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The Characterization of Dental Mesenchymal Stem Cells from Human Dental Pulp and Periodontal Ligament Based on Cell Surface Markers

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The Characterization of Dental Mesenchymal Stem Cells from Human Dental Pulp and Periodontal Ligament Based on Cell Surface Markers

A dissertation submitted in partial satisfaction for the requirements for the degree Doctor of Philosophy in Oral Biology

by

Ruth Alvarez

2016

ABSTRACT OF DISSERTATION

The Characterization of Dental Mesenchymal Stem Cells from Human Dental Pulp and Periodontal Ligament Based on Cell Surface Markers

by

Ruth Alvarez

Doctor of Philosophy in Oral biology

University of California, Los Angeles, 2016

Professor Cun Yu Wang, Chair

Mesenchymal stem cells (MSCs) are a promising tool in regenerative medicine due to their capacity to differentiate into multiple lineages. In addition to MSCs isolated from bone marrow (BMSCs), adult MSCs are isolated from craniofacial tissues including dental pulp (DP) and periodontal ligament (PDL) tissues using various stem cell surface markers. However, there has been a lack of consensus on a set of surface makers that are reproducibly effective at isolating putative multipotent dental mesenchymal stem cells (DMSCs). In this study, we used different combinations of surface markers (CD51/CD140α, CD271, and STRO-1/CD146) to isolate homogeneous populations of DMSCs from heterogeneous dental pulp cells (DPCs) and periodontal ligament cells (PDLCs) and compared their capacity to undergo multilineage differentiation. Fluorescence-Activated Cell Sorting (FACS) revealed that 27.3% of DPCs were

CD51+/CD140α+, 10.6% were CD271+, and 0.3% were STRO-1+/CD146+; whereas 24% of PDLCs were CD51+/CD140α+, 0.8% were CD271+, and 2.4% were STRO-1+/CD146+. Sorted cell populations were further assessed for their multipotent properties by inducing odonto/osteogenic, chondrogenic, and adipogenic differentiation. All three subsets of isolated DMSCs exhibited differentiation capacity into odonto/osteogenic and chondrogenic lineages but with varying degrees. CD271+ DMSCs demonstrated the greatest odonto/osteogenic potential with strong induction of odonto/osteogenic and periodontal markers such as DLX5, RUNX2, BGLAP, DMP1, DSPP, and PLAP-1. Based on these results, the role of CD271 in odontogenic differentiation was further evaluated. CD271 is one of the receptors which Nerve Growth Factor (NGF) exerts its biological effects through. Although NGF is known for its role in the the development and maintenance of the nervous system, it's capability to promote odontogenic differentiation has made it an interesting candidate for its role as a mineralizing agent in regenerative therapies. We found that exogenous treatment of NGF further increased odonotogenic potential of CD271+ DMSCs from DP with induction of odontogenic markers DLX5, RUNX2, and BGLAP, and increased MAPK, AKT, MTOR, and NF-κB signaling pathways in a time dependent manner. Furthermore, knockdown of tropomyosin trkA tyrosine kinase receptor ($trkA^{NGFR}$) and low affinity nerve growth factor receptor ($p75^{NTR}$/CD271/LNGFR) decreased odontogenic differentiation of CD271+ DMSCs. Our study provides important insights into the use of DMSCs and growth factors for regenerative therapies in dentistry, and improves our understanding of the molecular mechanisms involved in odontogenic differentiation

The dissertation of Ruth Alvarez is approved.

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To those who have believed in me,

Thank you.

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1. **Alvarez R**, Lee HL, Hong C, Wang CY; Single CD271 marker isolates mesenchymal stem cells from human dental pulp, IJOS 2015
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1 INTRODUCTION

1.1 Specific Aims

Dental caries and periodontal disease are the most common oral diseases affecting Americans today.[1] 85% of adults aged 18 and older are affected with dental caries, while 47.2% of Americans suffer from mild, moderate or severe periodontitis.[1] This increases to 70.1% for those 65 and older with periodontal disease.[1] Both diseases can lead to infections, bone and tooth loss, and a lower quality of life. Current therapies for dental and periodontal disease focus on managing the disease, use of bio-inert materials for replacement of lost tissues, debridement, antimicrobial treatments, surgery, or consequently extractions. A promising approach to restoring tissue structure and function is the use of tissue engineering. The activation of signal transduction pathways conducive to tissue regeneration would be achieved by identifying and understanding; cells, signals, and biomaterials: the triad of tissue engineering. The discovery of dental stem cells is offering the possibility of using a patient's own stem cells for regenerative therapies.[2] This is of great interest to dentistry since it may provide an innovative generation of clinical material and/or tissue regeneration.[2] Specifically, mesenchymal stem cells (MSCs) have been found in dental tissues: dental pulp, periodontal ligament, apical papilla, and the dental follicle.[2] These dental mesenchymal stem cells (DMSCs) can be isolated and grown under defined conditions and can be used for tissue engineering, including uses for: dental tissue, nerves, and bone regeneration.[2] DMSCs are characterized by the positive expression of stromal associated markers and lack expression of hematopoietic antigens.[3] The identification of novel surface markers that consistently isolate putative DMSCs and growth factors that can lead to the development of therapeutics for regenerative medicine and dentistry is of great importance.

Currently, numerous cell surface markers including STRO-1, CD29, CD44, CD73, CD90, CD105, CD106, and CD146 have been utilized to isolate homogenous and multipotent MSC populations.[4,5] However, a consensus on surface markers to isolate DMSCs with high differentiation potential is lacking. In this study we investigated the use of CD51/CD140α, CD271, and STRO-1/CD146 for the isolation of DMSCs. All three surface marker combinations have been used for the identification of BMSCs, yet CD51/CD140α and CD271 have not been used in the isolation of DMSCs. [6,7,8] Although our data demonstrates that all combinations were able to identify DMSCs from dental pulp (DP) and periodontal ligament (PDL), CD271 was sufficient to isolate a strong odonto/osteogenic population. Based on these findings we decided to investigate the role of CD271 in odontogenic differentiation of DMSCs by further assessing the effects of Nerve growth factor (NGF) on CD271+ DMSCs from DP. It is well documented that NGF plays an important role in the survival and differentiation of neurons, but there is now evidence that it plays important roles in tooth development and might modulate the proliferation and differentiation of developing epithelial and mesenchymal cells.[9] We found that exogenous treatment of NGF on CD271+ DMSCs increased odontogenic activity and activated MAPK, AKT, MTOR and NF-κB signaling pathways. Furthermore, knockdown of CD271 significantly decreased odontogenic potential of DMSCs. Although the identification DMSCs are important for the development of regenerative therapies in dentistry, the identification of signals such as NGF are essential for understanding signaling pathways during odotongenic differentiation and consequently tissue regeneration.

In the current study we hypothesized that CD271 is a sufficient marker for the isolation of a multipotent population of DMSCs from DP and PDL, and exogenous NGF treatment leads to increased odontogenic potential of DMSCs through activation of signaling pathways important for cell survival and differentiation. Furthermore, CD271 is crucial for odontogenic differentiation

and for NGF signaling pathways in DMSCs. Our hypothesis will be addressed in the following specific aims

Specific Aim 1: Investigate multilineage capacity of purified CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs from DP and PDL *in-vitro*.

DMSCs were purified by fluorescent activated cell sorting (FACS) from human DP and PDL. For *in-vitro* differentiation assays, sorted cells were induced into odonto/osteogenic, chondrogenic, and adipogenic lineage cells. Differentiation potential was determined by alkaline phosphatase (ALP) assay, Alizarin Red Staining (ARS), Alcian Blue Staining, Oil Red O staining, and mRNA levels of various lineage specific genes.

Specific Aim 2: Investigate effect of NGF on odontogenic differentiation of CD271+ DMSCs from human DP *in-vitro.*

FACS purified CD271+ DMSCs from human DP were induced into odontogenic lineages supplemented with NGF *in-vitro*, odontogenic potential was determined by ALP assay, ARS, and mRNA levels of specific odontogenic marker genes. In order to investigate the molecular signaling involved with NGF in CD271+ DMSCs protein expression of select candidates were investigated by western blot. Furthermore, $TrkA^{NGFR}$ and $P75^{NTR}$ siRNA were used to investigate their role in odontogenic differentiation, and in NGF signaling of DMSCs.

1.2 Mesenchymal Stem Cells

1.2.1 Definition of Mesenchymal Stem Cells

Stem cells have the ability to renew themselves and to differentiate into various specialized tissues, making them an attractive treatment modality in regenerative medicine.[10] Embryonic Stem Cells (ESCs) isolated from embryos have provided a powerful tool for biological research, and are considered the most promising cells for cell based therapy.[10] However, due to ethical concerns of isolating and using ESCs, great interest has focused on adult mesenchymal stem cells (MSCs), which are also characterized by their self-renewal, multipotent capacity, and lack of teratoma formation.[10,11,12] A subset of non-hematopoeitic adult stem cells, MSCs originate from the mesoderm and have the ability to differentiate not only into the mesoderm lineages: chondrocytes, osteocytes, and adipocytes, but also into ectodermic and endodermic cells. [10] MSCs exist in almost all tissues, and can be easily isolated from bone marrow, adipose tissue, umbilical cord, fetal liver, muscle, and lung and successfully expanded *in-vitro*. [10] MSCs are believed to be responsible for growth, wound healing, and replacement of cells that are lost through daily wear and tear as well as pathological conditions, suggesting that they are important for tissue repair and maintenance.[10] Although MSCs have multilineage potential, they also have the ability to secrete a broad spectrum of bioactive macromolecules that are both immunoregulatory and serve to structure regenerative microenviornments in fields of tissue injury. [10,11]

1.2.2 Definition of Dental Mesenchymal Stem Cells

While MSCs derived from bone marrow (BMSCs) are the most widely recognized and studied, alternate sources for MSCs have been investigated due to the complications associated with harvesting BMSCs, such as pain, morbidity, and low cell number. Evidence has suggested that MSCs may be present in virtually any vascularized tissue in the body.[13,14] In

recent years, MSCs derived from craniofacial tissues including dental mesenchymal stem cells (DMSCs) have been identified as a putative alternative.[2] Similar to BMSCs, these DMSCs are multipotent progenitor cells with: (1) the capability to self-renew and differentiate into a variety of cell types (2) ease of isolation, and (3) lack of immunogenicity.[15] When compared to BMSCs, DMSCs may be more advantageous for regenerating craniofacial defects because they are readily available without risk as DMSCs can be easily isolated from discarded tissues such as third molars.[16] Approximately 70% of dental patients have third molars that require extractions consequently providing an abundant source of DMSCs that can be cryopreserved for potential future use.[17] Currently DMSCs can be obtained from dental pulp tissues (DP), exfoliated deciduous teeth, periodontal ligament (PDL), apical papilla, dental follicle, and gingiva stem cells.[3,4]

1.3 Dental Pulp

1.3.1 Definition of Dental Pulp

The tooth is mainly composed of mineralized dentin; surrounded by enamel in the crown of the tooth and cementum in the roots.[5] DP is the only soft tissue in the tooth and it maintains its own homeostasis and that of dentin.[5] Characterized by a heterogeneous cell reservoir, DP consists of odontoblasts and interstitial fibroblasts located on its blood vessels and nerve endings.[5] DP originates from the dental papilla, which originates from cranial neural crest cells.[5,18] The function of dental pulp cells (DPCs) are to regenerate dentin and provide it with oxygen, nutrition and innervation, whereas the hard dentin can protect soft DP tissue. Together they maintain the integrity of tooth shape and function.[2]

1.3.2 Dental Mesenchymal Stem Cells Isolated from Dental Pulp

DMSCs isolated from DP have a fibroblastic morphology with the ability to adhere to solid surfaces, good proliferative potential and capacity to differentiate *in-vitro*, and the ability to repair tissues *in-vivo*.[3] DMSCs can differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, neurons, and hepatocyte lineages *in-vitro.* [3] Their differentiation capacity is determined by the components of the local microenvironment, such as growth factors, receptor molecules, signaling molecules, transcription factors and extracellular matrix protein.[2] They are characterized by the positive expression of markers such as CD90, CD29, CD73, CD105, CD44; negative expression of CD45, CD34, CD14. [3] In addition, they also express STRO-1, collagen, vimentin, laminin, and fibronectin. [3] In addition, some of the pluripotent stem cell markers, such as Oct4, Nanog, Sox2, Klf4, SSEA4, & c-myc have been reported to express on DPSCs. [3]

