneration.

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## 1. Introduction

Multipotent MSCs found in bone marrow were first described by Friedenstein et al. (1). After the characterization of Bone marrow-derived mesenchymal stem cells (BMSCs), MSCs have been searched in other tissues based on the characteristics of established BMSCs (1-5). In addition to bone marrow, MSCs have also been found in many other tissues, such as umbilical cord blood, synovium, the liver, adipose tissue, the lungs, amniotic fluid, tendons, placenta, skin, and breast milk (6-16). The MSCs found basically possess self-renewal and multi-differentiation potential, as well as immunomodulatory function and potent tissue regeneration properties (17). Because of those features, MSCs have been actively used in tissue regeneration studies.

The first type of dental stem cell was isolated from the pulp tissue of the human permanent teeth and termed ‘postnatal dental pulp stem cells’ (DPSCs) (18). Since the detection of DPSCs, other dental MSCs have also been reported, such as stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), alveolar bone-derived MSCs (ABMSCs), stem cells from apical papilla (SCAP), tooth germ progenitor cells (TGPCs) and gingival MSCs (GMSCs) (19-25). Among the dental MSCs, DPSCs have been widely studied. The most striking feature of DPSCs is their ability to regenerate dentin-pulp-like complex. This highlights the formation of mineralized matrix with dentin-like tubule with odontoblast, other vascular structure arranged very similarly as the natural human dental pulp (18).

The dental pulp is a soft tissue of ectomesenchymal and mesenchymal origin, developing from the neural crest cells (26). Dental pulp is a highly innervated and vascularized connective tissue, and it is responsible for several important functions in teeth, such as formation of dentin, nourishment, and sensory function (27). Despite satisfactory clinical outcomes, root

canal treatment cannot restore any of the original functions of pulp tissue, and the lack of sensory and defensive functions resulting from the treatment may lead to tooth fracture and reinfection (28). Thus, many researchers have been paying great attention to pulp regeneration, and a lot of studies has actually been done.

Traditionally, pulp regeneration studies are first prepared by isolating and manipulating stem cells. Then, the cells were loaded on to scaffold incorporated with or without signaling molecules and transplanted into the root canal of *ex vivo* tooth slides or *in situ* tooth (29). *In vitro* and *in vivo* animal studies have demonstrated great potential of DPSCs for pulp regeneration**.** A dentin-pulp like structure containing vascularized pulp-like tissue, and it surrounded by a layer of odontoblast-like cells was generated by transplanting DPSCs into immunocompromised mice with hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold *ex vivo* (18, 30). Another report indicated that DPSCs formed mineralized nodules with a reparative dentin-like tissue on the surface of human dentin *in vivo* (30), and it has been proven that many other scaffolds can be used *in vivo* to generate dentin-pulp like complex, such as calcium phosphate (31), polylactic acid (32), and puramatrix (17). Also, a small recent scale Phase I clinical trial was conducted by Misako Nakashima in Japan to evaluate the feasibility of pulp regeneration with DPSCs. The trial is to transplant autologous mobilized DPSCs to the teeth of patients with irreversible pulpitis, which express prolonged sensitivity to certain stimuli. After 25 weeks, the treated teeth show recovery from pulpitis symptoms and regenerated soft tissue determined by magnetic resonance imaging (MRI) without any adverse effects (33). These studies have primarily demonstrated the potential of complete pulp regeneration.

For such treatment, however, a healthy pulp tissue must be obtained from the patient's own healthy teeth. Also, such treatment requires multistep process; DPSCs should be isolated

from the healthy pulp tissue, grown in *in vitro* setting, and then can be used for the treatment. Therefore, this poses a limitation of cell-based endodontic cases, even if extracted teeth or primary teeth are available to obtain DPSCs.

It has been reported that human gingival tissue also has an MSCs population which is Gingival tissue-derived Mesenchymal Stem Cells (GMSCs). In *in vitro* studies, GMSCs exhibit clonogenicity, self-renewal, multipotent differentiation capacity, and immunomodulatory properties (25). Also it express CDs and display positive signals for Oct4, Sox2, Nanog, Nestin, SSEA4, and Stro-1 (25, 34, 35). Its High expression levels of OCN, OPN and Col I indicates the potential of GMSCs for *in vivo* bone regeneration (25). In the study about the embryonic origin of GMSCs, it is reported that 90% of GMSCs isolated from mice were positive for the neural crest-associated surface markers, while 10% were from mesoderm (36). Surprisingly, in an *in vivo* study in which dexamethasone-treated GMSCs were transplanted subcutaneously into immunodeficient mice (SCID mice), GMSCs formed the bilineage (mesodermal and ectodermal) mixed tumor that included fetal fat, striated muscle, cartilage, bone, epithelial tissue, and neural tissue (37). This finding suggests that GMSCs are capable of generating tissues originated form neural crest cells *in vivo* during embryogenesis, and MSCs in human gingival tissue can be utilized for pulp regeneration.

Recently, we have proposed tissue-based therapy utilizing minced pulp tissue, a new method for pulp regeneration to overcome the weaknesses of cell-based therapy. Through this new approach for pulp regeneration, cell culture process and associated regulatory requirements might be bypassed for clinical application (38).

We herein provide evidence about the feasibility of a new method "Gingival tissue grafting" for pulp regeneration. We characterized MSC features of outgrowing cells from gingival tissue explant and proved the odontogenic differentiation of the cells in dentin slice model mimicking the environment of pulp space *in vitro*. This study demonstrates that gingival tissue, the most commonly and easily obtained from intraoral tissue, is a source of MSCs that can be used for regenerative endo treatment.

## 2. Materials and Methods

### 2.1. Preparation of gingival tissues and cells.

Human gingival tissues were obtained from eleven freshly extracted third molars (patient age 18 to 25 years, no history of periodontal disease) which were collected from the UCLA Oral and Maxillofacial Surgery clinic. After extraction, the teeth and tissues were stored in sterile tubes containing a-MEM medium (Invitrogen, Carlsbad, CA) containing 3% Antibiotic-Antimycotic (Life Technologies, CA), and the tubes were stored in a box filled with ice until the culture. The primary culture of GMSCs and MG-MSCs were maintained with primary culture media, a-MEM medium containing 20% fetal bovine serum (FBS) (Invitrogen), 15 mg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA), and 20 mmol/L L-glutamine (Invitrogen). All cells after primary culture were maintained with basal media containing a-MEM with 10% FBS and 5 mg/mL gentamicin sulfate (18).

The gingival tissues detached from the extracted teeth were removed from the epithelium and divided into two groups. For the conventional enzymatic culture of GMSCs, the tissues were chopped into very small pieces and treated with a solution of 3 mg/mL type I

collagenase (Thermo Fisher) and 4 mg/mL Dispase (Roche, South San Francisco, CA) for 120 minutes in 37℃. For the gingival tissue explant, the tissues were minced into 1mm sized pieces and seeded one by one in 48 well plates. The minced gingival tissues were maintained with primary culture media for 7 to 14 days until the outgrowing cells from the tissue showed 80% confluency.

Primary GMSCs and MG-MSCs were passaged on a new plate when they showed 80% confluency. Cumulative population doublings (PDs) and replication kinetics were calculated from the number of cells initially seeded on the plate and the number of end of each passages as previously described (39).

The senescence associated b-galactosidase activity of GMSCs and MG-MSCs were stained at different PDs as described in another report (39). The result was quantified under the light microscopy.

### 2.2. Immunophenotype Characterization

Cells were pelleted and resuspended in flow buffer which consists of PBS with 0.1% sodium azide and 1% BSA. The Cells were incubated with different concentrations of antibody according to the manufacturer's method. The antibodies against CD44 (hyaluronan receptor), CD90 (thymocyte antigen), CD34, CD45 (hematopoietic stem/progenitor lineage marker), CD105, CD146 (endothelial lineage), CD73 (T lymphocyte differentiation antigen), STRO-1 (Stem cell marker) were used for the experiment.

### 2.3. Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol reagents (Invitrogen). Clear aqueous phase containing RNA was transferred to a clean tube after centrifugation, and 2-propanol was

added to precipitate RNA followed by centrifugation. RNA pellet was quickly rinsed with 75% ethanol and NanoDrop Spectrophotometer (Thermo Fisher Scientific) was used to measure RNA concentration and quality. Using 1mg of extracted RNA cDNA was synthesized via the SuperScript first-strand synthesis system (Invitrogen). Each gene of interest was amplified with SYBR Green I Master Mix (Roche) with LightCycler 480 II real-time polymerase chain reaction system (Roche) following the manufacturer's protocol. Each qPCR was performed in triplicate with initiation of heat denaturation at 95℃ for 10 minutes, then 45 cycles of 95℃ for 10 seconds followed by 58℃ for 45 seconds and 72℃ for 10 seconds. Total 50 cycles of qPCR reactions were taken, and second derivative quantification cycle value determination method value was used to compare fold differences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

The name of genes and DNA Sequence of Primers used in Real-Time Quantitative Polymerase Chain Reaction.

| Name of gene | Sequence |
|---|---|
| Hs nanog F | 5'- AAA GAA TCT TCA CCT ATG CC - 3' |
| Hs nanog R | 5' - GAA GGA AGA GGA GAG ACA GT - 3' |
| Hs Sox2 F | 5' - GGG AAA TGG GAG GGG TGC AAA AGA GG - 3' |
| Hs Sox2 R | 5' - TTG CGT GAG TGT GGA TTG GTG - 3' |
| Hs Oct3/4 F | 5' - GAC AGG GGG AGG GGA GGA GCT AGG - 3' |
| Hs Oct3/4 R | 5' - CTT CCC TCC AAC CAG TTG CCC AAA C - 3' |
| Hs OCN F | 5' - GCA GGT GCG AAG CCC AGC GGT GCA GAG - 3' |
| Hs OCN R | 5' - GGG CTG GGA GGT CAG GGC AAG GGC AAG - 3' |

| | |
|---|---|
| Hs DMP1 F | 5’ - GTG AGT GAG TCC AGG GGA GAT AA - 3’ |
| Hs DMP1 R | 5’ - TTT TGA GTG GGA GAG TGT GTG C - 3’ |
| Hs OPN F | 5’ - TTT CGG AGA CCT GAC ATCC - 3’ |
| Hs OPN R | 5’ - GGC TGT CCC AAT CAG AAGG - 3’ |
| Hs DSPP F | 5’ - TCA CAA GGG AGA AGG GAA TG - 3’ |
| Hs DSPP R | 5’ - TGC CAT TTG CTG TGA TGT TT - 3’ |
| Hs GAPDH F | 5’ - AGC CAC ATC GCT CAG ACA C - 3’ |
| Hs GAPDH R | 5’ - GCC CAA TAC GAC CAA ATC C - 3’ |

### 2.4. Migration assays

Cells were seeded in transwell inserts with 8 μm pores (Corning, NY) and the outside of the inserts were filled with a-MEM with 1% FBS. After 24 hours and 48 hours, the non-migrated cells inside the insert were removed with a cotton swab and cells migrated outside the insert were stained with 1% crystal violet.

### 2.5. Odontogenic Differentiation Assay

DPSCs, GMSCs, and MG-MSCs were cultured with basal media until 80% confluency and treated with osteogenic media which consists of a-MEM containing 20% FBS, 100 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 9 mM KH2PO4, 10 mM b-glycerol phosphate, and 9.8 nM dexamethasone (Sigma-Aldrich). Basal media were used for control group. All cells were stained with the ALP Staining Kit (Sigma-Aldrich) for ALP activity and were stained with the Alizarin Red S. (Sigma-Aldrich) for mineralization at different day, using the protocol described previously (40). ALP absorbance was measured by spectrophotometer with 405nm wavelength.

### 2.6. Adipogenic Differentiation Assay

DPSCs, GMSCs, and MG-MSCs were cultured with basal media until 80% confluency and treated adipogenic media which consists of Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS, 60 mM indomethacin (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1 mM dexamethasone (Sigma-Aldrich), and 5 mg/mL insulin (Invitrogen) for 21 days. Basal media were used for control group. All cells were fixed with 10% neutral formalin for 30 min, and cytoplasmic lipid droplets were stained with Oil Red O solution as previously described (41). Culture plates were observed under high magnified microscope view after drying it.