1.3.3 Potential Biological Uses of Dental Mesenchymal Stem Cells from Dental Pulp

Dentin matrix protein 1 (DMP1) a non-collagen extracellular matrix protein extract from dentin, can significantly promote odontoblastic differentiation of DMSCs and formation of reparative dentin over the exposed pulp tissue. [2] Transforming growth factor β1 (TGFβ1) alone or in combination with fibroblast growth factor (FGF2) have been shown to induce DMSCs into the odontoblast lineage. [2] DMSCs express bone markers such as bone sialoprotein, osteocalcin, alkaline phosphatase, and type 1 collagen, indicating their differentiation commitment to bone tissues. [2] *In-vivo* transplantation of DMSCs into immunocompromised mice demonstrated that ability to generate functional tissue in the form of dentin/pulp complexes. [2] This provides a new alternative for the regeneration of pulp tissue for endodontic diseases. Interestingly, DMSCs express neural markers and differentiate into functionally active neurons suggesting their potential as cell therapy for neuronal disorders. [2]

1.4 Periodontal Ligament

1.4.1 Definition of Periodontal Ligament

The PDL is a dynamic and specialized connective tissue derived from the dental follicle that originates from neural crest cells.[19,20] PDL tissues contain a heterogeneous population of cells, including fibroblasts, epithelial cells, endothelial cells, cementoblasts, osteoblasts, and neural cells.[21] Embedded between the cementum and the inner wall of the alveolar bone socket, the PDL's primary functions are to anchor the teeth to the alveolar bone and to provide them with protection against mechanical loads generated by mastication.[22] In addition to mechanical support, the PDL has many critical biological functions including providing tooth nutrition and regenerating periodontal tissues damaged by inflammatory periodontal disease or mechanical trauma.[22]

The role of the PDL is especially important in repair after periodontal disease, which can have acute, chronic, or systemic manifestations, ultimately leading to destruction of periodontal tissue, progressive alveolar bone loss, and eventual tooth loss.[23,24,25,26,27] This periodontal regeneration is challenging due to the complexity of the PDL attachment apparatus requiring finely orchestrated formation of new cementum, bone, and PDL fibers followed by the insertion of these fibers into the bone and cementum.[28]

1.4.2 Dental Mesenchymal Stem Cells from Periodontal Ligament

Putative periodontal mesenchymal progenitor cells that present properties similar to BMSCs have been characterized from parental PDL cells (PDLCs).[4,19,29,30,31,32,33,34] These cells were shown to differentiate into various distinct cell types, such as osteoblasts, fibroblasts, chondrocytes, cementoblasts, adipocytes, and neural like cells.[4,19,29,30,31,32,33,34] They express MSC surface markers such as STRO-1, CD146, STRO-3, CD13, CD29, CD44, CD90, CD105,

CD106, and CD166.[31,35,36,37] In addition, progenitor cells from the PDL express higher levels of scleraxis than MSCs from other tissues including bone marrow and dental pulp, making them a unique population of MSCs.[31]

1.4.3 Potential Biological Uses of Dental Mesenchymal Stem Cells from Periodontal Ligament

DMSCs selectively isolated from the PDL with high odonto/osteogenic potential are therefore expected to be the best-suited source of progenitor cells for regenerative periodontal therapy.[38,39] Additional uses of DMSCs from the PDL to improve clinical outcomes in dentistry include regeneration of PDL on the root surface of extracted or avulsed teeth and on titanium implants.[40,41,42]

1.5 Isolation of Mesenchymal Stem Cells

1.5.1 Isolation of Mesenchymal Stem Cells with Surface Antigens

MSCs are a heterogeneous population of cells with varying magnitudes of differentiation potential among single clones of MSCs.[43] It was documented that *in-vitro* single cell cloning of human MSCs demonstrated that approximately 30% of the clonal cells were multipotent and thus “true” MSCs.[43] Currently, there is no consensus on a single surface molecule to identify MSCs from various sources. The minimum criteria of MSCs include: (a) remain plastic-adherent under standard culture conditions; (b) express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14, or CD11b, CD79a or CD19, and HLA-DR; (c) differentiate into osteoblasts, adipocytes, and chondrocytes *in-vitro*. Other surface antigens generally expressed by MSCs and used for the isolation of homogenous and multipotent populations include CD13, CD29, CD44, and CD10, CD105, CD106, CD146, STRO-1, Oct4, Nanog, and Nestin [4,5,14]

1.5.2 Sole Markers versus Stem Cell Markers

Currently, the markers proposed for MSCs fall into two categories: sole markers and stemness markers. A sole marker is an alternative MSCs selection tool to plastic adherence, which alone is sufficient to identify or purify MSC like cells from their *in-vivo* environment. A stemness marker is able to identify a subset of MSCs with high CFU-Fs and trilineage potential or even identify ESC-like population. [14] Based on these two different types of markers, the sole markers are normally highly expressed, while the stemness markers may be moderately detected. [14] The majority of MSC markers are identified in BMSCs, whether these markers can be applied to other sources is unclear. [14] Consequently, a consensus on surface markers to isolate MSCs from various sources with high differentiation potential is lacking. As the future of successful craniofacial defect repair is dependent on the ability to isolate specific subsets of DMSCs with potent differentiation capacity into appropriate cell types, the identification of markers that isolate multipotent DMSCs effectively is critical.

1.5.3 Surface marker combinations CD51/CD140α, CD271, STRO-1/CD146

Recent studies showed that surface marker combinations, CD51/CD140α and CD271/CD90/CD106, isolate highly enriched clonogenic cells from human bone marrow.[6,7] Intermediate filament protein Nestin labels population of stem/progenitor cells, including self-renewing MSCs, which is a major constituent of the HSC niche. Pinho *et al*, identified that combination CD51/CD140α characterized a large population of Nestin+ cells, containing most fibroblastic CFUs, mesenspheres, and self-renewal capacity after transplantation.[6] CD51+/CD140α+ subset of Nestin+ cells were also enriched in major HSC maintenance genes, supporting the notion that niche activity co-segregates with MSC activity.[6]

Mabuchi *et al*, found that the use of CD271/CD90/CD106 allowed for the selection of highly enriched clonogenic cells.[7] Their group found that CD271+/CD90+ cells demonstrated cellular heterogeneity among the clones.[7] In addition, rapidly expanding clones exhibited robust multilineage differentiation and self-renewal potency, and uniquely also expressed CD106; leading to the conclusion that CD271/CD90/CD106 can be used selectively to isolate the most potent and genetically stable MSCs.[7]

Previously, STRO-1 and CD146 have been used for the isolation of MSCs. STRO-1 is a cell membrane single pass type 1 protein that translocates from the endoplasmic reticulum to the cell membrane in response to the depletion of intracellular calcium.[14] Although STRO-1 is not universally expressed in all MSCs, it has been used for the isolation of DMSCs.[14] While, CD146 is a key cell adhesion protein in vascular endothelial cell activity and angiogenesis.[14] Interestingly the expression of CD146 is consistently found not only in BMSCs but in all other sources of MSCs.[14] STRO-1/CD146 combination has previously been used to obtain DMSCs from PDL.[32] No previous attempts have been made to isolate DMSCs from DPCs or PDLCs using CD51/CD140α and CD271.

1.6 Growth Factors

1.6.1 Definition of Growth Factors

Growth factors are poplypeptides or proteins that bind to specific receptors on the surface of target cells.[44] These can initiate a cascade of intracellular signaling and my act in an autocrine or paracrine manner.[44] Cytokines are sometimes used interchangeably with growth factors because they share similar actions with growth factors. Compared with hormones which have systemic effects, growth factors and cytokines typically act on locally target cells.[44]

1.6.2 Growth factors and Regeneration of Dental Pulp-like Tissues

It has been documented that regeneration of dental pulp like tissues occur *in-vivo* with the delivery of dental and non-dental stem cells. [44] Yet an alternative approach would be to combine the use of these cells with growth factors, which are important to the homing of host endogenous cells. Growth factors which have been implicated in dental pulp regeneration are: Platelet-Derived Growth Factor (PDGF), transforming growth factor-β (TGF-β), Bone Morphogenic Protein (BMP), Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Insulin-like Growth Factor (IGF), NGF, Stromal Cell-Derived Factor-1 (SDF-1). [44] The use of growth factors in dentistry offer the possibility of regenerating dental-pulp dentin like tissues by cell homing and with direct delivery, an important advancement towards clinical translational science. It is important to fully understand the actions of these growth factors, and their how their signaling pathways are involved in the regeneration of dental tissues. [44]

1.6.3 Nerve Growth Factor

NGF is the first discovered member of the neurotrophin family. [45] It is well known for its ability to stimulate growth, differentiation, survival, and maintenance of peripheral sensory and sympathetic neurons during development and after injury.[46,47] Like other neurotrophins, NGF is produced in the nervous system by accessory cells, yet it has also been found to be produced and utilized by several non-nervous cell types, including immune inflammatory cells, epithelial cells, keratocytes, and smooth muscle cells, indicating that NGF may have roles outside the nerouvs system.[46,48,49,50] Because of its activities outside the nervous system, NGF is considered a pleiotropic factor.

1.6.4 Nerve Growth Factor Signaling

NGF exerts its effect through two receptors: tropomyosin trkA tyrosine kinase receptor (termed p140trkA^{NGFR} or simply trkA^{NGFR}) and the p75 pan-neurotrophin receptor (named p75^{NTR}).[46,51,52,53] P75^{NTR} binds all neurotrophins with equal affinity (1nm kd), trkA^{NGFR} is very specific in its binding (10pm Kd). In the presence of of trkA^{NGFR}, p75^{NTR} can participate in the formation of high affinity binding sites resulting in enhanced NGF responsiveness, as well as selective binding, in which duration and magnitude depend on the ratio of p75^{NTR} and trkA^{NGFR} on the cell surface.[46,54,55] trkA^{NGFR} is a transmembrane glycoprotein belonging to the family of receptor typrosine kinase, called trks. The cascade include the MAPK-RAS-ERK pathway, phospholipase Cy1, P3I kinase and SNT proteins. [46,54,55] Interestingly the activation of the Smad signal transduction pathway, a typical pathway used by members of the TGFB family.[46,56] Most NGF biological activates are due to ligand-dependent trkA^{NGFR} autophosphorylation and subsequent activation of several signal transduction cascades. Whereas the role of p75^{NTR} is more complex and clearly defined. It is a type1 transmembrane protein whose extracellular domain contains four repeated modules of six cysteines, a typical structure of the TNFR superfamily (FAS/TNF receptor superfamily), and devoid of intrinsic catalytic activity. P75^{NTR} signaling involves activation of NF-kB and the phosphorylation of the transcription factor c-Jun kinase (JNK), as well as increased ceramide, leading to gene transcription or programmed cell death.[46,51,53]

1.6.5 Role of Nerve Growth Factor on Odontogenic Differentiation

Although it has been extensively studied for its physiological role in neurobiology, NGF's importance in non-neural cells has broadened its therapeutic potential.[45,58] Previously, NGF was found to increase proliferation and differentiation of DP cells during mineralization, and promote

differentiation of odontoblast-like cells of rat apical papilla cells.[59,60] The expression of NGF and p75NTR increases in dental pulp cells at the injury site.[44,61] Also, during development NGFs play a role in regulating tooth morphogenesis and tooth innervation in rat tooth development.[44,62] These findings offer the possibility of using NGF for pulpal regeneration, in order to stimulate reparative dentin formation.[59] Yet the molecular mechanisms whereby NGF activates odontogenic differentiation need to be to elucidated in order to understand its therapeutic potential.

1.6.6 Signaling pathways implicated in odontogenic differentiation

Identifying signaling pathways in odontogenic differentiation is important for the development of regenerative therapies in dentistry. Interestingly, downstream signaling implicated in the differentiation of odontoblasts is not well defined. Previous studies have found that dentin phosphoryn (DPP), an extracellular matrix protein synthesized by odontoblasts, activates AKT and MTOR signaling which further leads to the activation of NF-κB. von Kossa staining and Alizarin red staining indicated that pre-odontoblasts had terminally differentiated to odontoblasts with DPP. [63] Wu *et al*., also found that Dentin Matrix phosphorotein 1 (DMP1) which is expressed in bone and dentin, activates downstream effectors of the MAPK pathways, namely ERK and JNK, after DMP1 treatment.[64] These studies provide a basis for understanding the molecular mechanisms occurring during the odotogenic differentiation of DMSCs, and the possible candidates by which NGF is activing as a mineralizing agent.

2 MATERIALS AND METHODS

2.1 Cell Isolation and Culture

Primary dental pulp cells (DPCs) and periodontal ligament cells (PDLCs) were isolated from the DP and PDL of extracted adult third molars (IRB#13-000241-CR-00001) as previously described.[34] DPCs and PDLCs were cultured in alpha modified Eagle's medium (α-MEM) (Invitrogen, Carlsbad, CA, USA) containing 20% FBS, non-essential amino acids, 100 units/mL penicillin, and 100 units/mL streptomycin, in a humidified 5% CO2 incubator at 37°C (all reagents were from Invitrogen, Carlsbad, CA, USA). Media was changed every 2 days, and cells were passaged at 80–90% confluency. DMSCs used in this study were from passages 4–8.

2.2 Fluorescent Activated Cell Sorting (FACS)

Expression of stem cell surface markers in DPCs and PDLCs was determined by FACS analysis. The cells were detached using trypsin in 0.25% EDTA. After neutralization, single-cell suspensions were washed with phosphate-buffered saline (PBS) supplemented with 2% FBS and 0.01% NaN_3 (FACS buffer). Quantities of 1×10^6 cells were incubated with direct conjugated antibodies for 20 minutes on ice in the dark. After washing, fluorescence intensity was measured on FACS Aria II cell sorter (BD Biosciences, USA). The following anti-human antibodies were used: Phycoerythrin (PE)- CD271 (Miltenyi Biotec, Auburn, CA), Fluorescein Isothiocyanate (FITC)-CD90 (Biolegend Inc, San Diego, CA, USA), allophycocyanin (APC)-CD106 (Biolegend Inc, San Diego, CA, USA) or dual color combinations APC- STRO-1/PE-CD146 (Both from: Biolegend Inc, San Diego, CA, USA), and PE-CD51 (Biolegend Inc, San

Diego, CA, USA) /APC-CD140α (BD Biosciences, USA). PE-IgG was used as a negative control.

2.3 Cell Proliferation Assay

Sorted DMSCs were plated at 5 × 10^3 cells per cm^2 in 96 well plates in α- MEM with 10% FBS. Medium was refreshed every 2 days. On days 1, 3, 5, and 7,cells were incubated for 4 h with 10μl of 5mg/mL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) (Promega, Madison, WI). Cells were washed with PBS and followed with the addition of 200μl DMSO and gentle shaking for 10 min so that the complete dissolution was achieved. Absorbance was recorded at 570nm using the microplate reader.

2.4 Induction of Odonto/Osteogenic Differentiation

Sorted DMSCs were plated at 1×10^5 cells/well into 12-well plates. To induce odonto/osteogenic differentiation into odonto/osteogenic lineages, sorted DMSCs were grown in odonto/osteogenic induction medium (OIM). OIM contained α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 50 μg/mL ascorbic acid, 5 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma-Aldrich, St Louis, MO, USA). For experiments involving human recombinant β-nerve growth factor (NGF) (R&D Systems, Minneapolis, MN) 2ng/ml and 10ng/ml was supplemented into media. OIM was changed every 2-3 days. For ALP staining, after odonto/osteogenic induction for 7 days, cells were fixed with 4% paraformaldehyde and incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue BB (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.1 M Tris buffer (pH 9.3). ALP activity assay was preformed using an ALP kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol and normalized based on protein

concentrations. To detect mineralization potential cells were induced for 2 weeks using OIM, fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (Sigma-Aldrich, St Louis, MO, USA). For quantification, Alizarin Red was destained with 10% cetylprydiniumcholoride in 10mM sodium phosphate for 30 min at room temperature. Optical absorbance was measured at 562nm using a multiplate reader with a standard calcium curve in the same solution. The final calcium level in each group was normalized with the total protein concentrations prepared from a duplicate plate.

2.5 Induction of Chondrogenic Differentiation

Sorted DMSCs were plated at 1 x 10^5 cells/well into 12-well plates. To induce differentiation into chondrogenic lineages, sorted DMSCs were grown in chondrogenic inducing media (CIM) containing α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 100 mM sodium pyruvate, 40ug/mL proline, 100 nm dexamethasone, 200 μM ascorbic acid (all from Sigma-Aldrich, St Louis, MO, USA), and 10ng/mL TGF-β3 (R&D systems). Culture medium was changed every 2-3 days. After 4 weeks of differentiation *in vitro*, Alcian blue staining (Sigma-Aldrich, St Louis, MO, USA) was performed as previously described.[35] For quantification, stained Alcian blue was eluted with 6M guanidine hydrocholoride for 8hrs at room temperature. The optical absorbance was measured at 620 nm using a microplate reader.

2.6 Induction of Adipogenic Differentiation

Sorted DMSCs were plated at 1 x 10^5 cells/well into 12 well plates. To induce differentiation into adipogenic lineages, sorted DMSCs were grown in adipogenic inducing media (AIM) containing 90% Dulbeco's Modified Eagle's Medium (DMEM) high glucose

(Invitrogen, Carlsbad, CA, USA), 10% FBS (Invitrogen), 1 μM dexamethasone, 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2 mM indomethacin (all from Sigma-Aldrich, St Louis, MO, USA). Culture media was changed every 3 days. After 3 weeks of differentiation in vitro, Oil Red O (Sigma-Aldrich, St Louis, MO, USA) was performed as previously described. [35] For quantification, stained Oil Red O was eluted with 75% ethanol for 30 minutes at room temperature. The optical absorbance was measured at 500nm on a microplate reader.

2.7 Quantitative RT-PCR

The total RNA was isolated from DMSCs using Trizol reagents (Invitrogen, Carlsbad, CA, USA). Two-μg aliquots of RNAs were used to synthesize cDNAs using random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The real-time PCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and the Icycler IQ Multicolor Real-time PCR Detection System (Bio-Rad). The primers that were used are listed on Table 1.

2.8 Western Blotting

Proteins was isolated from cells using CelLytic MT solution (Sigma), supplemented with protease inhibitor cocktail (PIC, Promega, Southampton, USA). 40μg aliquots or protein were separated by SDS-polyacrylamide (PAGE) gel and transferred to a PDVF membrane. The primary antibodies rabbit-anti: Phospho-p44/42 MAPK (ERK1/2) (THr202/Tyr204); p44/42 MAPK (ERK1/2), Phospho-AKT (Ser473) (D9E) XP, AKT, phospho-MTOR (Ser2448) (D9C2), MTOR, phospho-NF-κB p65 (Ser536) (93H1), NF-κB p65, (all from Cell Signaling Technology, Danvers, MA USA) and mouse anti-tubulin (Santa Cruz Biotechnology; Dallas, TX USA). Secondary antibodies for mouse anti-Rabbit-IgG-horseradish peroxidase (HRP) were used

(Promega, USA). Detection was performed with the Luminal/Enhancer Solution and Super Signal West Stable Peroxide Solution (Thermo).

2.9 Matrigel Invasion Assay

The migration ability of CD271 DMSCs with NGF was determined using 24-well Matrigel invasion chambers (BD Biosciences Discovery Labware). Cells were seeded into upper inserts at 1×10^5 (24-well) per insert in serum-free α-MEM. Outer wells were filled with α-MEM containing 2ng/mL or 10ng/mL of NGF as chemoattractant. Cells were incubated for 24hrs, and then non-invading cells were removed by swabbing top layer of Matrigel with a cotton swap. Membranes containing invading cells were stained with a HEMA-3 kit (Fisher). The invading cells on the entire membrane were counted under light microscope.

2.10 si-RNA transfection

DMSCs were transfected with non-specific scrambled siRNA or siRNA targeting human TrkA (sc-36726), NGFR p75 (sc-36058), AKT (sc-29195) (Santa Cruz Biotechnology; Dallas, Texas USA) at a concentration of 10nM using Lipofectamine reagents (Invitrogen) according to the manufacture's instructions. DMSCs were induced to undergo odontogenic differentiation after 24 hours post-transfection, and harvested at 48, 72hrs post transfection for mRNA isolation. For protein isolation, after 24hrs post-transfection DMSCs were treated with NGF for 4 hours.

Genes	Forward (5'-3')	Reverse (5'-3')
DLX5	GCTCTCAACCCCTACCAGTAT	CTTTGGTTTGCCATTCACCATTC
RUNX2	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
BGLAP	AGCAAAGGTGCAGCCTTTGT	GCGCCTGGTCTCTTCACT
DMP1	GACAGCAAGGGTGACTCTCA	AGATGACAGGTTGGCCTCTT
DSPP	TCAACAGCAAGAGAAATGGG	TCGTCTTCATCCTCATCTGC
PLAP-1	TATTCAACAACCCGGTGAA	ACATTCCAAAGTTCCCAAGC
SOX9	CACACAGCTCACTCGACCTT	CAAAGGGAATTCTGGTTGGT
COL2A1	CAAGAAGAACTGGTGGAGCA	CATAGCTGAAATGGAAGCCA
ACAN	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
PPARy	GGGATCAGCTCCGTGGATCT	TTGCACTTTGGTACTCTTGAAGTT
trkA^{NGFR}	GCCACACGCAACTGTCTAGT	GCCTCCCACACGGTAATAGT
P75^{NTR}	CCTCATCCCTGTCTATTGCTCC	GTTGGCTCCTTGCTTGCTTGTTCTGC

Table 1. Human primer sequences used for quantitative RT- PCR

3 SINGLE CD271 MARKER ISOLATES MESENCHYMAL STEM CELLS FROM HUMAN DENTAL

3.1 Isolation of DMSCs with surface markers CD51/CD140α, CD271, and STRO-1/CD146 from DPCs

Many available MSC surface markers fail to represent MSCs *in vivo* as markers are not homogenously expressed across cultures.[65] Recent studies showed that specific combinations of surface markers including CD51/CD140α and CD271/CD90/CD106 isolated highly enriched clonogenic cells from human bone marrow, respectively.[6,7] In addition, STRO-1/CD146 combination has successfully characterized DMSCs from dental tissues such as periodontal ligament and apical papilla.[32,66] However, whether these verified combination of surface markers are capable of isolating multipotent and self-renewing progenitor cells from human DP remains to be investigated. Using two surface combination of CD51/CD140α or STRO-1/CD146, FACS revealed that 27.3% of DPCs were CD51+/CD140α+ and 0.3% of DPCs were STRO-1+/CD146+ (Fig. 3.1A). Interestingly, we found that the majority (99%) of DPCs expressed CD90 (Fig. 3.1B), indicating that CD90 might not be useful. The combination of CD271/CD106 yielded extremely few positive cells (Fig. 3.1C). Therefore, we decided to use single CD271 marker to isolate MSCs from DPCs and found that 10.6% of DPCs were CD271+. In addition, the proliferation of sorted cell combinations was assessed, and CD271+ DMSCs had a higher proliferation capacity compared to the other two combinations. (Figure 3.2)

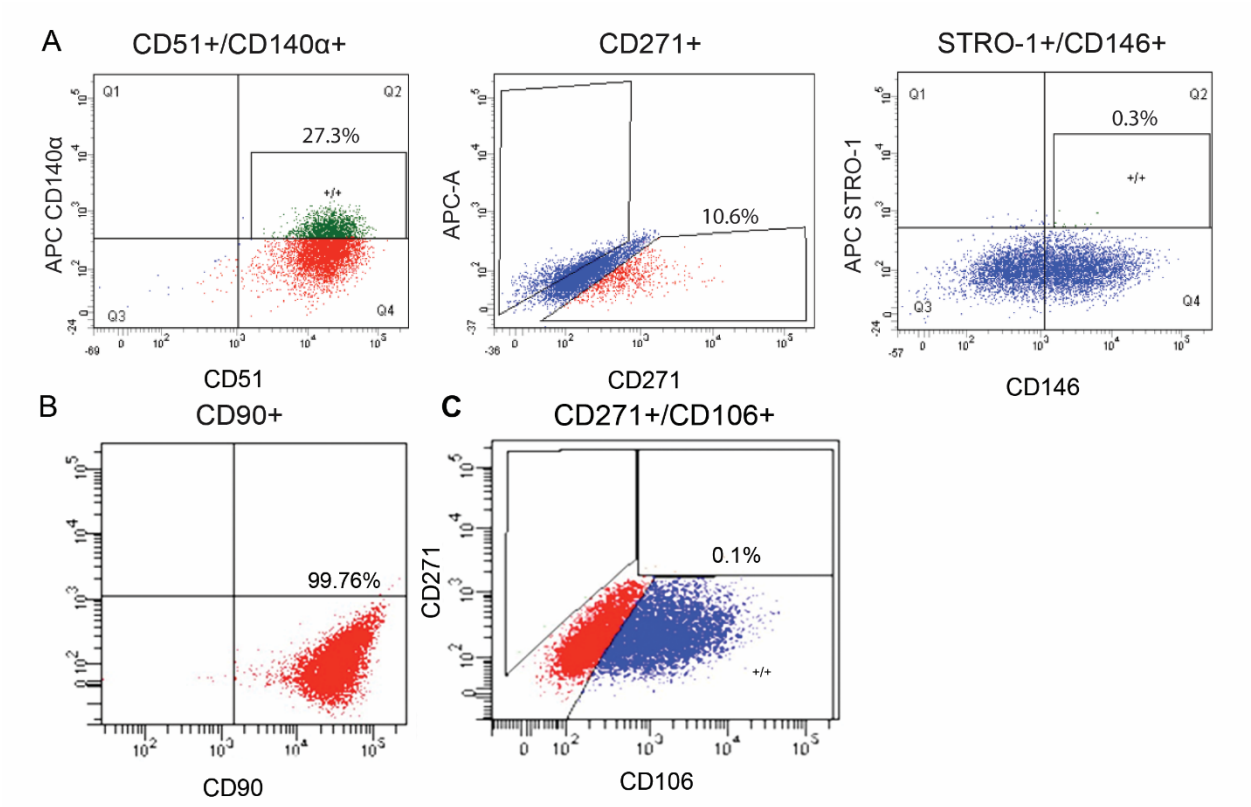

Figure 3.1 The expression profiles of stem cell surface markers in human primary cells from dental pulp tissues determined by FACS. (A) CD51/CD140α, CD271, and STRO-1/CD146 (B) CD90 (C) CD271/CD106. Cells were isolated from dental pulp of adult third molars and stained with antibodies.