### 2.7. Dentin Slice/Scaffold Model

The cemento-enamel junction of the collected molars was cut horizontally with diamond bur to obtain dentin slices with 1 mm thickness. The pulp space of dentin slices was filled with porous poly-L-lactic acid (PLLA) (Goodfellows, Coraopolis, PA) scaffold or PuraMatrix™ hydrolgel (BD Biosciences). Three MG tissues were seeded onto the fully polymerized PLLA and cultured for 21 days. The dentin slice / scaffold samples were stained with the Alizarin Red S. (Sigma-Aldrich) to measure mineralization, or sectioned with 5 mm thickness and stained with hematoxylin and eosin. After filling the pulp space of each tooth slice with 0.5% PuraMatrix™ which were made by mixture of 1% PuraMatrix™ and 10% sucrose at a ratio of 1:1, freshly MG tissue was seeded in the middle portion of PuraMatrix™ and maintained in the cell incubator for 7 to 21 days. Dentin slice/scaffold samples were then stained with the Alizarin Red S. (Sigma-Aldrich) for analysis of mineralization.

### 2.8. Immunofluorescent staining

The paraffin embedded MG tissue was sectioned with 5 mm thickness and deparaffinized in a 65 ° oven for 30 min. After rehydrating it with alcohol for 30 min, the tissue was unmasked with citrated buffer overnight at 60 ° C and blocked with 3% hydrogen peroxidase for 15 minutes. The tissue was blocked with PBS containing 10% FBS for 30 minutes and incubated with a primary antibody for 1.5 hours. Then, the tissue was incubated for 1 hour with a secondary antibody labeled with fluorescence. The secondary antibody used was Alexa 488 (green). Slides were mounted in Prolong Gold w/DAPI (Invitrogen). Images were taken on a confocal microscope.

## 3. Results

### 3.1. Tissue and cell culture.

From March to December 2017, gingival tissues attached to the extracted 3$^{rd}$ molars of 11 patients were used in the experiment. Patients were 18 to 25 years of age with no history of periodontal disease. The obtained gingival tissues were divided into two groups and half of them were used for general GMSCs culture using enzyme. The other half of the tissues were cut into 1-mm-sized tissue and placed into a 48-well plate for tissue culture (1-A). After about 5-6 days, we could see the outgrowing cells from the minced gingiva and named it Minced Gingiva-derived Mesenchymal Stem Cells (MG-MSCs) to make a distinction from well-known GMSCs. Occasionally, the mesh-like layer extended from the MG tissues, but the cells from the well were excluded because they did not correspond to the three MSC morphologies and matched the morphology of the epithelial cells (42). To rule out variability due to genetic makeup of patients, cells derived from the same patients (DPSCs, GMSCs, and MG-MSCs) were used for each

experiment. Early passage cells prior to passage 3 were used to reduce the effect of *in vitro* culture on intact cell condition, and all experiments were triplicated.

### 3.2. Exhibition of plastic adherent property and mesenchymal stem cell morphology of outgrowing MSCs from minced gingiva (MG-MSCs).

MG-MSCs from MG tissue have fulfilled the basic criteria of MSCs, which exhibit plastic adherent properties and spindle-shaped MSCs morphology (Fig 1-A, B) (4). To prove the presence of cells and investigate the cellularity in MG tissue, we detached MG tissues from Day 0 and Day 15 culture and performed hematoxylin-eosin staining. The MG tissue contained many cells at Day 0 and at Day 15 days, and it was confirmed that many cells were clustered with some parts thought to be attached to the plate (*arrows*) (Fig 1-C). DAPI staining was performed to more clearly visualize the change in cellularity with time difference (Fig 1-D). After 15 days, the MG tissue showed a significantly lower cellularity than the Day 0 in the overall area. This demonstrates that cells of all tissue areas attempt to move out of tissue, not just cells from the periphery or from one particular area. In addition, all the MG tissues changed into smaller, rounded shapes over time (Fig 1-A), and the changes in volume and shape of these tissues also prove cell actively migrate to outside.

### 3.3. Distinct replicative phenotype of MG-MSCs from GMSCs

The replication pattern of GMSCs, which grows more rapidly than the well-known MSCs, BMSCs, is already well studied (25). MG-MSCs showed faster replication in the beginning than GMSCs, but replication arrest also happened early. In the initial 1 to 3 passages, MG-MSCs showed higher replication than GMSCs, but afterwards it was similar to GMSCs (Fig 2-A). A similar pattern was also observed in the study of MP-MSCs (38). The initial state of

MG-MSCs from tissue indicates a higher replication pattern and this pattern disappears as cells grew in *in vitro* condition longer. Also, this rapid replication arrest of MG-MSCs was further demonstrated by senescence-associated b-galactosidase staining showing cell senescence (Fig 2-B).

### 3.4. Migration capacity of MG-MSCs compared to DPSCs and GMSCs.

Higher migratory phenotype is one of the major characteristic of MSCs because of the nature of MSCs that must move to wound tissue to perform their roles (43). Compared to cell-based therapy where cultured MSCs are transplanted, the source of MSCs in tissue-based therapy is the migrating cells from the transplanted tissues. Therefore, sufficient migration capability of MG-MSCs is important for successful tissue regeneration. Migration ability of DPSCs, GMSCs, and MG-MSCs was evaluated by Transwell assay. 20,000 cells were seeded and the number of migrated cells was measured at Day 1 and Day 2. MG-MSCs showed much higher migration capacity than DPSCs, the main cell type used in the pulp regeneration study (Fig 2-A, B). This suggests that tissue-based therapy using MG tissue may also have sufficient therapeutic efficiency.

### 3.5. Osteogenic/odontogenic differentiation capacity of MG-MSCs compared to DPSCs and GMSCs.

Multipotent differentiation potential is a major criterion of MSCs (4). To test the multi-differentiation potential of MG-MSCs, we examined the osteogenic differentiation of MG-MSCs, which is one of the important lineages of MSCs. The osteogenic differentiation capacities of early passage (P2) DPSCs, GMSCs and MG-MSCs were compared after treatment of osteogenic induction media or basal media. Because ALP is an early marker for osteoblasts, ALP

staining was performed at early dates (Day 5 and Day 7) compared to ARS. Compared to DPSCs, GMSCs and MG-MSCs showed stronger ALP staining on both days (Fig3-A), and quantification of ALP absorbance at Day 7 showed a significant increase in ALP level of MG-MSCs compared to the other two cell lines (Fig3-B). ARS measures the mineralization and stains free calcium and certain calcium compounds. Similar to the results of ALP staining, the plate wells treated with conditioned media showed heavier red staining (Fig3-C). Interestingly, in the MG-MSCs wells, the area estimated to be occupied with dense deposits was observed, and small blackish red dots were found in the magnified microscope view (Fig3-C, D). When we checked some of the particles off the plate after washing, we confirmed that it was a hard mineralization matrix. This demonstrates the strong mineralization of MG-MSCs compared to the other two cells lines. Our data suggest that MG-MSCs have higher osteogenic differentiation potential compared to the DPSCs and GMSCs.

### 3.6. Adipogenic differentiation capacity of MG-MSCs compared to DPSCs and GMSCs

Also, we measured adipogenic differentiation capacity, which is another criterion of the multipotency of MSCs (4). In the wells treated with adipogenic induction media, many red lipid droplets made from adipocytes were found (Fig 5). On the other hand, lip droplet was not found in the wells treated with basal media. This data indicates that MG-MSCs have higher adipogenic differentiation potential.

Overall, MG-MSCs have sufficient differentiation potentials, and in particular, it has much higher osteogenic differentiation capacity compared to the other two cell lines.

### 3.7. mRNA expression patterns of genes associated with stemness, osteogenic differentiation, and odontogenic differentiation.

To further study, we measured the expressions of three well-known stemness markers in MG-MSCs. Interestingly, MG-MSCs showed significantly increased mRNA expression in all three stemness markers compared to the other two cell lines (Fig 6-A).

Next, to determine the odontogenic differentiation capacity, the most important capability for pulp regeneration, we measured the mRNA expression of four well-known genes involved in dentinogenesis. Cell plates wells were divided into two groups: induction media treated group and basal media group (44). The mRNA expressions of OCN and OPN, the markers for early stage of dentinogenesis, were significantly higher in the osteogenic induction media group than the basal media group (Fig 6-B). In particular, mRNA expression of OCN was significantly higher in MG-MSCs than in the other groups (45). The mRNA expression of DMP1 and DSPP, markers for late stage of dentinogenesis, were also significantly higher in the plate wells treated with induction media (Fig 6-B). Interestingly, we found that mRNA expression of DSPP, odontogenic specific marker, was significantly higher in MG-MSCs (45).

This data suggests that MG-MSCs have higher stemness and odontogenic differentiation capacity than other two cell lines, which were prepared with series of enzymatic digestion in *in vitro* environment.

**3.8. MG-MSCs exhibit the immunophenotypic profile of mesenchymal stem cells.**

Immunophenotypic pattern is also one of the important criteria of MSCs (4). To characterize the immunophenotype of MG-MSCs, we measured the expression level of cell surface marker by flow cytometry. Expression levels of CD44, CD73, CD90, CD105, CD146, STRO-1, well-known MSCs surface markers, and hematopoietic stem cells surface markers, CD34 and CD45, were measured (17). The expression pattern of MG-MSCs was not

significantly different from that of DPSCs and GMSCs which are well established (Fig 7-A, B). MSC surface markers showed high expression levels and hematopoietic stem cells surface markers, which should not be expressed in MSCs, were scarcely expressed in all three cell lines (Fig 7-A, B). This demonstrates that MG-MSCs have the same immunophenotypic profile as MSCs.

Taken together all the previous data, the cells that grow out from MG tissue can actually be defined as mesenchymal stem cells.

### 3.9. MG tissues are able to attach to porous poly-L-lactic acid scaffold in dentin slice and generate migrating MG-MSCs

Cells, scaffolds, and signaling molecules are three important factors for tissue engineering. Among the three components, scaffolds provide structural support for cell attachment and subsequent tissue regeneration (46). In the previous experiments on cell plates, we identified that MG tissues have sufficient MSCs population and the MG-MSCs have high osteogenic/odontogenic differentiation potential. For clinical trials, we validated that these characteristics are indeed reproducible in scaffolds.

Poly L-lactic acid (PLLA) is one of the most widely used scaffolds in biomedical field This has the advantages of biodegradability, biocompatibility, thermal plasticity and suitable mechanical properties (47). It was reported that PLLA scaffolds promoted dental pulp cell differentiation into odontoblasts (48). Dentin slice model is a well-established model for mimicking the pulp space of clinical application.

To test whether MG tissue can be a cell source in 3-D environment, three MG tissues were placed onto the PLLA in the pulp area of dentin slice and the dentin slice was cultured for 3

weeks. Migrating MG-MSCs from the MG tissues to PLLA were observed in the magnified microscope view (*white arrow*) (Fig 8-A). A portion of the MG tissue extending to attach to the PLLA was also found (*black arrow*) (Fig 8-A). For histological analysis, tissue slides embedded with paraffin were examined with H-E staining. Interestingly, there are plenty of extracellular matrix (ECM)-like structures with MG-MSCs (Fig 8-B). To visualize the presence of MG-MSCs more clearly, we confirmed it with DAPI staining and densely populated MG-MSCs were detected in the ECM-like structures.

This data suggests that MG tissues attach to PLLA successfully and generate enough outgrowing cells, MG-MSCs. Therefore, this suggests that MG tissues can be a clinically feasible cell source for pulp regeneration.

**3.10. Mineralization of the migrating MG-MSCs from MG tissues attaching on porous poly-L-lactic acid scaffold in dentin slice, and odontogenic marker DSP expression of the MG-MSCs in contact with dentin surface.**

To test whether MG-MSCs have sufficient osteogenic differentiation potential even after migrating to PLLA scaffold in dentin slices, we examined the osteogenic differentiation capacity of MG-MSCs in PLLA scaffold. After 3 weeks of osteogenic induction, ARS was performed. PLLA scaffold in the dentin slice without MG tissues was also tested with ARS as a negative control. Reddish areas and numerous red dots were observed around MG-MSCs in PLLA scaffold compared to control group (*arrow*) (Fig 9-A).

Furthermore, to investigate the efficacy of odontogenic differentiation of MG-MSCs grown in dentin slice model with PLLA, we examined the dentin sialoprotein (DSP) expression of MG-MSCs. The tissue slides embedded with paraffin were examined with immuno-

fluorescence staining. MG tissues were cultured on PLLA/dentin slice model for 3 weeks with basal media. MG-MSCs on the dentinal surface showed strong DSP expression, and the cells processes extending to the dentin matrix were confirmed with the expression of DSP (Fig 9-B).

Odontogenic differentiation of DPSCs adjacent to the dentinal surface was already reported with DSP expression (49). Because DSP is one of the accepted specific markers of odontoblast differentiation, the strong expression of DSP in MG-MSCs indicates their odontogenic differentiation (45).

This data suggests that MG tissues with PLLA scaffold can be a viable option for clinical trial of pulp regeneration.

### 3.11. Ability of MG-MSCs to attach to PuraMatrix™ scaffold on dentin slice and generate migratory cells with osteogenic capacity.

Although PLLA is well-established scaffold for tissue regeneration, Chloroform solution is used in order to solidify PLLA. Because this is a powerful anesthetic chemical, it requires almost one day for the extra chloroform to fully evaporate. In addition, close contacts between dentinal surface and cells is very important for odontogenesis as demonstrated in other studies (49, 66), however, the pre-solidified PLLA cannot create firm contacts between them.

PuraMatrix™ is an injectable self-assembled synthetic peptide hydrogel. The porocity (5-200 nm) and the nanofiber structure (thinner than the cell and less than 10 nm in diameter) of PuraMatrix™ allow cells to grow in the gel. Thus, the nano fibers of PuraMatrix™ surround the cells to provide an environment similar to the natural ECM. In addition, it is an injectable solution that enables close contact between the cell and the dentin surface. Moreover, PuraMatrix™ has no remaining growth factors, undefined constituents or non-quantified

substances since this is not an animal derived material (50). Taking these advantages into consideration, we tested PuraMatrix™ as a new scaffold option.

To investigate whether the MG tissue can attach well to the PuraMatrix™ and the cells can grow outward, MG tissues capsulized with PuraMatrix™ were cultured in basal media. After 14 days in basal media treatment, the cell migration pattern was examined with H-E staining. MG tissues were successfully attached to PuraMatrix™ and migrating cells from MG tissue to PuraMatrix™ were detected at the borderline between them. (*arrow*) (Fig 10-A). Similar to the PLLA/dentin slice model, we made a model in which the PuraMatrix™ was filled in the dentin slice to mimic the pulp space environment of clinical trial. The model with MG tissue capsulized in PuraMatrix™ was cultured with Basal media. After 14 days, we confirmed that MG-MSCs migrated from MG tissue filled almost all areas of the PuraMatrix™ (*arrow*) (Fig 10-B).

Next, osteogenic differentiation capacity of MG-MSCs in PuraMatrix™ was checked through ARS. After 3 weeks of osteogenic induction, the wells treated with induction media showed stronger red staining compared to the basal media treated wells, and mineralized deposits were found in the outlying area near the dentinal surface (Fig 10-B).

This Indicates that PuraMatrix™ can be an appropriate scaffold option for clinical trial of pulp regeneration with MG tissues.

## 4. Discussion

Since the detection of MSCs population in pulp tissue, DPSCs, pulp regeneration has been one of the most actively studied topics in the field of Dentistry. There are two main approaches to pulp regeneration: infusion of exogenous MSCs and mobilization of host MSCs

(51). A representative example of host MSCs mobilization is revascularization which is a method for pulp regeneration of immature teeth. However, most of the tissues generated after pulp revascularization have been histologically proven to be non-pulp like tissues consisting of cementum, periodontal, and bone-like tissues (29). Thus, the majority of pulp regeneration studies have been conducted on transplanting DPSCs with scaffolds, however, this cell-based approach has also limitations we described (38).

In our previous studies, we proposed a new method for regenerating the pulp-dentin complex, pulp tissue grafting, which may allow to bypass the limitations associated with cell preparation *in vitro* and overcome the constraints for clinical application (38). However, the new approach also cannot be free from the most fundamental question, “Where do you get pulp tissue for pulp regeneration?”

Therefore, we searched for a new tissue that would be a cell source to replace pulp tissue for pulp regeneration and considered three prerequisites to find the tissue.
First, obtaining the tissue should not be too invasive and should be easy. Second, the tissue should have MSCs population which have sufficient stemness. Third, migrated cells from the tissue should be capable of odontogenic differentiation.

Gingival tissue has easy accessibility among the oral tissues, and this tissue can be easily obtained from biological samples to be discarded (17). Also, Sufficient MSC characteristics of GMSCs have been well studied and reported (25). However, the characteristics of cells migrating out of the gingival tissue explant have not been studied precisely, unlike the pulp tissue explant.

Thus, in this study we identified the MSC characteristic of the cell from the MG tissue explant and named it MG-MSCs to distinguish it from the GMSCs. MG-MSCs showed higher differentiation capacity and proliferation compared to GMSCs. In addition, MG-MSCs showed a

much higher expression level in mRNA expression of embryonic stem cell marker (OCT 3/4, NANOG, SOX2) than GMSCs, which is consistent with previous pulp tissue explant studies (52).

There is a possible reason why MG-MSCs have stronger stemness than the two cell types isolated by enzymatic process. The tissue itself possibly maintains the immature state of MSCs, and the MSCs from tissues can be considered to sustain the cell's original abilities in the natural 3D microenvironment of tissue compared to the MSCs exposed to the stresses of the enzymatic process and the 2D culture environment *in vitro* (52-54). This is consistent with the high stemness of sphere MSCs in sphere assays replicating the 3D structure of the MSCs in the original microenvironment (55).

Surprisingly, in all experiments, GMSCs and MG-MSCs showed higher gene expression of stemness markers and both differentiation and migration capacity than DPSCs, which had been mainly used in the pulp regeneration study. This suggests that MSCs populations in gingiva are more immature, potent, and active than MSCs in pulp tissues, which can be associated with rapid healing of oral gingival wounds (56, 57). Although GMSCs' modulation of wound microenvironment has been reported, the superior abilities of MSCs in gingiva demonstrated in this study also can be considered to contribute to the rapid healing (58-61).

In this study, we also demonstrated the possibility of MG tissue as a cell source with two scaffolds *in vitro*, PLLA and Puramatrix$^{TM}$, for clinical trials.

PLLA had already been used in previous stem cell transplantation study *in vitro* for pulp regeneration. This study demonstrated successful growth and odontogenic differentiation of

DPSCs in PLLA (48). Our previous study has also shown that stem cells from pulp tissue differentiate into odontoblasts on PLLA (38).

Puramatrix™ is a scaffold more suitable for clinical application than PLLA. Puramatrix™ is injectable, can self-assemble to create intimate contact with the dentin surface, and does not require additional preparation time. Many studies using stem cells and Puramatrix™ have shown the differentiation of stem cells into odontoblasts and regeneration of pulp-like tissue (62-64).

In the *in vitro* studies using these two scaffolds, we first confirmed that MG-MSCs successfully grew out from MG tissue and proliferated on the scaffold. Second, we confirmed the osteogenic differentiation potential of the proliferated MG-MSCs on the scaffold. Lastly, we demonstrated that the MG-MSCs differentiate into odontoblasts at the dentin surface.

Osteogenic differentiation capacity of stem cells is more important for pulp regeneration than the other types of differentiation capacity because of the similarity between odontoblast and osteoblast. Dentin and bone produced by odontoblasts and osteoblasts are very similar in composition, and the protein expressions in the process of producing the mineralized matrix are also very similar, such as OCN, OPN, DMP-1 (44, 65). The high osteogenic differentiation capacity of MG-MSCs identified in this study supports the high ability of MG-MSCs to differentiate into odontoblasts. In addition, the differentiation of MG-MSCs contacting with dentin surface into odontoblasts in the experiment with PLLA is consistent with the study of DPSCs, and it suggests that the reason is the signaling molecules released in the dentin matrix have a potential effect that leads to odontogenic differentiation (49, 66).

In conclusion, we have demonstrated that MG tissue can be a sufficient cell source for the pulp regeneration. This study characterizes and proves the MSC features of MG-MSCs which migrate from MG tissue, and demonstrates the sufficient potential in clinical trials for pulp regeneration of MG tissue transplantation by confirming the differentiation of MG-MSCs into odontoblasts in the dentin slice model. Through this study, we present a new approach for pulp regeneration “gingival tissue grafting” that extends beyond “pulp tissue grafting”, which could only be used when viable pulp tissues remained, such as irreversible pulpitis and partial necrosis, to complete pulp necrosis. Based on the evidences studied in this study, it will be necessary to identify the regeneration of dentin-pulp complexes *in vivo* and furthermore, and to establish detailed protocol for clinical trial.

## 5. Figure Legends and Figures

**Figure 1. Exhibition of plastic adherent property and mesenchymal stem cell morphology of outgrowing MSCs from minced gingiva (MG-MSCs).**

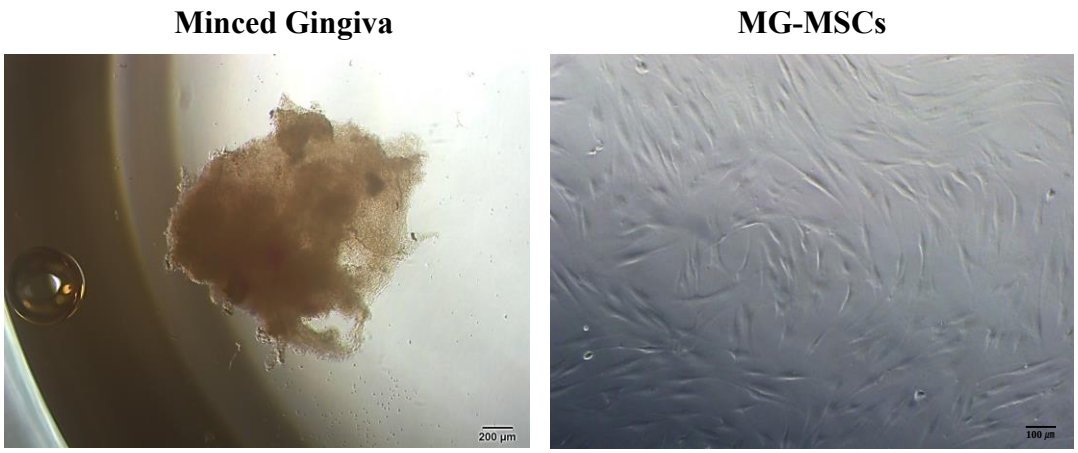
(A) Morphology of minced gingival tissue at Day 0 and MG-MSCs at passage 2.

(B) Phase-contrast view of MG-MSCs that grow out from minced gingival tissue after 5 days and 8 days of explantation.