3.2 Isolated DMSCs exhibited differential odontogenic potential depending on surface markers

To evaluate and compare differentiation capacity, isolated CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs were induced to undergo odontogenic differentiation. All three types of isolated DMSCs exhibited the capacity to differentiate into the odontogenic lineage as demonstrated by ALP staining on the seventh day (Figure 3.3A). Quantification of ALP activity revealed the most significant increase of ~5 fold in induced CD271+ DMSCs compared to noninduced CD51+/CD140α+. Induced CD51+/CD140α+ DMSCs exhibited 2.5 fold increase in ALP activity while induced STRO-1+/CD146+ DMSCs showed 1.25 fold increase (Figure 3.3B). All three isolated DMSC groups had formation of mineralized nodules after prolonged treatment with odontogenic induction media for 14 days as demonstrated by ARS (Figure 3.3A). The quantification of ARS also showed significant mineralization potential in CD271+ DMSCs (4.5 fold) followed by CD51+/CD140α+ DMSCs (2.75 fold) and STRO-1+/CD146+ DMSCs (1 fold) (Figure 3.3C).

Using these isolated DMSCs, we further confirmed their odontogenic potential by examining mRNA expression of several odontogenic marker genes including *DLX5*, *RUNX2, BGLAP, DMP1*, and *DSPP* at different time points: 0, 3, 7, and 10 days after odontogenic induction. Consistent with ALP and ARS results, odontogenic marker gene expression was upregulated for all three subsets of DMSCs. In particular, marked increase of *DLX5* expression was found in CD51+/CD140α+ DMSCs at day 7 of induction compared to CD271+ and STRO-1+/CD146+ DMSCs (Figure 3.4A). The expression of *RUNX2* in both CD51+/ CD140α+ and CD271+ DMSCs were more significantly upregulated in a time dependent manner at day 3 and day 7 of induction (Figure 3.4B) compared to STRO-1+/CD146+ DMSCs. Significant *BGLAP* expression induction was found in CD271+ DMSCs at day 10 of odontogenic induction (Figure

3.4C). Lastly, in all three isolated DMSC populations, *DMP1* and *DSPP* expression were significantly upregulated at day 10 of induction (Figure 3.4D & E).

Collectively, this data suggested that homogenous populations of DMSCs isolated using FACS with different surface marker combinations are capable of differentiating into odontogenic lineages, but at different magnitudes.

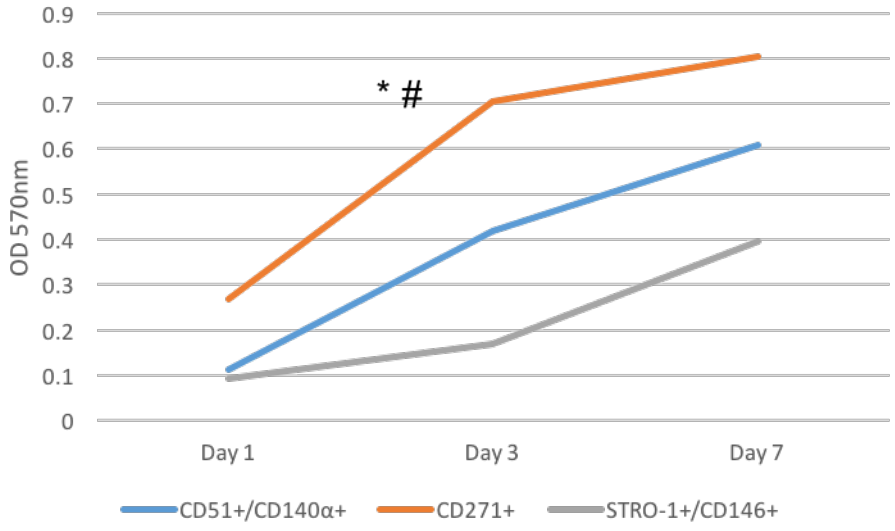

Figure 3.2 Proliferation of isolated CD51+/CD140α+, CD271+, and STRO-1/CD146+ DMSCs from DP *p<0.05 versus CD51+/CD140α+, and #p<0.05 versus STRO-1+/CD146+ by Two-Way ANOVA

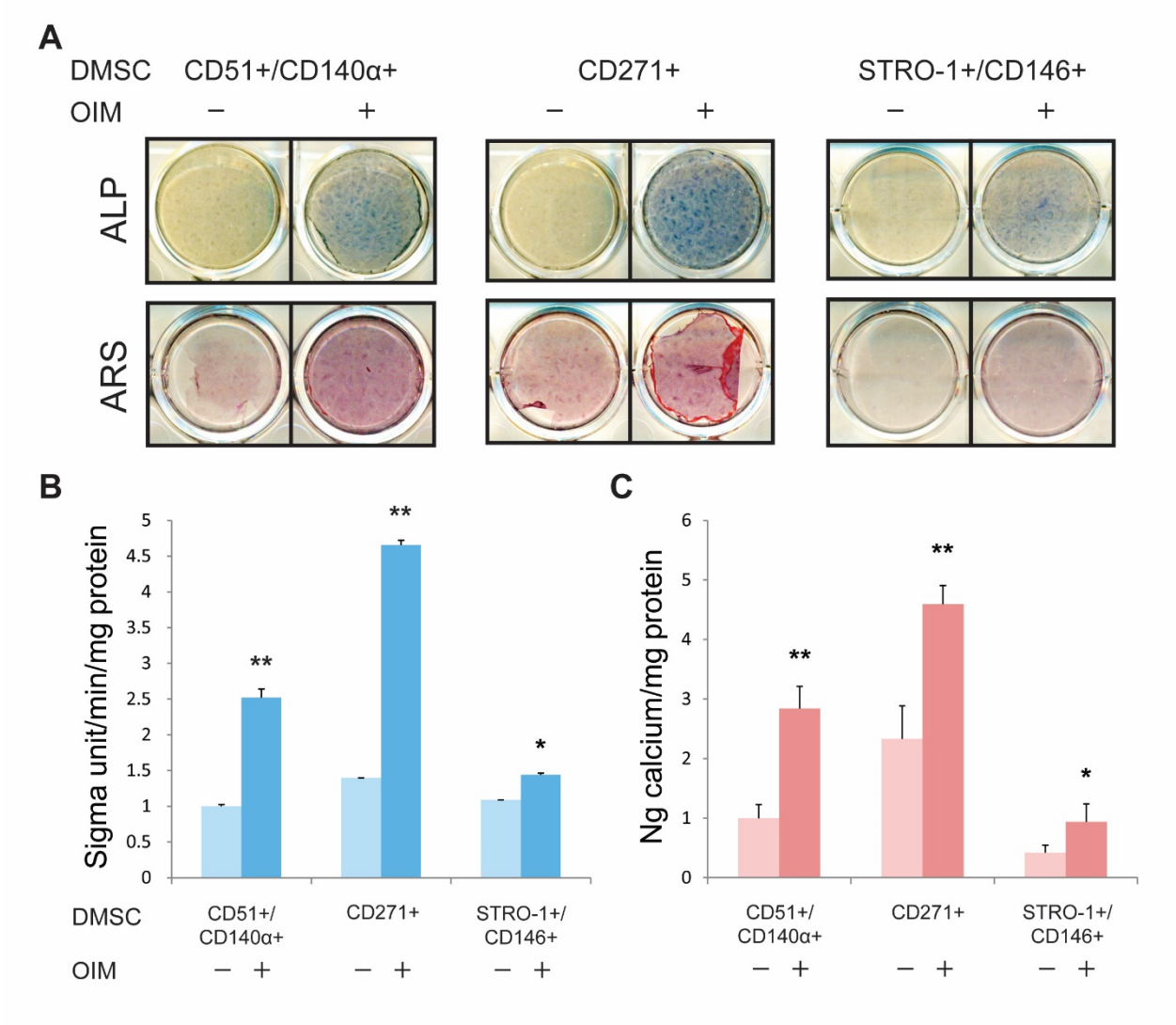

Figure 3.3 Odontogenic differentiation of isolated CD51+/CD140α+, CD271+, and STRO-1/CD146+ DMSCs. **(A)** ALP staining and Alizarin Red staining after these cells were induced to differentiate into odontogenic lineages for 7 days and 14 days respectively. **(B)** Quantification of ALP activity **(C)** Quantification of ARS. Values were normalized to non-induce d CD51+/CD140α+ DMSCs. * $p<0.05$, ** $p<0.001$.

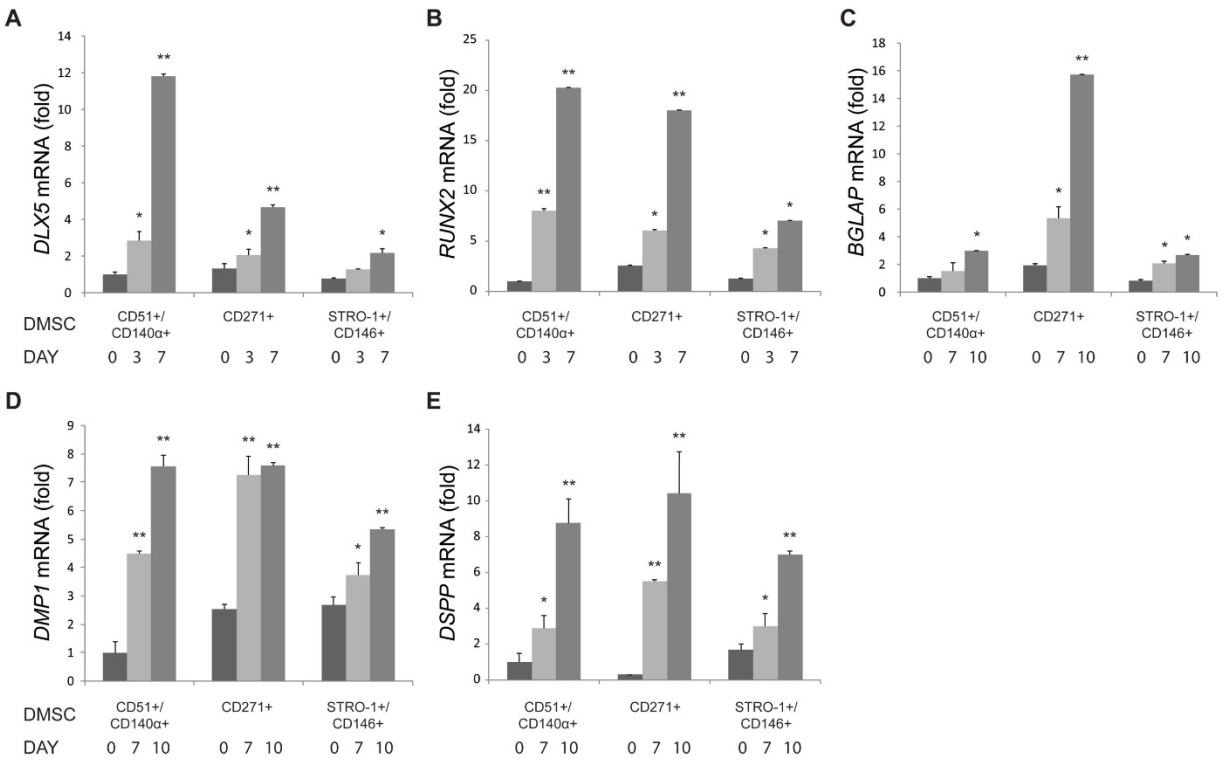

Figure 3.4 mRNA expression of odontogenic genes after 0, 3, 7, and 10 days of odontogenic induction in CD51+/CD140α+, CD271+ and STRO-1+/CD146+ DMSCs. **(A)** DLX5 **(B)** RUNX2 and **(C)** BGLAP **(D)** DMP1 and **(E)** DSPP. Values were normalized to CD51+/CD140α+ DMSCs at day 0. * $p<0.05$, ** $p<0.001$.