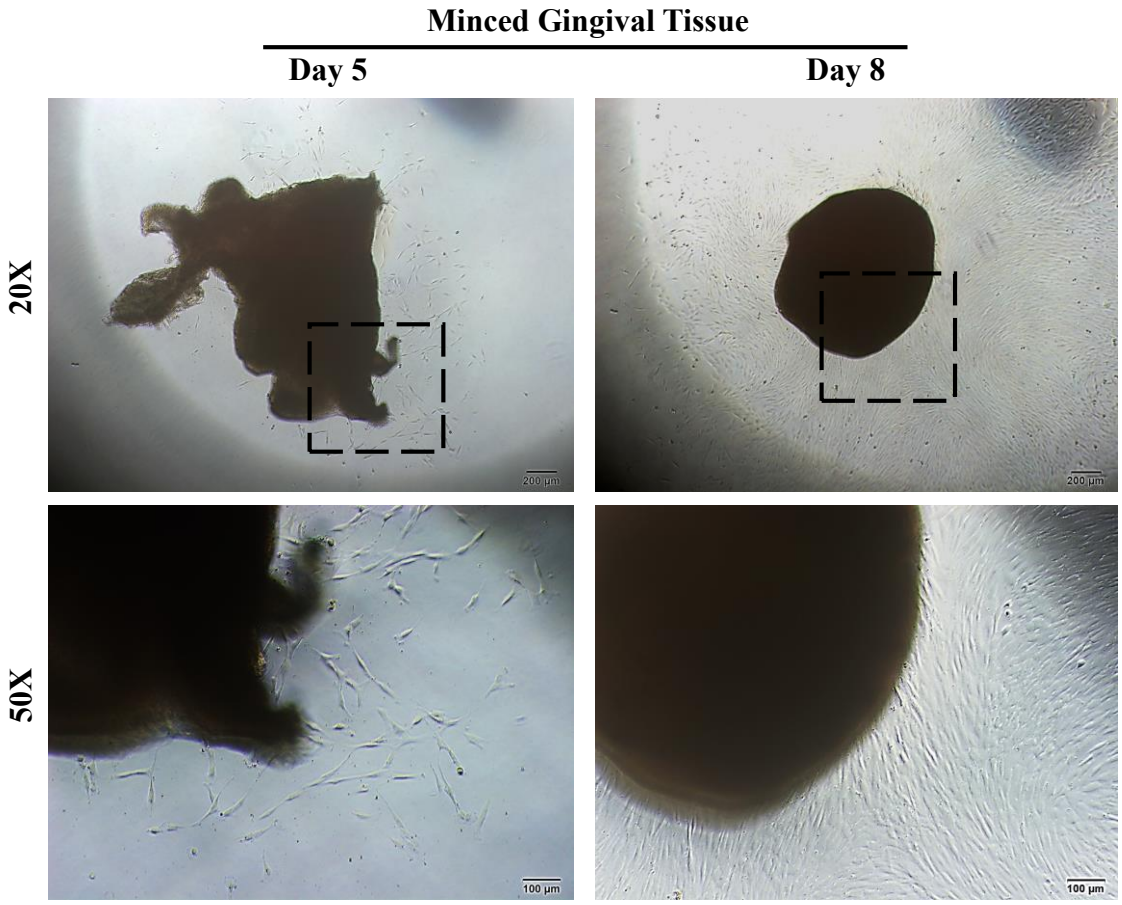
(C) MG tissues were histologically examined with hematoxylin-eosin staining before and after 15 days of explantation. MG-MSCs. Many cells are observed in the tissue part, which is supposed to be attached to the plate (*arrows*).

(D) DAPI (4’,6-diamidino-2-phenylindole) staining of the tissue section to visualize the cellularity inside the MG. A tendency of cells to go out of tissue is observed (*arrows*).

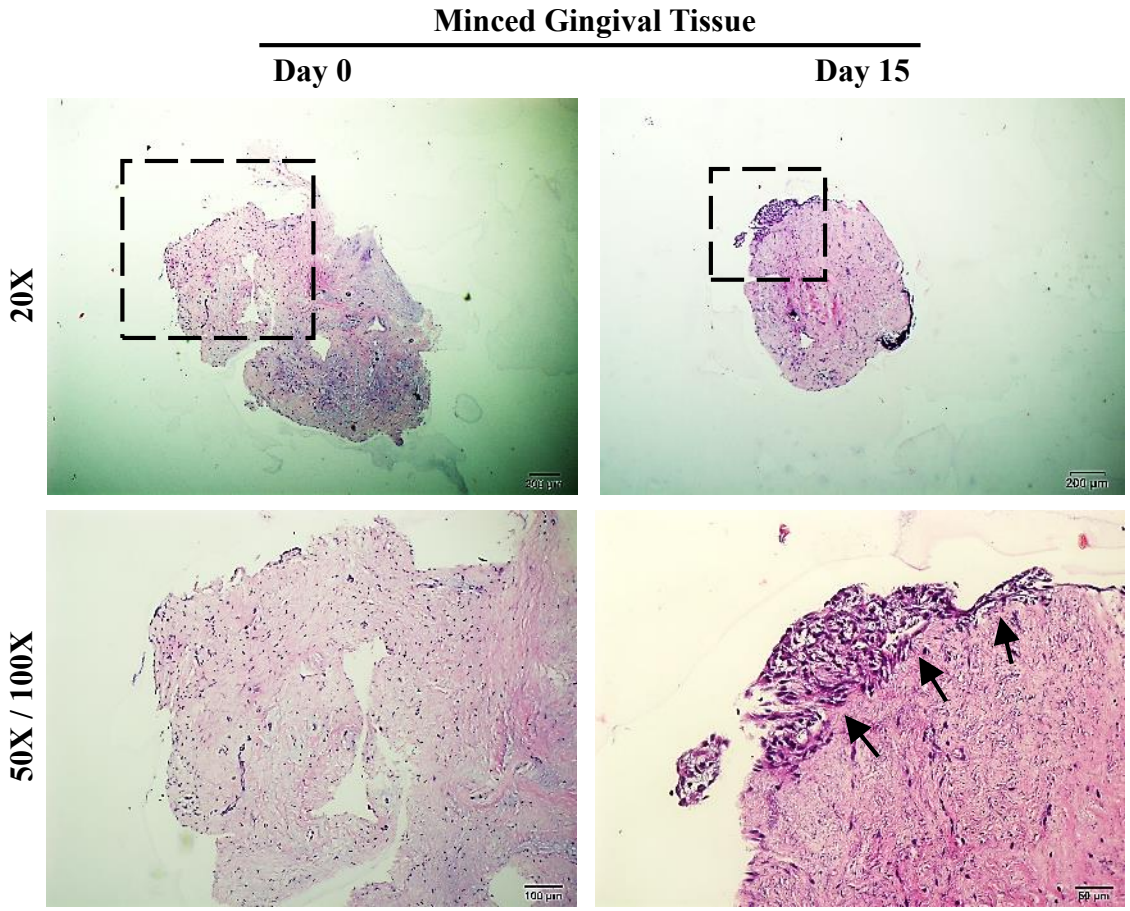
**A**


Minced Gingiva
MG-MSCs
200 µm
100 µm

**B**


Minced Gingival Tissue
Day 5
Day 8
20X
50X
200 µm
200 µm
100 µm
100 µm

C




D

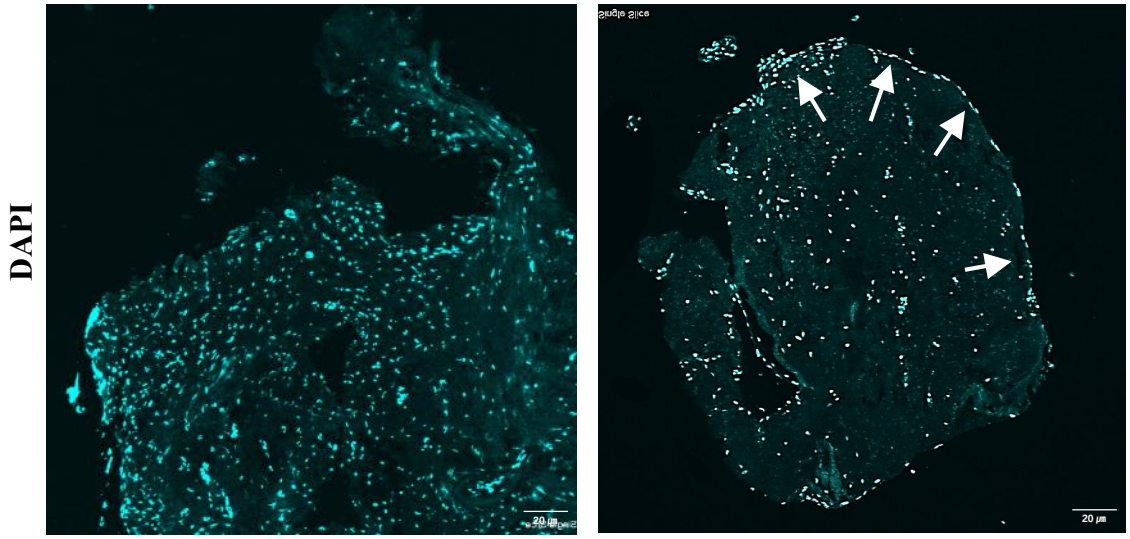


**Figure 2. Distinct replicative phenotype of MG-MSCs from GMSCs**

(A) The population doublings (PDs) were measured to examine the replication kinetics of MG-MSCs at each culture day and compared to GMSCs.

(B) The percentage of Senescence-Associated β-Galactosidase (SA-β-Gal) positive cells of MG-MSCs was quantified under microscope view at different PD levels and compared to GMSCs. * $p<0.05$, Data are expressed as the means ± SD of 5 determinations.

(C) The SA-β-Gal staining of MG-MSCs and GMSCs at different PD levels.

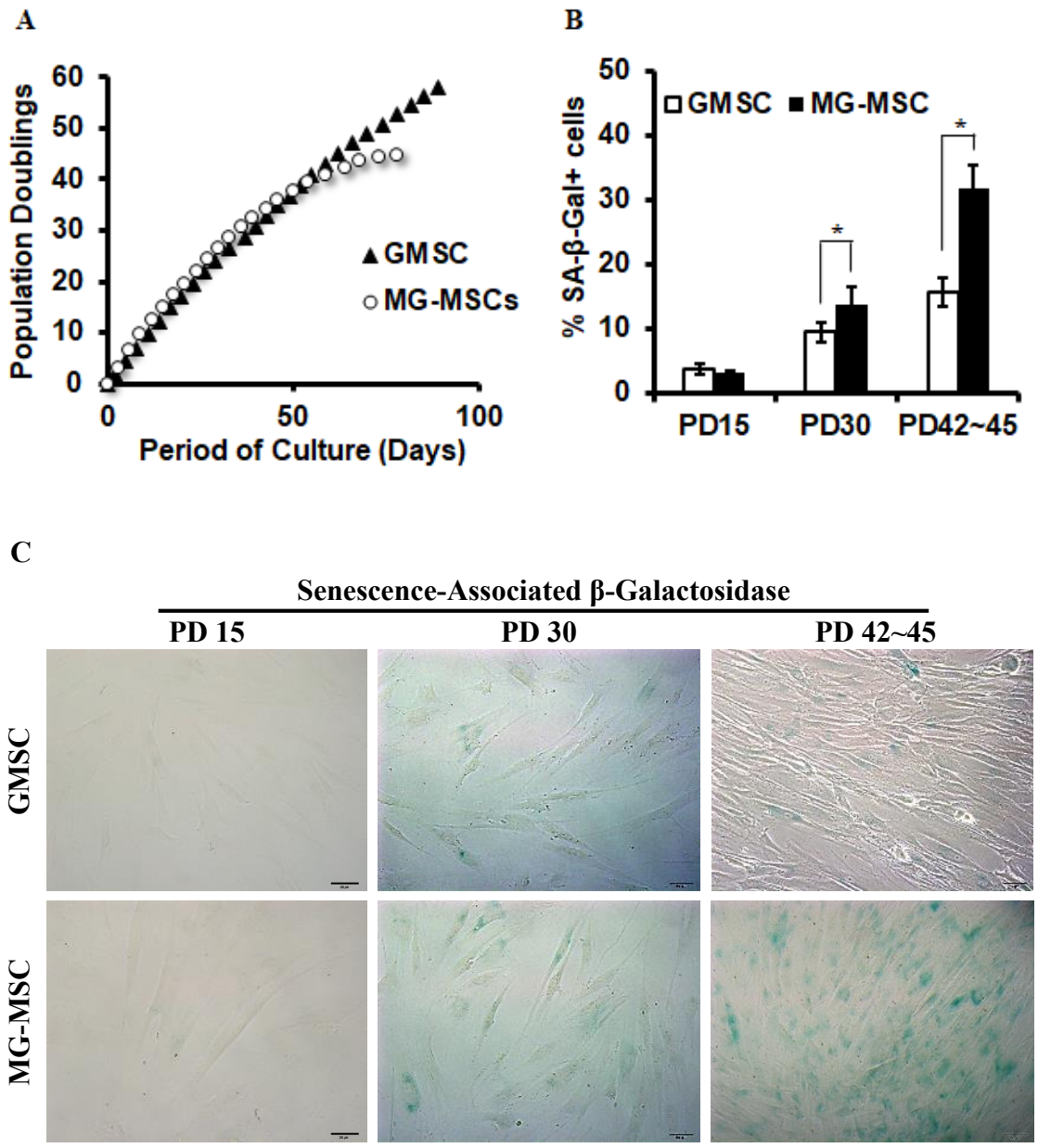

A
Population Doublings
60
50
40
30
20
10
0
0
50
100
Period of Culture (Days)
GMSC
MG-MSCs
B
% SA-β-Gal+ cells
50
40
30
20
10
0
GMSC
MG-MSC
*
*
PD15
PD30
PD42~45
C
Senescence-Associated β-Galactosidase
PD 15
PD 30
PD 42~45
GMSC
MG-MSC

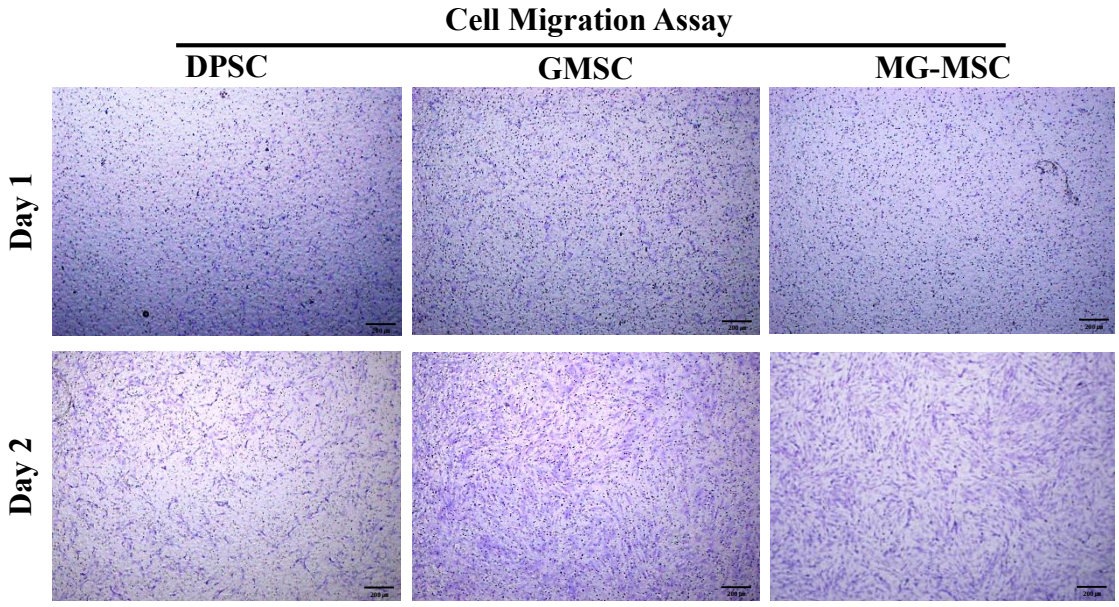
**Figure 3.**

**Migration capacity of MG-MSCs compared to DPSCs and GMSCs.**

(A) The migration capacities of MG-MSCs, DPSCs and GMSCs were measured by transwell migration assay after 1 day and 2 days of cell seeding.

(B) The migrating cell number of DPSCs, GMSCs, and MG-MSCs were counted under microscope view. * $p<0.05$, Data are expressed as the means ± SD of 5 determinations.

A


Cell Migration Assay
DPSC
GMSC
MG-MSC
Day 1
Day 2

B

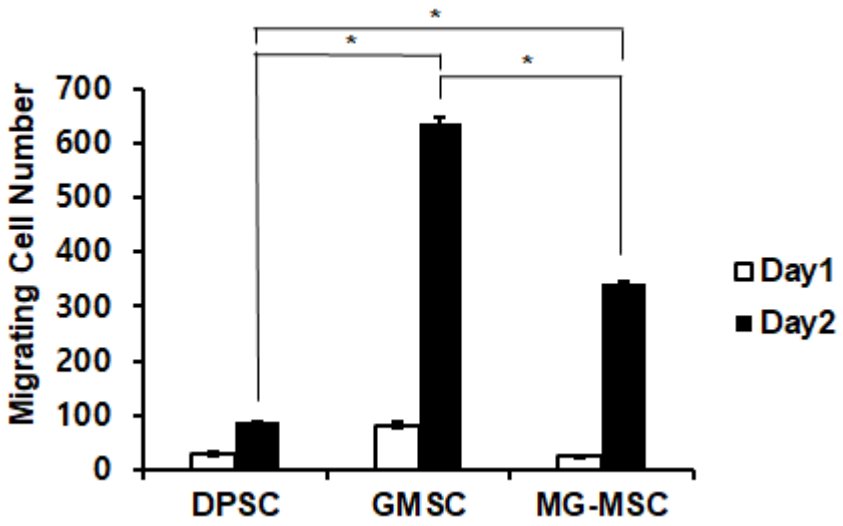

Migrating Cell Number
700
600
500
400
300
200
100
0
DPSC
GMSC
MG-MSC
Day1
Day2
*
*
*

**Figure 4. Osteogenic/odontogenic differentiation capacity of MG-MSCs compared to DPSCs and GMSCs.**

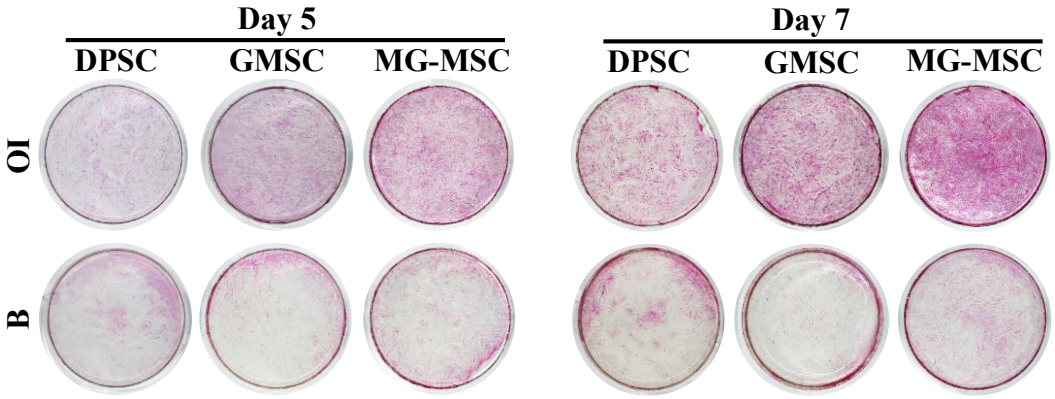
(A) Alkaline phosphatase (ALP) staining after treatment of osteogenic induction media or basal media for 5 days or 7 days. Each media was changed every two days at the same day.

(B) ALP absorbance was measured by spectrophotometer with 405nm wave length. * $p<0.05$, Data are expressed as the means ± SD of 3 determinations.

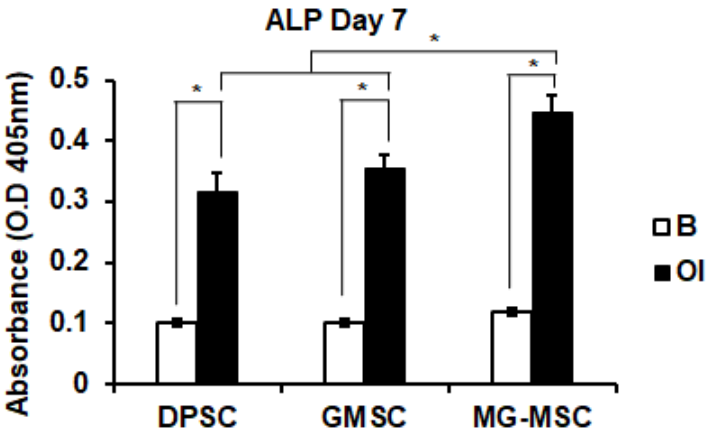
(C) Alizarin red staining after treatment of osteogenic induction media or basal media for 14 days or 21 days. Each media was changed every three days at the same day. Dense mineralization areas with black surface were observed.

(D) Magnified microscope view of dense mineralization area of MG-MSCs.

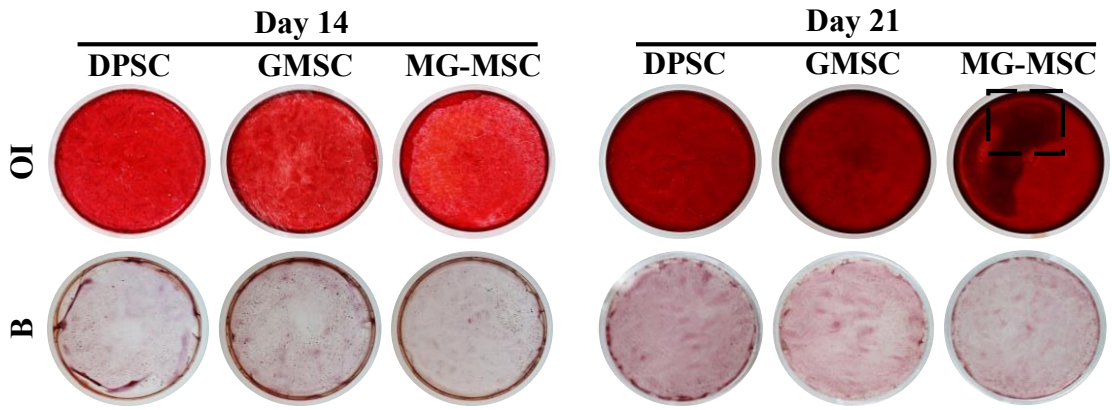
**A**


Day 5
Day 7
DPSC
GMSC
MG-MSC
DPSC
GMSC
MG-MSC
OI
B

**B**


ALP Day 7
Absorbance (O.D 405nm)
0.5
0.4
0.3
0.2
0.1
0
DPSC
GMSC
MG-MSC
B
OI
*
*
*
*

C


Day 14
Day 21
DPSC
GMSC
MG-MSC
DPSC
GMSC
MG-MSC
OI
B

D

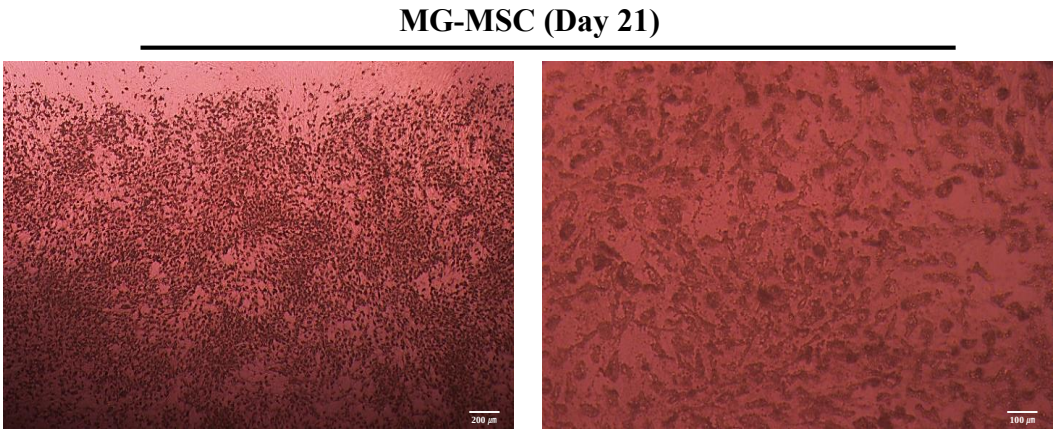

MG-MSC (Day 21)
200 µm
100 µm
20x
50x

**Figure 5. Adipogenic differentiation capacity of MG-MSCs compared to DPSCs and GMSCs.**

Lipid droplets of each cell lines were stained by Oil Red O staining 21 days after treatment of adipogenic induction media or basal media. The 100X magnification images on upper panel were taken after the plates were dried. The 200X magnification images on lower panel were taken immediately after staining. Each media was changed every three days at the same day.