3.3 Isolated DMSCs exhibited differential chondrogenic potential depending on surface markers

We further evaluated and compared chondrogenic differentiation capacity of isolated DMSCs under chondrogenic conditions. All three isolated DMSC groups showed the presence of glycosaminogylcans as demonstrated by Alcian Blue staining after prolonged treatment with chondrogenic induction media for 28 days (Figure 3.5A). The quantification of Alcian Blue staining exhibited the greatest chondrogenic differentiation in CD271+ DMSCs, followed by STRO-1+/CD146+ DMSCs and CD51+/ CD140α+ (Figure 3.5B). mRNA expression of *Sox9,* the master gene of chondrogenic differentiation, was significantly upregulated confirming their chondrogenic potential in all three populations of isolated DMSCs (Figure 3.5C).

3.4 Isolated DMSCs exhibit weak adipogenic potential

We evaluated and compared the adipogenic differentiation capacity of isolated DMSCs under adipogenic conditions. All three isolated DMSC groups showed weak presence of lipid droplets as demonstrated through Oil Red O staining after prolonged treatment with adipogenic induction media for 21 days (Figure 3.6A). The quantification of Oil Red O staining did not exhibit significant differentiation of sorted DMSCs (Figure 5B). Interestingly, mRNA expression of *PPARγ,* the master gene of adipogenic differentiation, was significantly upregulated in CD51+/ CD140α+ and CD271+ DMSCs (Figure 3.6C). Further studies need to be completed to validate these results.

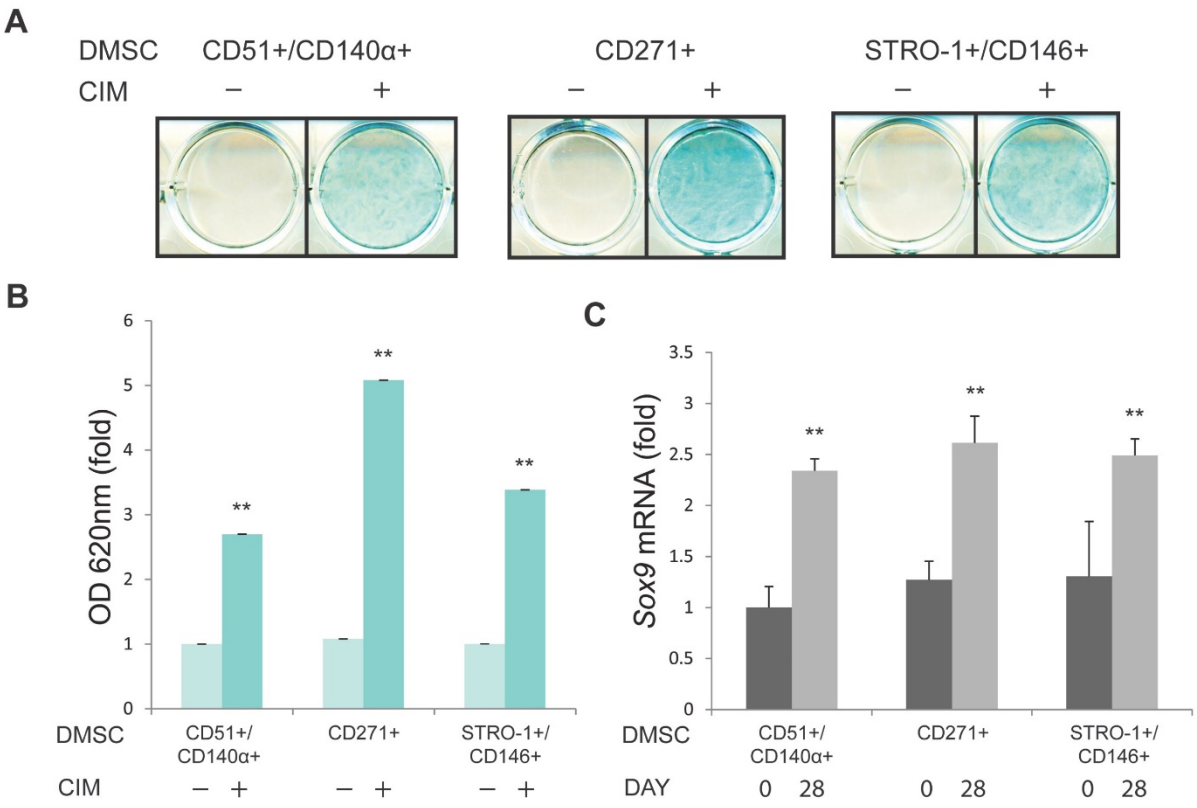

Figure 3.5 Chondrogenic differentiation of CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs. (A) Alcian Blue staining after cells were induced to differentiate into chondrogenic lineages for 28 days. **(B)** Quantification of Alcian Blue **(C)** mRNA expression of Sox9 after 0 and 28 days of chondrogenic induction. Values were normalized to CD51+/CD140α+ DMSCs at day 0. * $p<0.05$, **$p<0.001$.

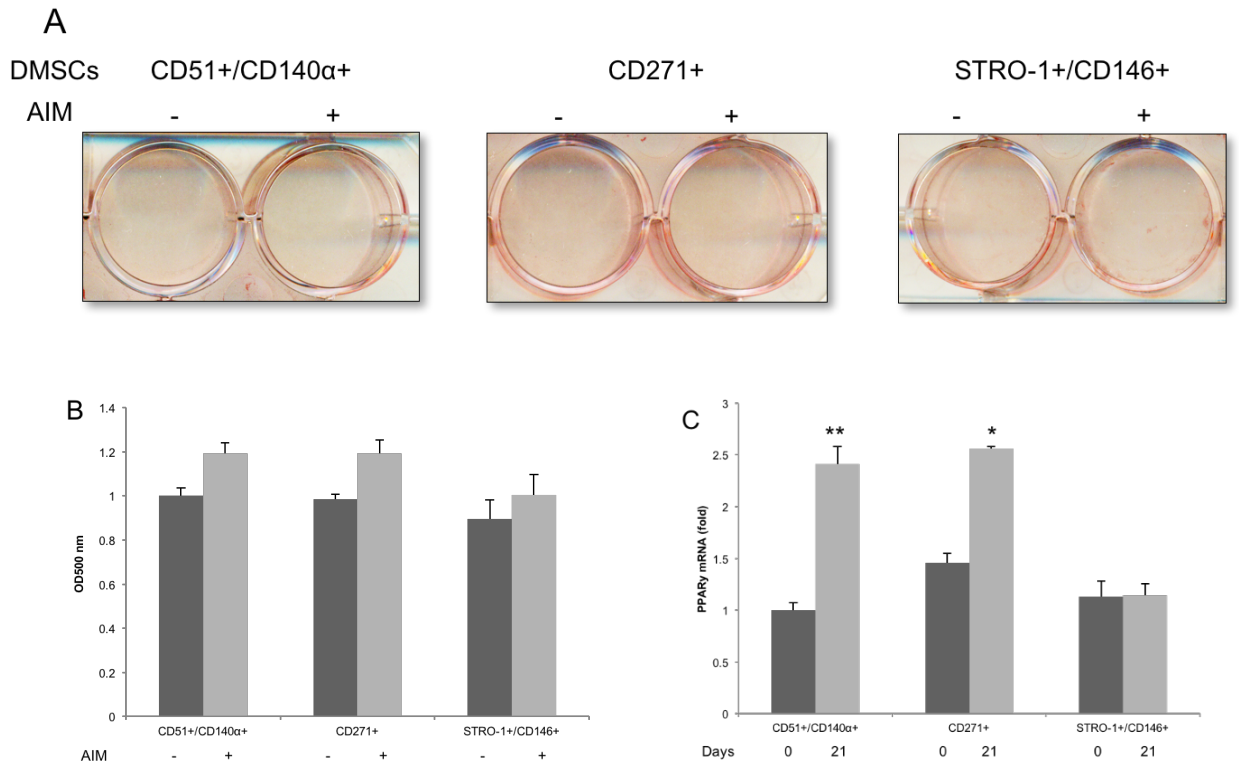

Figure 3.6 Adipogenic differentiation of CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs from DPCs. (A) Oil Red O staining after cells were induced to differentiate into adipogenic lineages for 21 days. **(B)** Quantification of Oil Red O **(C)** mRNA expression of PPARγ after 0 and 21 days of adipogenic induction. Values were normalized to CD51+/CD140α+ DMSCs at day 0. * $p<0.05$, ** $p<0.001$.

4 CHARACTERIZATION OF OSTEOGENIC POTENTIAL OF MESENCHYMAL STEM CELLS FROM HUMAN PERIODONTAL LIGAMENT BASED ON CELL SURFACE MARKERS

4.1 Isolation of DMSCs with surface markers, CD51/CD140α, CD271, STRO-1/CD146 from PDLCs

It has been established that the surface maker combination of STRO-1/CD146 has successfully isolated DMSCs from dental tissues such as the PDL and apical papilla using FACS analysis [32,66] Additional markers including CD51/CD140α and CD271/CD90/CD106 have been suggested for the purpose of identifying multipotent homogenous population of BMSCs and these marker combinations were not previously evaluated in DMSCs.[6,7] Using two surface marker combinations of CD51/CD140α and STRO-1/CD146, FACS revealed that 24% of PDLCS were CD51+/CD140α+ and 2.4% of PDLCs were STRO-1+/CD146+. (Figure 4.1A and C). The isolation of STRO-1+/CD146+ DMSCs is consistent with previous studies, which indicated a 2.6% isolation of DMSCs from PDL.[32] Interestingly, we found that the majority of PDLCs (99%) expressed CD90 (Figure 4.1D), indicating that CD90 is not sufficiently selective for sorting. On the contrary, FACS using dual markers, CD271 and CD106, revealed that an extremely low proportion of PDLCs were double positive for this combination. (Figure 4.1E). Therefore, we used a single maker, CD271, to isolate DMSCs and found that 0.8% of PDLCs were CD271+, representing the smallest proportion among our combinations (Figure 4.1B). Consistent with our results, a second patient sample expressed similar percentage of surface marker combinations (Fig 4.2A, B, C). Interestingly the expression CD271 in the other two combinations CD51+/CD140α+ and STRO-1+/CD146+ of DMSCs was low 4% and .2%

respectively, making isolation of cells difficult (Fig 4.2D, E). CD51+/CD140α+ DMSCs exhibited increased proliferation compared to the other two sorted combinations (Fig 4.3)

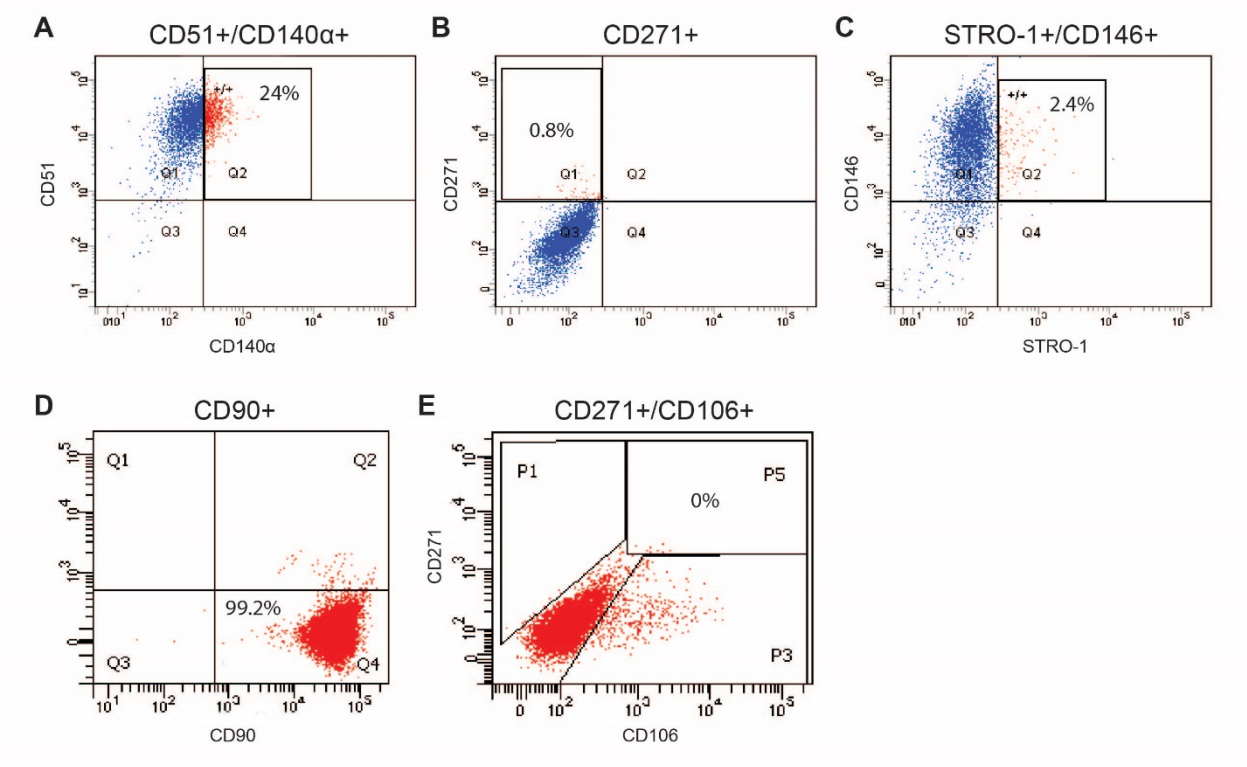

Figure 4.1 The expression profiles of stem cell surface markers in human primary cells from periodontal ligament tissues determined by FACS. **(A)** CD51/CD140α, **(B)** CD271, **(C)** STRO-1/CD146, **(D)** CD90, and **(E)** CD271/CD106. Cells were isolated from the periodontal ligament of adult third molars and stained with antibodies.