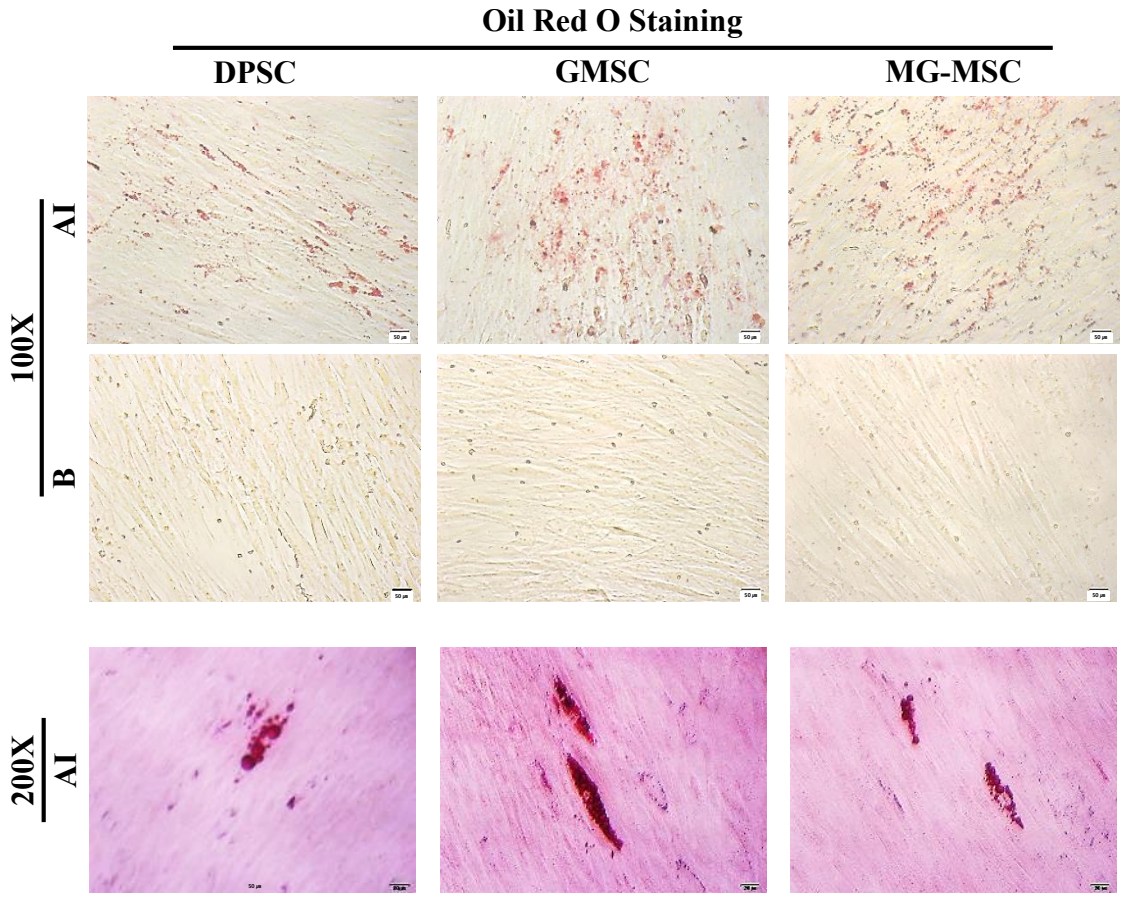

Oil Red O Staining
DPSC
GMSC
MG-MSC
100X
AI
B
200X
AI

**Figure 6. mRNA expression patterns of genes associated with stemness, osteogenic differentiation and odontogenic differentiation.**

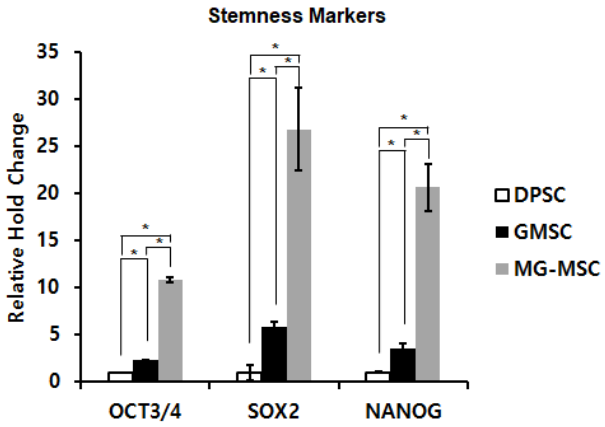
(A) Expressions of stemness-related genes; Oct3/4, SOX2, NANOG at cell passage 1. Stemness-related gene expression of MG-MSCs was significantly higher than that of other cell lines.

(B) Expressions of osteogenic/odontogenic genes; OCN (Osteocalcin), OPN (Osteopontin), DMP-1 (Dentin matrix acidic phosphoprotein-1) and odontogenic specific gene; DSPP (Dentin sialophosphoprotein) after treatment of osteogenic induction media or basal media for 21 days. mRNA expressions of all genes of three cell lines were significantly increased in osteogenic condition compared to control. Odontogenic-specific gene DSPP expression of MG-MSCs was significantly higher than that of other cell lines.

The experiments were repeated three times, and one representative data is presented. The measured values are cq values, and the relative hold change of each cell line is plotted with the value of DPSC as 1

* $p<0.05$, Data are expressed as the means ± SD of 3 determinations

**A**


Stemness Markers
Relative Hold Change
35
30
25
20
15
10
5
0
OCT3/4
SOX2
NANOG
DPSC
GMSC
MG-MSC

**B**

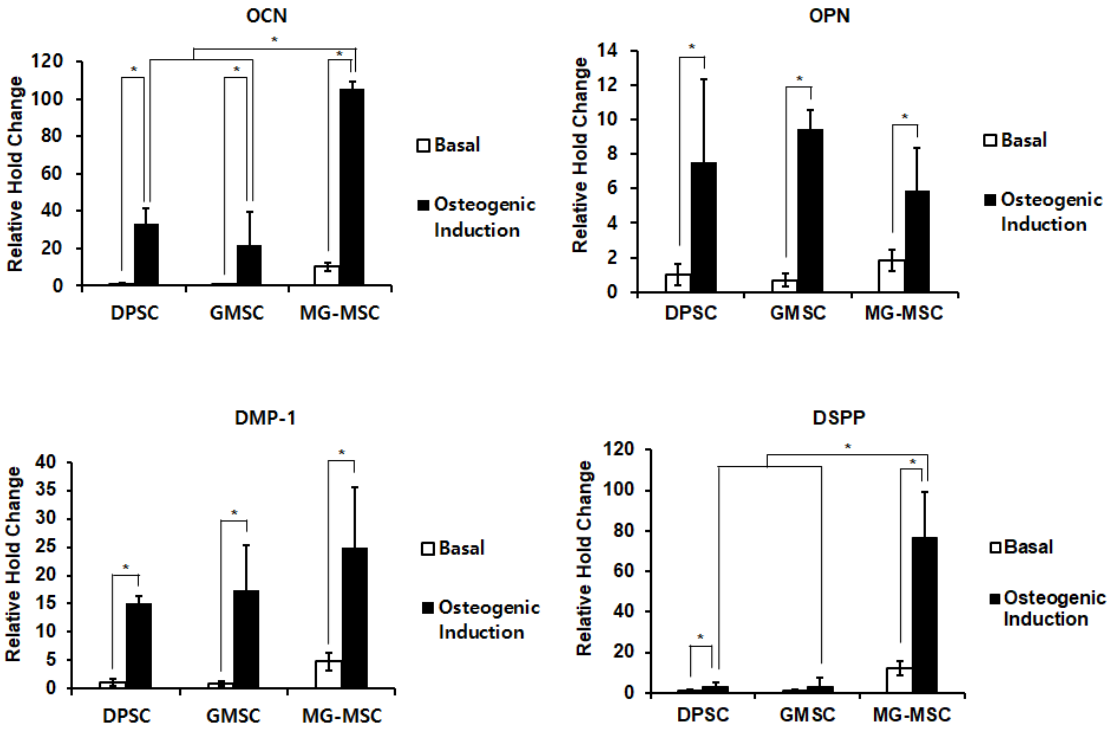

OCN
Relative Hold Change
120
100
80
60
40
20
0
DPSC
GMSC
MG-MSC
Basal
Osteogenic Induction
OPN
Relative Hold Change
14
12
10
8
6
4
2
0
DPSC
GMSC
MG-MSC
Basal
Osteogenic Induction
DMP-1
Relative Hold Change
40
35
30
25
20
15
10
5
0
DPSC
GMSC
MG-MSC
Basal
Osteogenic Induction
DSPP
Relative Hold Change
120
100
80
60
40
20
0
DPSC
GMSC
MG-MSC
Basal
Osteogenic Induction

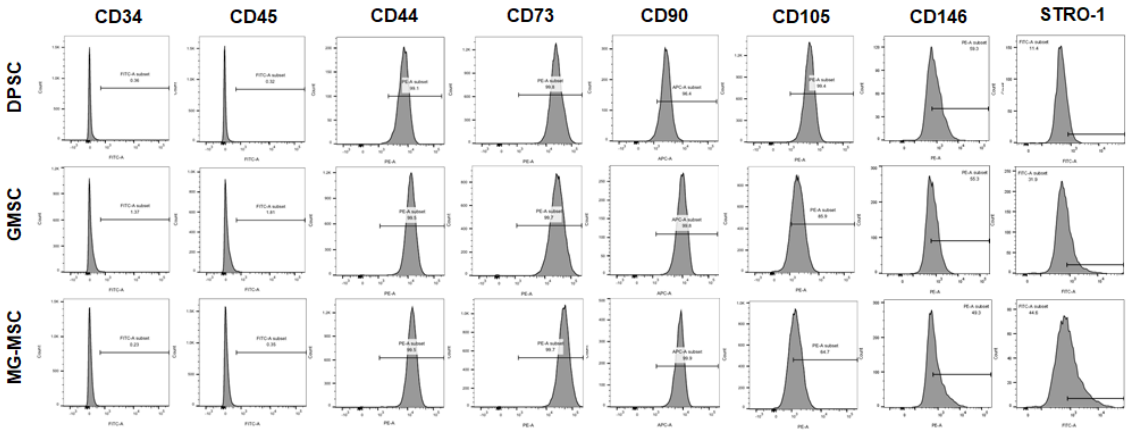
**Figure 7. MG-MSCs exhibit the immunophenotypic profile of mesenchymal stem cells.**

Flow cytometry analysis was performed to measure the expression of cell surface markers of MG-MSCs. DPSCs, well-established MSC, were used as positive control. A typical MSC marker pattern of MG-MSCs was confirmed. The expression levels of hematopoietic cell markers, CD34 and CD45, were expressed very low in all cell lines.

(A) Graph pattern of each cell surface marker.

(B) A graph to compare the expression level of surface markers of each cell line.