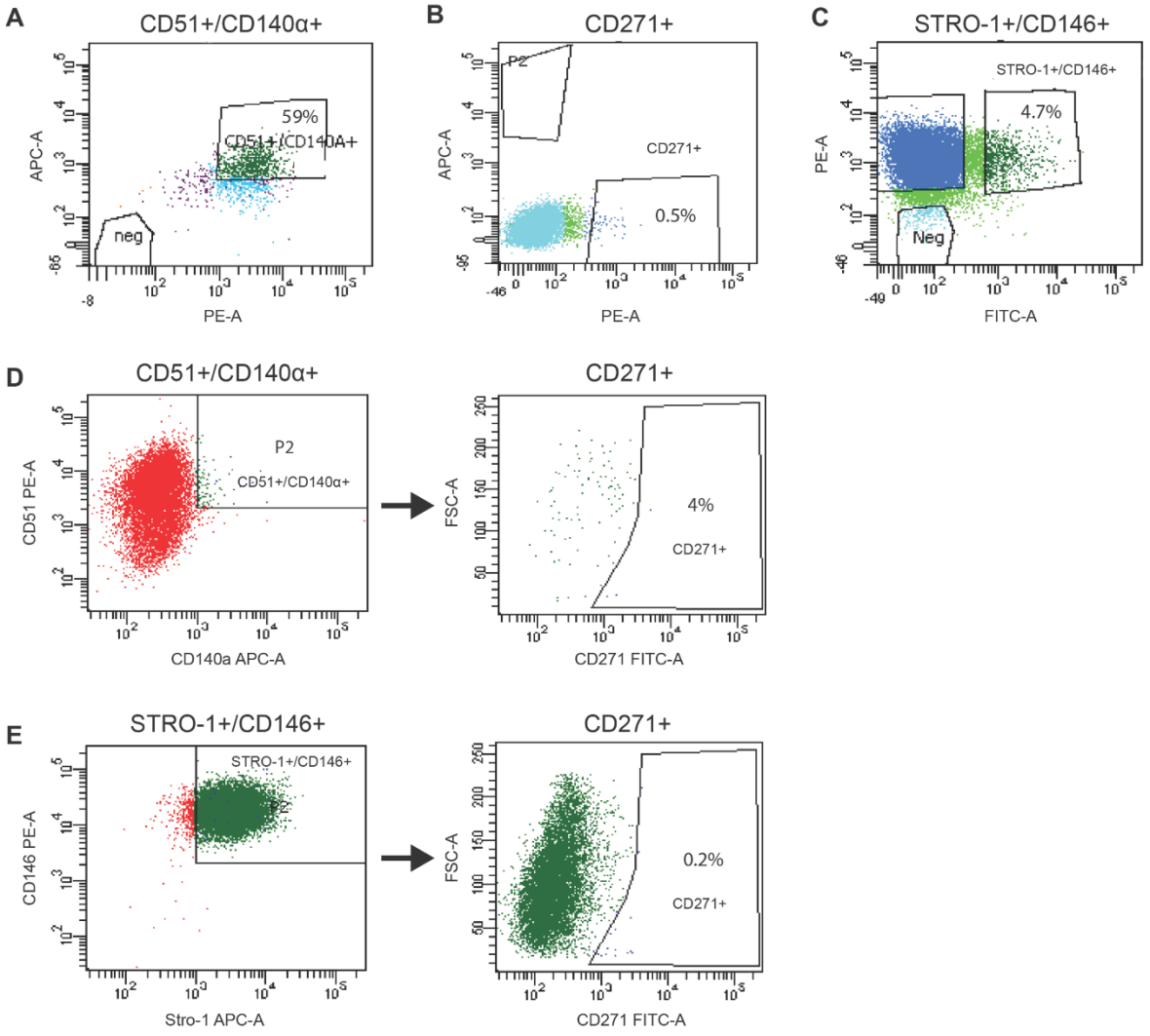

Figure 4.2 The expression profiles of stem cell surface markers in human primary cells from a second sample of periodontal ligament tissues determined by FACS. (A) CD51/CD140α, **(B)** CD271 **(C)** STRO-1/CD146 **(D)** CD271 in CD51/CD140α and **(E)** CD271 in STRO-1/CD146

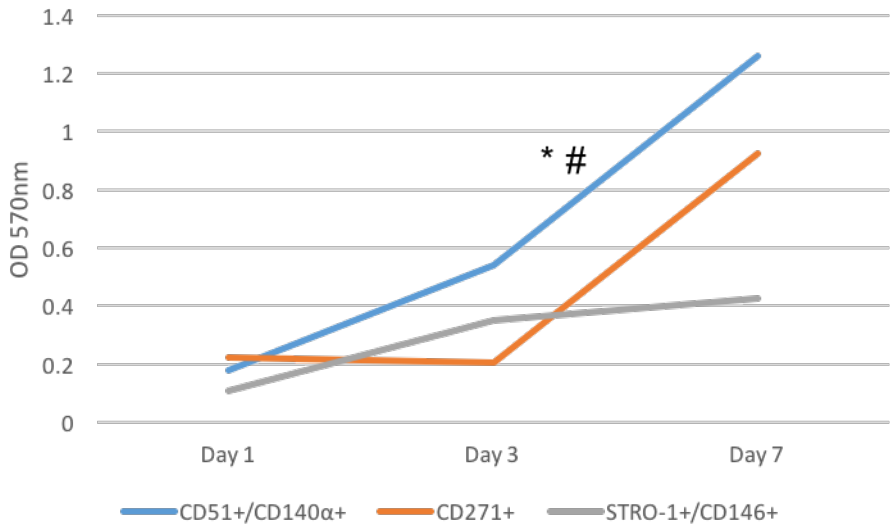

Figure 4.3 Proliferation of isolated CD51+/CD140α+, CD271+, and STRO-1/CD146+ DMSCs from PDL *p<0.05 versus CD271+ and #p<0.05 versus STRO-1+/CD146+ by Two-Way ANOVA

4.2 Sorted DMSCs exhibited significant differential odonto/osteogenic potential

To evaluate and compare differentiation capacity, isolated CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs were induced to undergo odonto/osteogenic differentiation. All three combinations of DMSCs displayed the capacity to differentiate into the odonto/osteogenic lineage as demonstrated by ALP staining on the seventh day (Figure 4.4A). Quantification of ALP activity revealed significant increase of approximately 5 to 9 fold in all three populations of induced isolated DMSCs compared to non-induced CD51+/CD140α+ cells (Figure 4.4B). In addition, all three isolated DMSC groups had formation of mineralized nodules after prolonged treatment with OIM for 14 days as demonstrated by ARS (Figure 4.4A). Quantification of ARS also showed significant mineralization potential in all induced DMSCs with a 2.5 to 4-fold increase compared to non-induced CD51+/CD140α+ DMSCs.

Using these isolated DMSCs, we further confirmed their odonto/osteogenic potential by examining mRNA expression of several odonto/osteogenic marker genes including *DLX5*, *RUNX2,* and *BGLAP,* at different time points: 0, 3, 7, and 10 days after odonto/osteogenic induction. Consistent with ALP and ARS results, odonto/osteogenic marker gene expression was significantly upregulated for all three subsets of isolated DMSCs. In particular, marked increase of *DLX5* and *RUNX2* expression was found in CD271+ DMSCs at day 7 of induction compared to CD51+/CD140α+ and STRO-1+/CD146+ DMSCs (Figure 4.5A, B). Significant *BGLAP* expression induction was found in CD271+ DMSCs at day 7 and 10 of osteogenic induction (Figure 4.5C). In addition, periodontal marker gene, periodontal ligament associated protein (*PLAP-1*), was assessed at 0, 7, 10 days after osteogenic induction. *PLAP-1* expression was significantly upregulated in all 3 subsets (Figures 4.5D).

Collectively, this data suggested that homogenous populations of DMSCs isolated using FACS with different surface marker combinations have capacity to differentiate into osteogenic lineages.

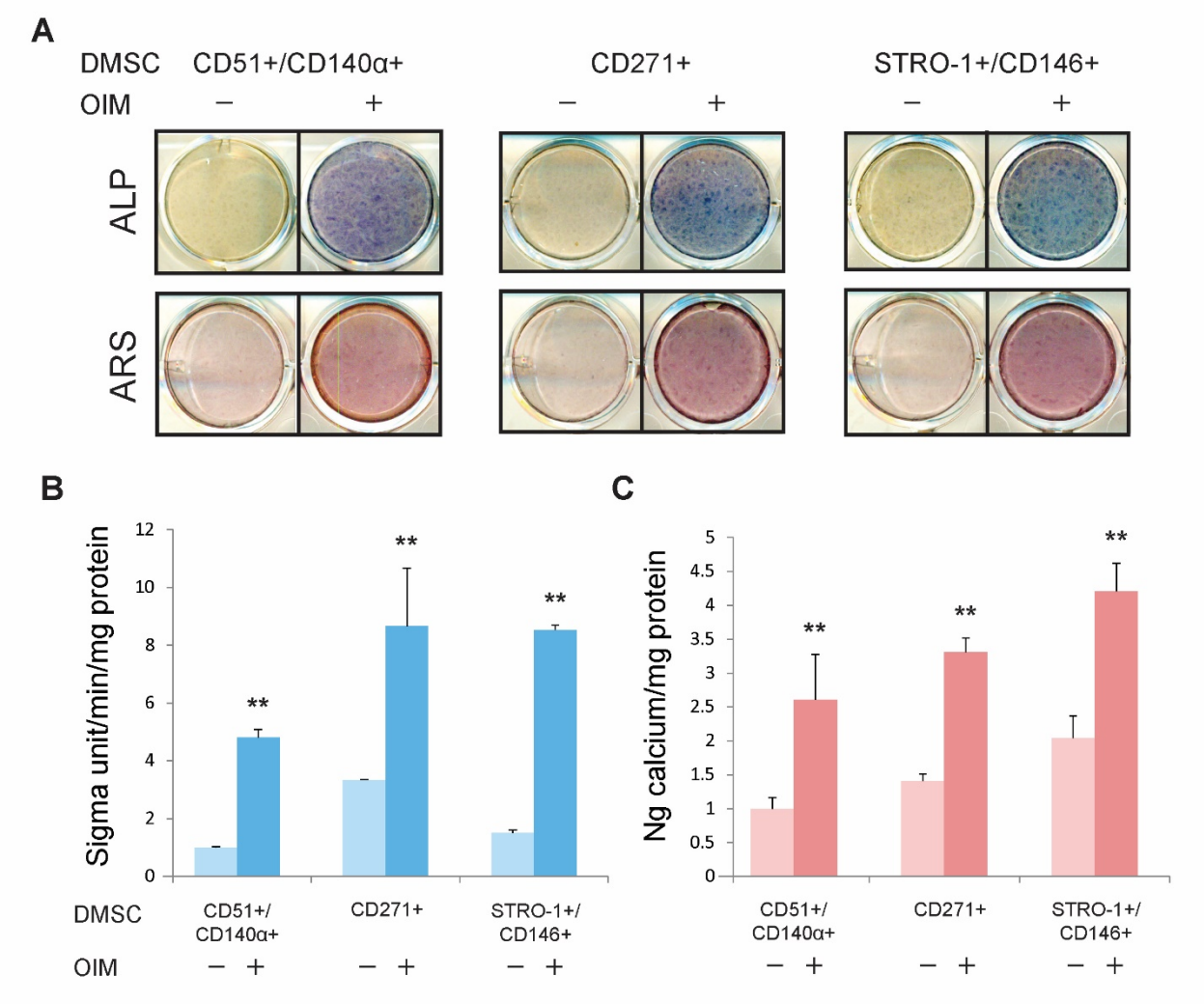

Figure 4.4 Odonto/Osteogenic differentiation of isolated CD51+/CD140α+, CD271+, and STRO-1/CD146+ DMSCs. (A) ALP staining and ARS after these cells were induced to differentiate into odonto/osteogenic lineages for 7 days and 14 days respectively. **(B)** Quantification of ALP activity (**C)** Quantification of ARS. Values were normalized to non-induced CD51+/CD140α+ DMSCs. * $p<0.05$, ** $p<0.001$

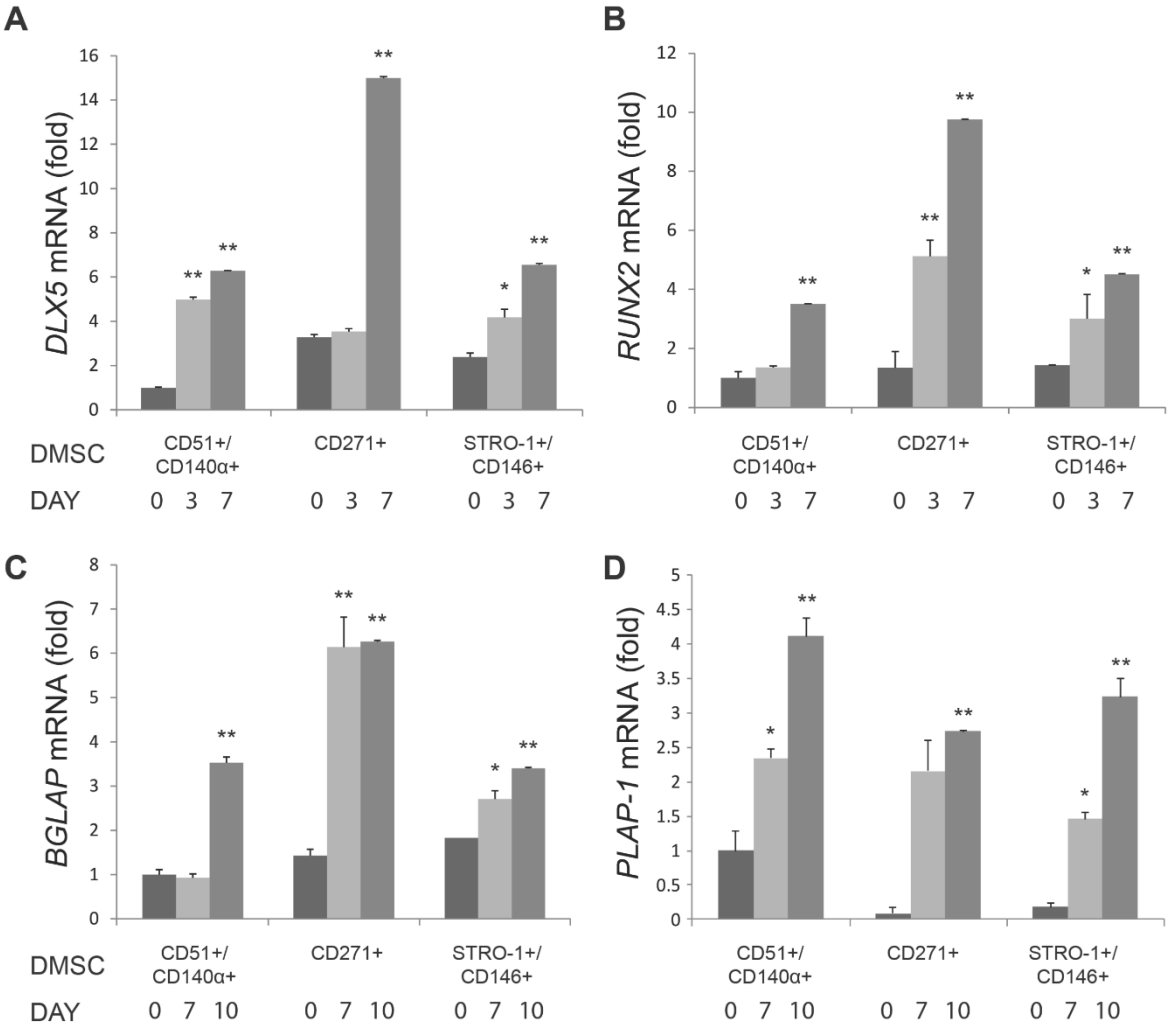

Figure 4.5 mRNA expression of odonto/osteogenic and periodontal genes after 0, 3, 7, and 10 days of odonto/osteogenic induction in CD51+/CD140α+, CD271+ and STRO-1+/CD146+ DMSCs. (A) DLX5 (B) RUNX2 and (C) BGLAP and (D) PLAP-1. Values were normalized to CD51+/CD140α+ DMSCs at day 0. * $p<0.05$, ** $p<0.001$.

4.3 Isolated DMSCs exhibited significant differential chondrogenic potential

Furthermore, we evaluated and compared chondrogenic differentiation capacity of isolated DMSCs under chondrogenic conditions. All three isolated DMSC groups showed the presence of glycosaminogylcans as demonstrated by Alcian Blue staining after prolonged treatment with CIM for 28 days (Figure 4.6A). Quantification of Alcian Blue staining exhibited a 3 to 4.5 fold increase in chondrogenic differentiation in all three isolated DMSC groups compared to non-induced CD51+/CD140α+ DMSCs (Figure 4.6B). mRNA expression of several chondrogenic genes including *SOX9*, collagen type 2 alpha 1 (*COL2A1*) and aggrecan (*ACAN*) were significantly induced confirming their chondrogenic potential (Figure 4.7A-C). In particular all three chondrogenic markers were significantly upregulated in STRO-1+/CD146+ DMSCs (Figure 4.7A-C).

4.4 Isolated DMSCs exhibited significant differential adipogenic potential

We evaluated and compared the adipogenic differentiation capacity of isolated DMSCs under adipogenic conditions. All three isolated DMSC groups showed presence of lipid droplets as demonstrated through Oil Red O staining after prolonged treatment with adipogenic induction media for 21 days (Figure 4.8A). The quantification of Oil Red O staining exhibited a 1 fold increase in STRO-1+/CD146+ DMSCs (Figure 4.8B). Interestingly, mRNA expression of *PPARγ* was significantly upregulated in CD271+ and STRO-1+/CD146+ DMSCs (Figure 4.8C). Since Oil Red O staining and mRNA findings are not consistent; further studies looking at additional adipogenic markers need to be completed.

4.5 Expression of combinations CD51+/CD140α+, STRO-1+/CD146+ , and CD271+ decreases with time

The expression of all three sorted combinations decreased with cell passage, but CD51+/CD140α+ decreased the less (Fig 4.9). This most likely is associated with the ability of CD51+/CD140α+ to isolate the largest population of cells from PDL.

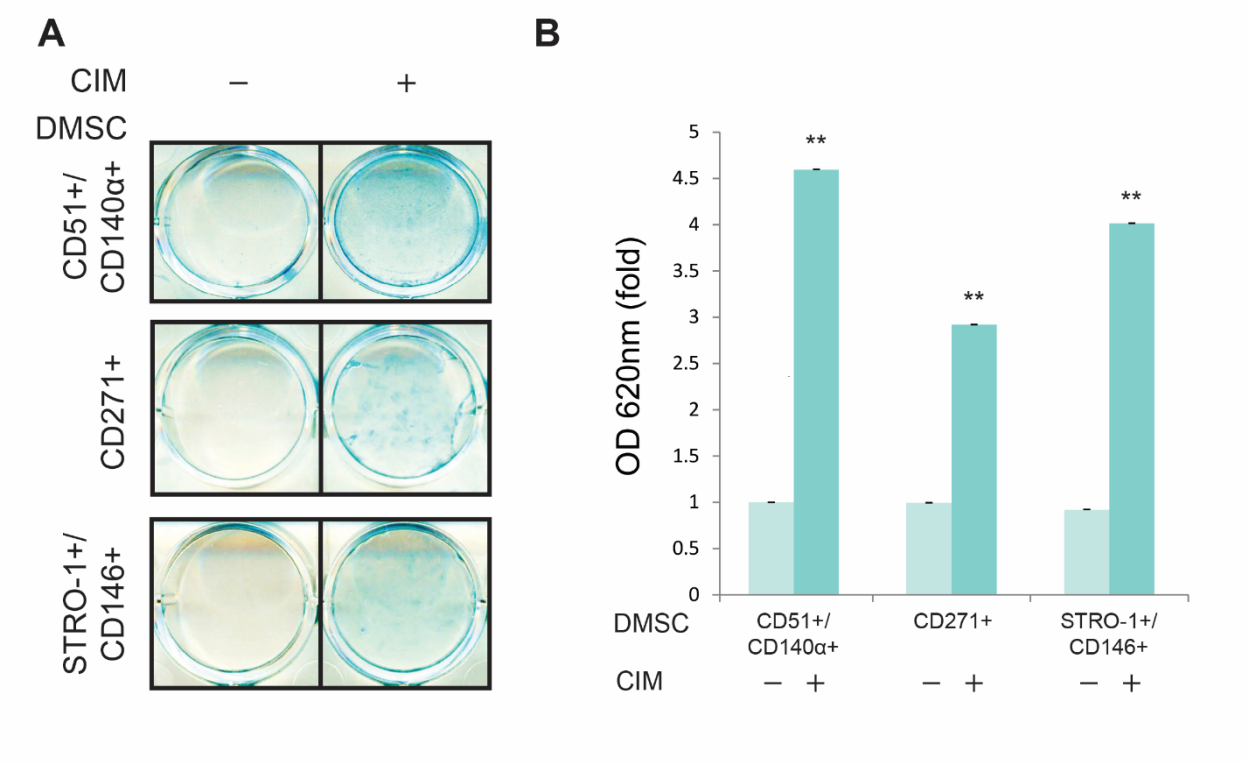

Figure 4.6 Chondrogenic differentiation of CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs. **(A)** Alcian Blue staining after cells were induced to differentiate into chondrogenic lineages for 28 days. **(B)** Quantification of Alcian Blue

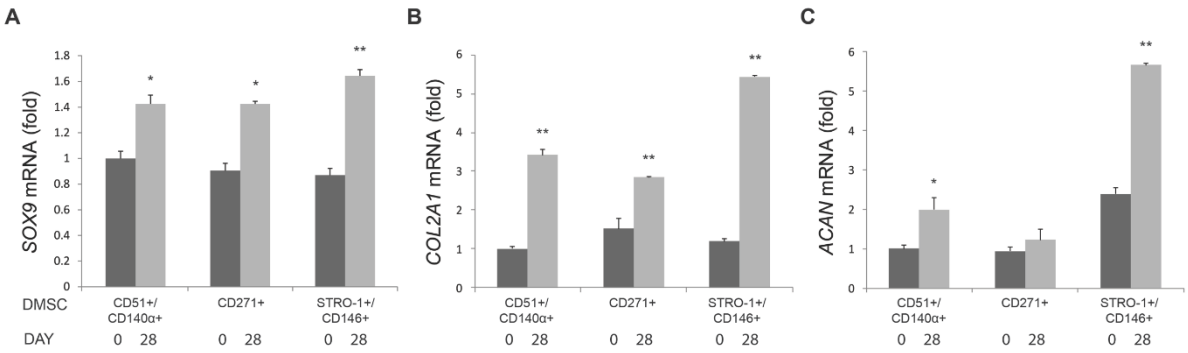

Figure 4.7 mRNA expression of chondrogenic genes after 0 and 28 days of chondrogenic induction in CD51+/CD140α+, CD271+ and STRO-1+/CD146+DMSCs (A) SOX9 **(B)** COL2A1 and **(C)** ACAN. Values were normalized to CD51+/CD140α+ DMSCs at day 0. *p<0.05, **p<0.001.