**A**


CD34
CD45
CD44
CD73
CD90
CD105
CD146
STRO-1
DPSC
GMSC
MG-MSC

**B**

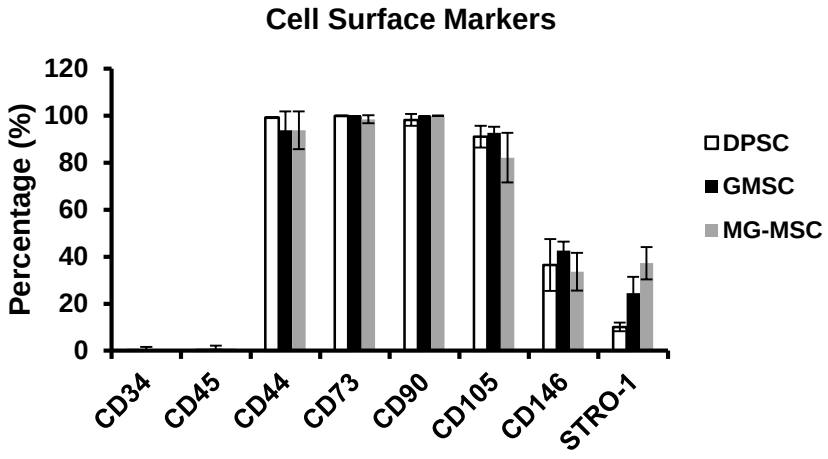
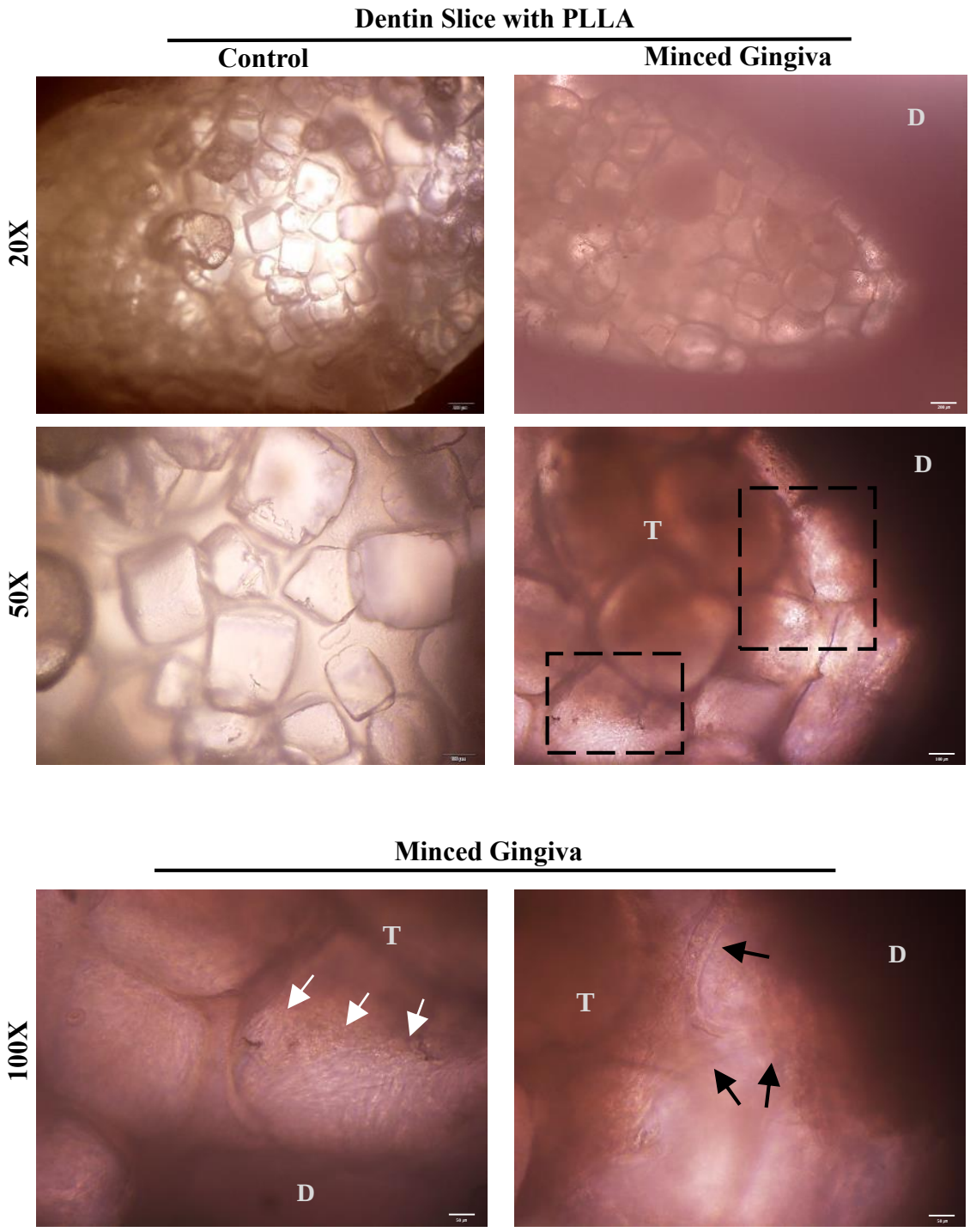

Cell Surface Markers
Percentage (%)
120
100
80
60
40
20
0
CD34
CD45
CD44
CD73
CD90
CD105
CD146
STRO-1
DPSC
GMSC
MG-MSC

**Figure 8. MG tissues are able to attach to porous poly-L-lactic acid scaffold in dentin slice and generate migrating MG-MSCs.**

(A) Phase-contrast view of MG tissues on porous PLLA scaffold in dentin slice. Three MG tissues were placed on the surface of porous poly-L-lactic acid scaffold. It appeared that the MG tissues were stretching for attachment (*Black arrow*s). Outgrwoing cells from MG tissue were confirmed (*White arrows*). T: MG tissue, D: Dentin slices

(B) ECM-like structures were observed with hematoxylin-eosin staining and densely populated MG-MSCs were detected by immuno-fluorescence staining (DAPI; 4',6-diamidino-2-phenylindole). D: Dentin slices

A


Dentin Slice with PLLA
Control
Minced Gingiva
20X
50X
D
T
Minced Gingiva
100X
T
D
T
D

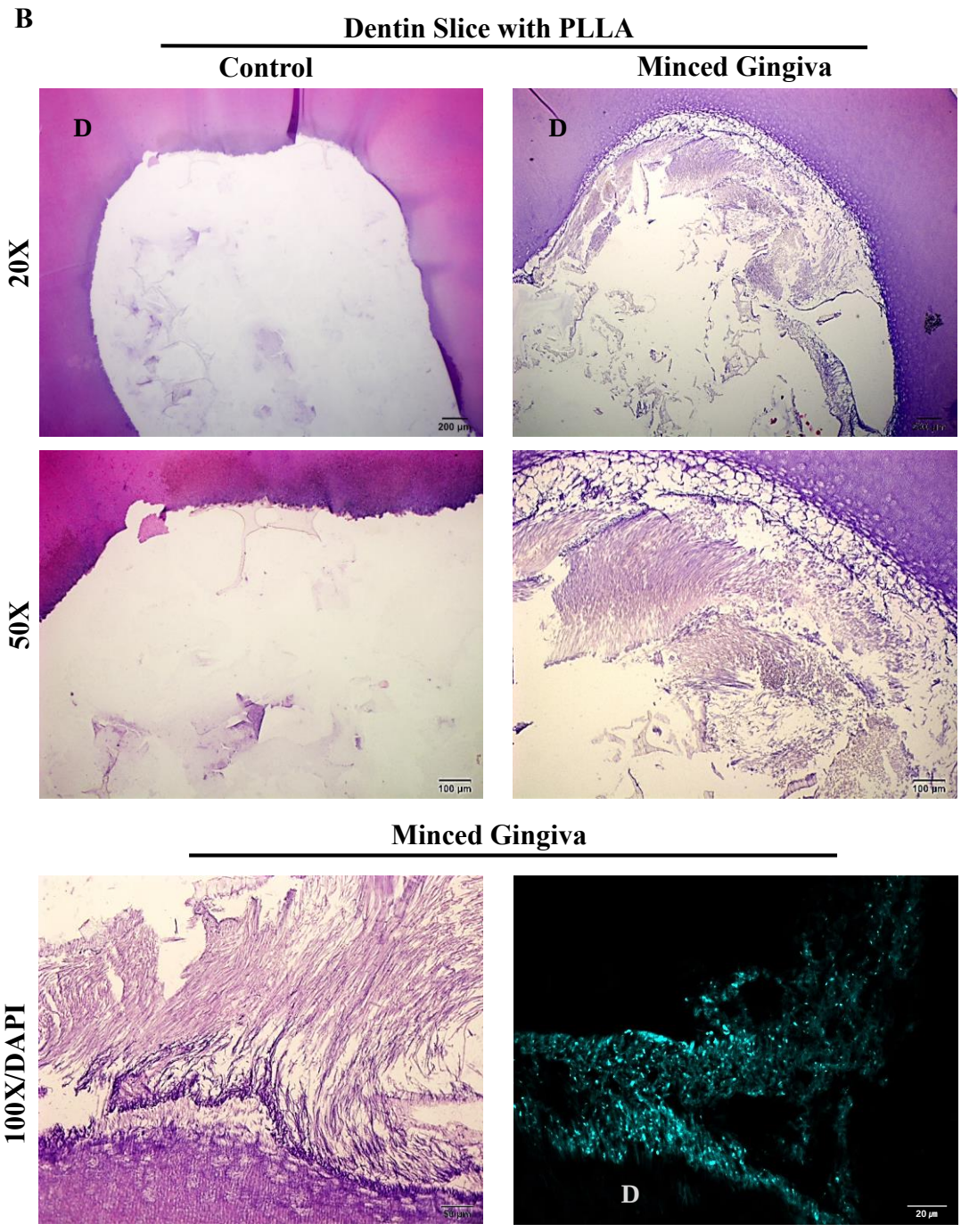
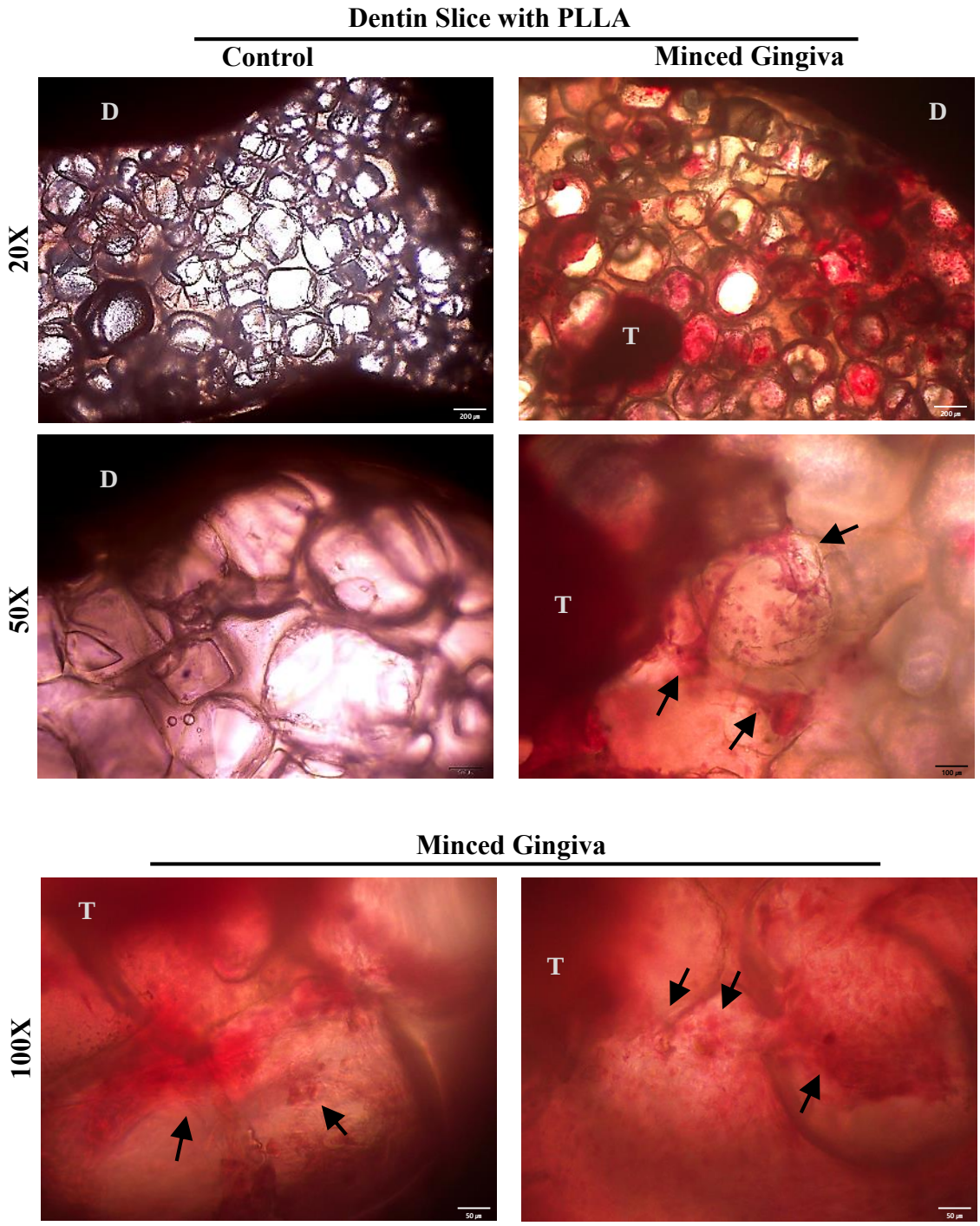

B
Dentin Slice with PLLA
Control
Minced Gingiva
20X
50X
D
D
200 µm
100 µm
100 µm
Minced Gingiva
100X/DAPI
D
20 µm

**Figure 9. Mineralization of the migrating MG-MSCs from MG tissues attaching on porous poly-L-lactic acid scaffold in dentin slice, and odontogenic marker DSP expression of the MG-MSCs in contact with dentin surface.**

(A) Alizarin red staining of MG-MSCs in porous poly-L-lactic acid scaffold in dentin slice which grow out from MG tissues. The staining was performed 21 days after treatment of osteogenic induction media. Mineralized deposits produced from MG-MSCs were stained as red (*arrows*). T: MG tissue, D: Dentin slices

(B) Immuno-fluorescence staining of DSP in the MG-MSCs in contact with dentin surface. The staining was performed 21 days after treatment of osteogenic induction media. In addition to the expression of DSP around the MG-MSCs, the cell processes extending to the dentin matrix were confirmed with the expression of DSP.

A


Dentin Slice with PLLA
Control
Minced Gingiva
20X
50X
D
T
Minced Gingiva
100X

**B**

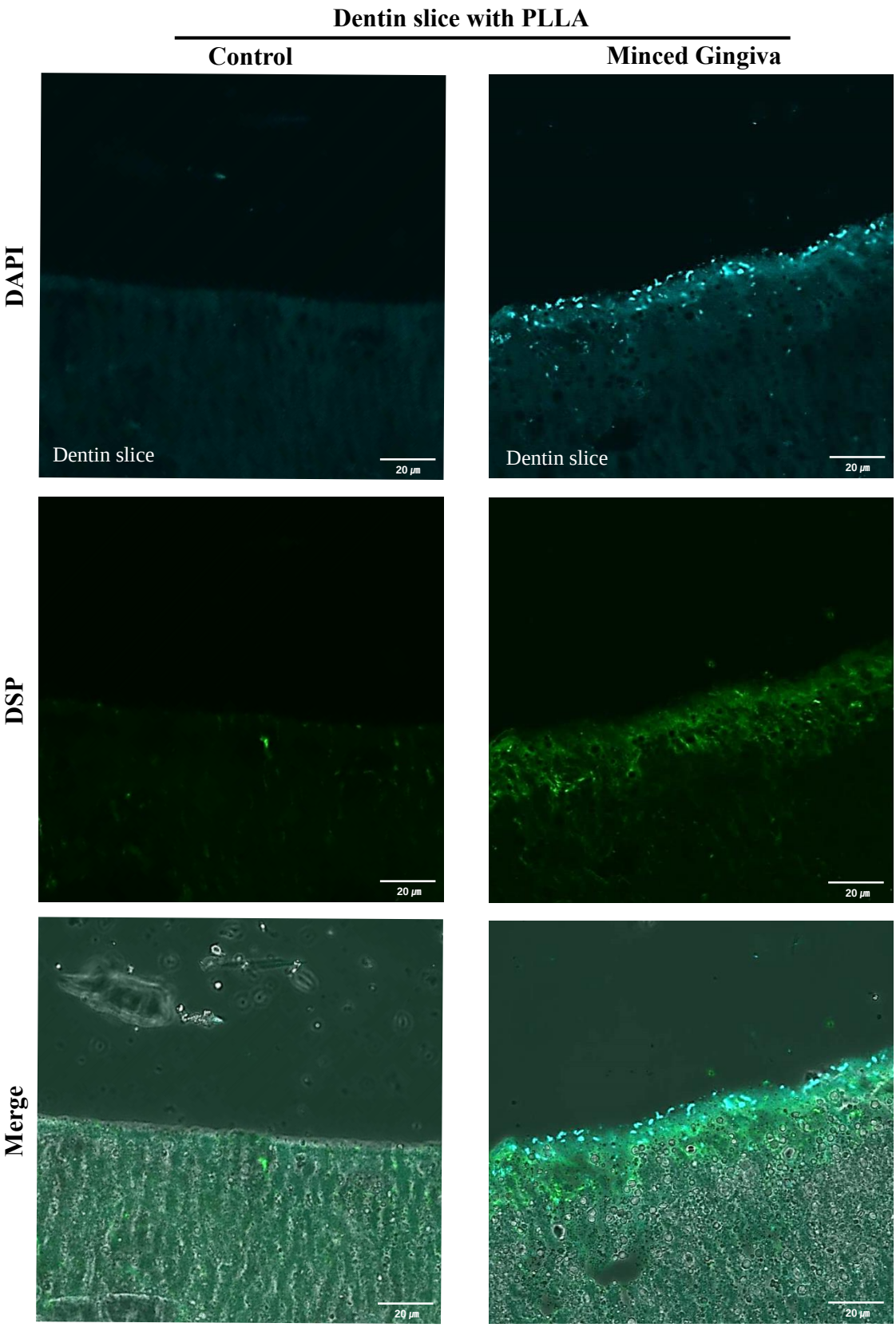

Dentin slice with PLLA
Control
Minced Gingiva
DAPI
DSP
Merge
Dentin slice
Dentin slice
20 μm

**Figure 10. Ability of MG-MSCs to attach to PuraMatrix™ scaffold in dentin slice and proliferate with osteogenic capacity.**

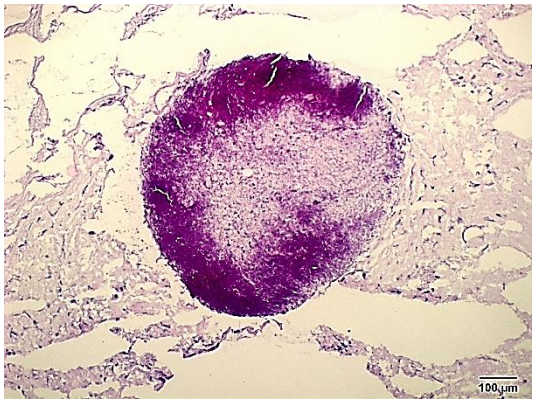
(A) MG tissues and PuraMatrix™ scaffold were frozen sectioned and histologically examined with hematoxylin-eosin staining 14 days after culture. MG-MSCs growing out from MG tissues to PuraMatrix™ were observed at the border (*arrows*).

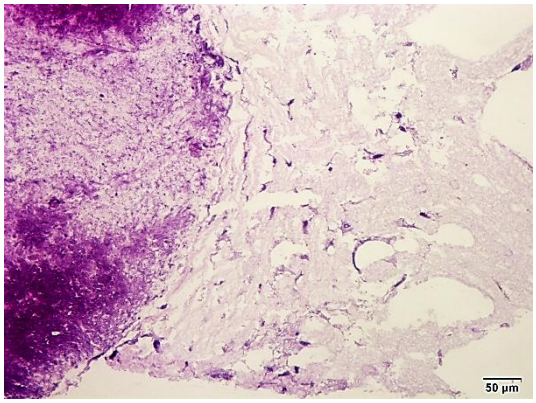
(B) Alrizarin red staining was performed to confirm mineralization of MG-MSCs migrating from MG tissues to PuraMatrix™ scaffold in dentin slices. This staining was performed 21 days after the treatment of osteogenic induction media. Mineralized deposits from MG-MSCs in PuraMatrix™ were stained as red dots (*arrows*). T: MG tissue D: Dentin slices

**A**

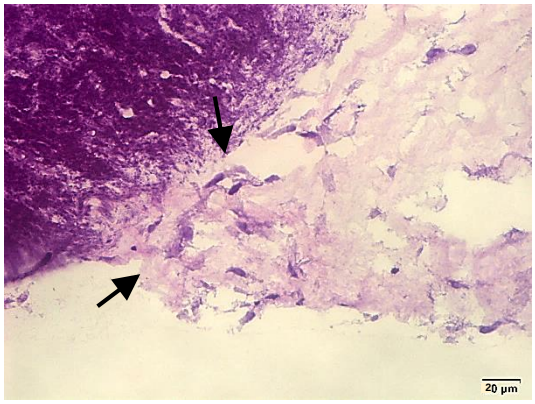
**PuraMatrix™ with minced gingiva**

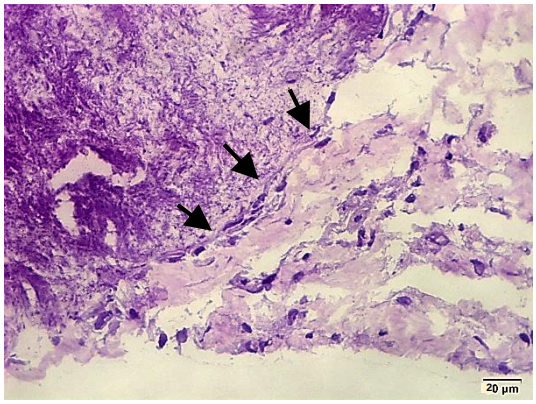
**50X / 100X**



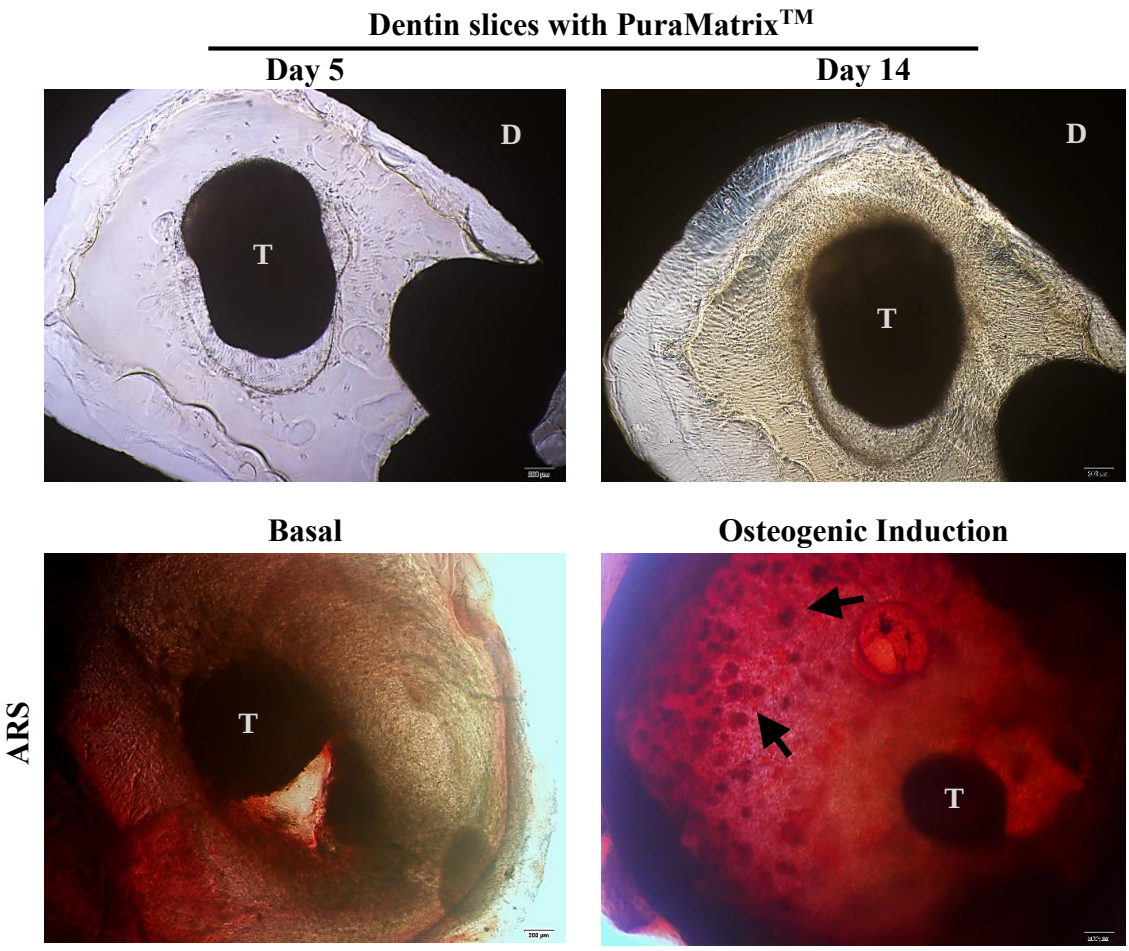




**200X**



**B**


Dentin slices with PuraMatrix™
Day 5
Day 14
D
T
D
T
Basal
Osteogenic Induction
ARS
T
T

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