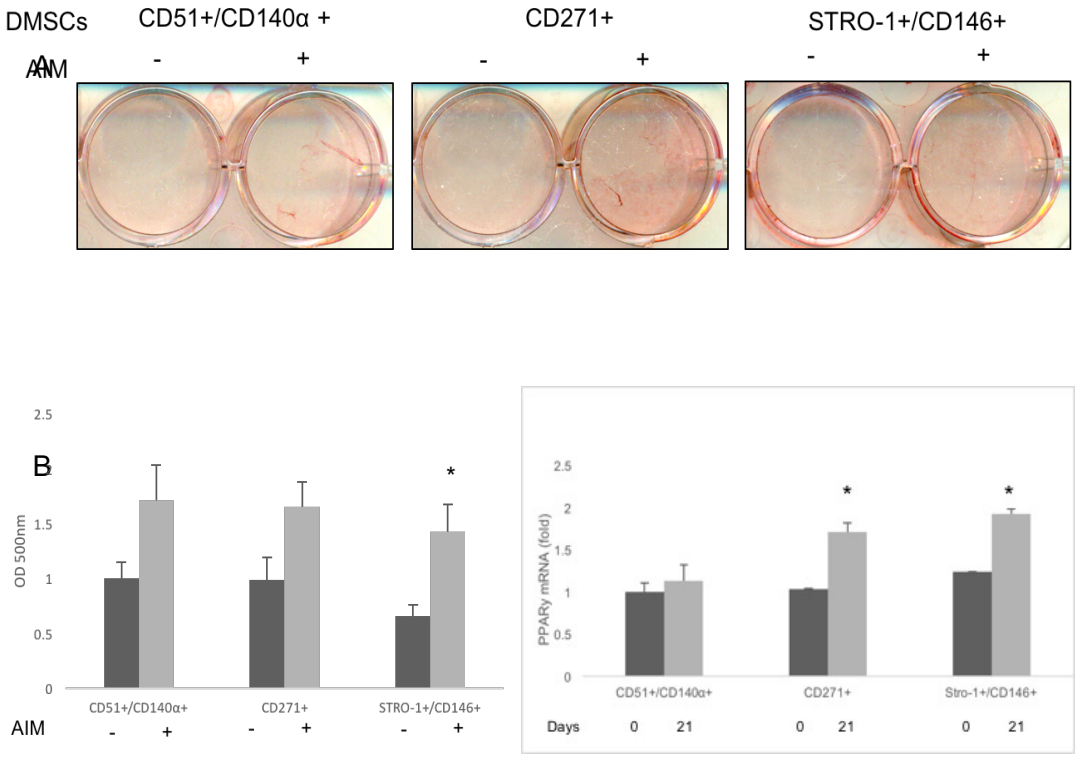

Figure 4.8 Adipogenic differentiation of CD51+/CD140α+, CD271+, and STRO-1+/CD146+ DMSCs from PDLCs. (**A**) Oil Red O staining after cells were induced to differentiate into adipogenic lineages for 21 days. **(B**) Quantification of Oil Red O **C**. mRNA expression of PPARγ after 0 and 21 days of adipogenic induction. Values were normalized to CD51+/CD140α+ DMSCs at day 0. * $p<0.05$, **$p<0.001$.

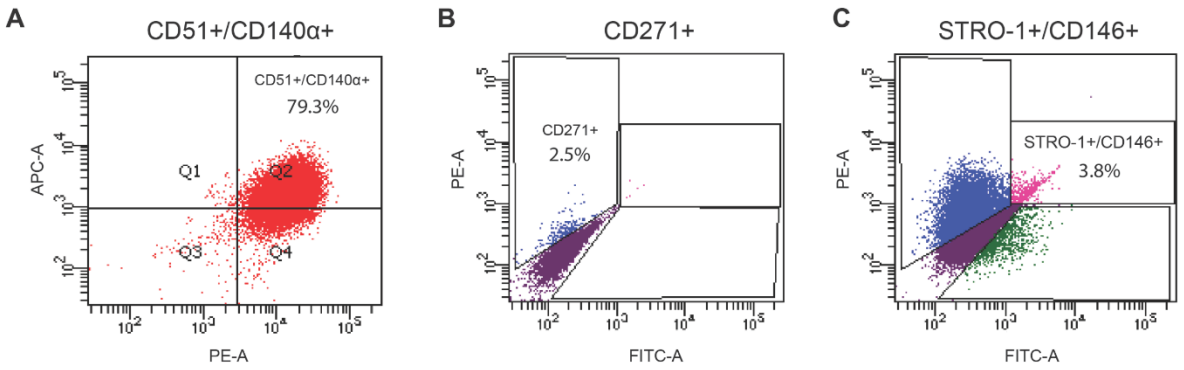

Figure 4.9 The expression profiles of stem cell surface markers in the positive selected DMSCs from PDL expanded in culture determined by FACS. (A) CD51/CD140α, **(B)** CD271 **(C)** STRO-1/CD146. Cells were evaluated at passage 9

5 NGF and CD271 PROMOTE ODONTOGENIC DIFFERENTIATION OF DMSCs

5.1 Isolated CD271+ DMSCs from DP exhibit increased differential odontogenic potential with exogenous NGF

To evaluate the effect of NGF on differentiation capacity, isolated CD271+ DMSCs from DP were induced to undergo odontogenic differentiation. Compared to OIM alone, 2ng and 10ng of NGF with OIM increased CD271+ DMSCs capacity to differentiate into the odontogenic lineage as demonstrated by ALP staining on the seventh day (Figure 5.1A). Quantification of ALP activity revealed significant increase of approximately 5 to 10 fold in CD271+ DMSCs treated with either 2ng or 10ng of NGF compared to OIM only (Figure 5.1B). In addition, isolated DMSCs treated with NGF had formation of mineralized nodules after prolonged treatment with OIM for 14 days as demonstrated by ARS (Figure 5.1A). Quantification of ARS also showed significant mineralization potential in NGF treated DMSCs with a 3.5 to 4-fold increase compared to only OIM induced DMSCs.

Using CD271+ isolated DMSCs, we further confirmed the effect of NGF on their odontogenic potential by examining mRNA expression of several odontogenic marker genes including *DLX5*, *RUNX2, BGLAP, DMP1, and DSPP* at different time points: 0, 3, 7, and 10 days after odontogenic induction. Consistent with ALP and ARS results, odontogenic marker gene expression was significantly upregulated for isolated CD271+ DMSCs treated with NGF (Figure 5.2). In particular, marked increase of *DLX5* and *RUNX2* expression was found with 2ng/mL of NGF at day 3 of induction compared to 10ng/mL of NGF (Figure 5.2A, B). At day 7 there was a marked increase of *DLX5*, *RUNX2*, and *BGLAP* expression with 2ng/mL of NGF,

but an almost 20-fold increase of *DLX5* with 10ng/mL of NGF (Figure 5.2A, B, C). Lastly, significant *DMP1* and *DSPP* expression induction was found at day 7 and 10 of induction, with increasing NGF concentrations (5.2D, E). In particular, there was a marked increase of DMP1 at Day 7 of odontogenic induction with 2ng/mL of NGF (5.2D).

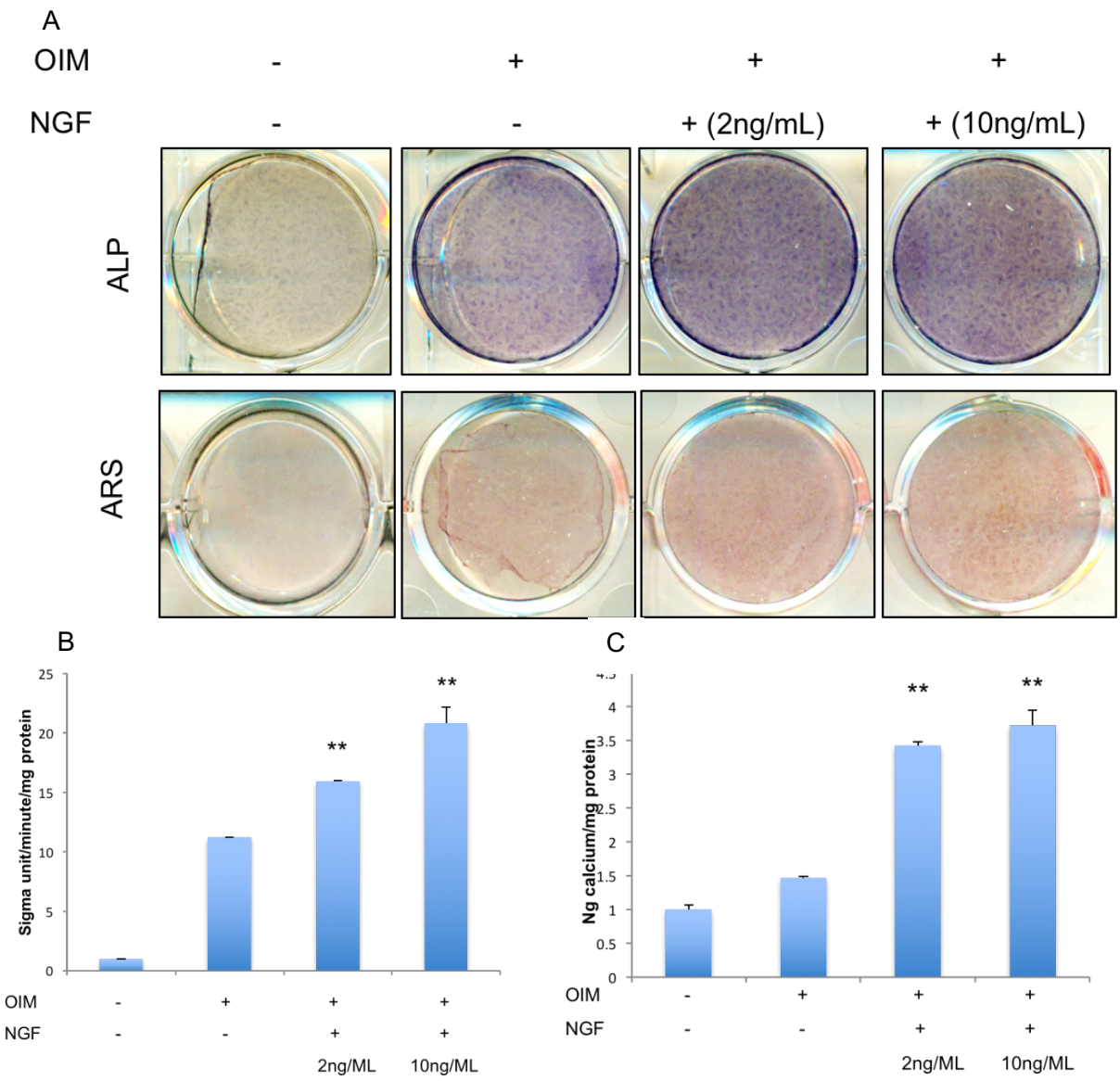

Figure 5.1 Odontogenic differentiation of isolated CD271+ DMSCs from DP with NGF treatment. (A) ALP staining and Alizarin Red staining after these cells were induced to differentiate into odontogenic lineages for 7 days and 14 days respectively. **(B)** Quantification of ALP activity **(C)** Quantification of ARS. Values were normalized to non-induce d CD51+/CD140α+ DMSCs. * $p<0.05$, ** $p<0.001$.

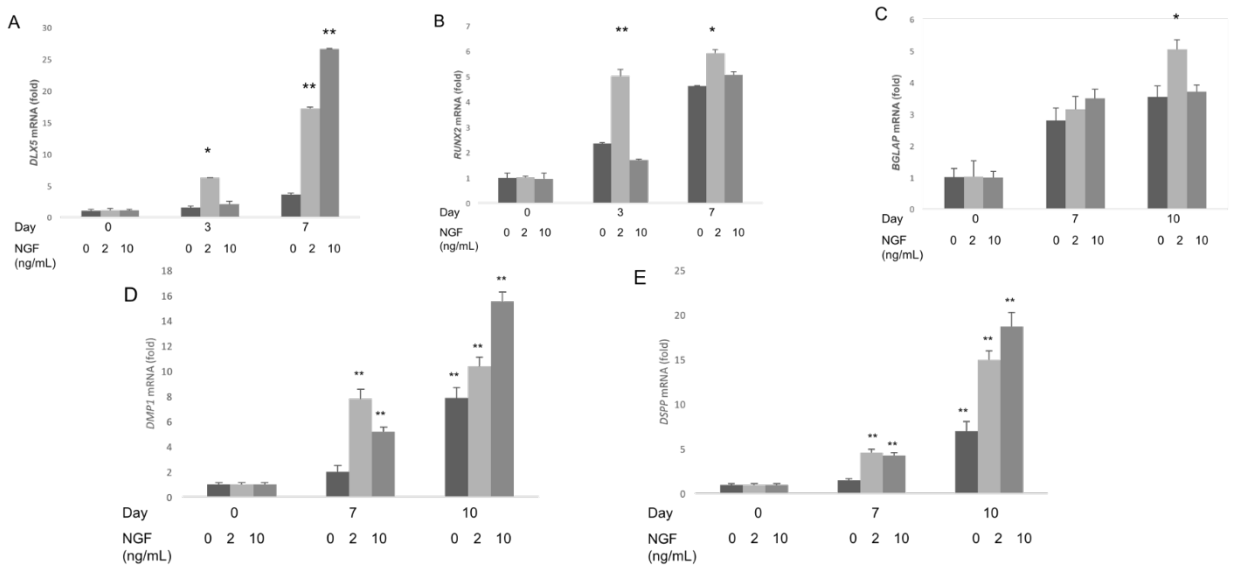

Figure 5.2 mRNA expression of odontogenic genes after 0, 3, 7, and 10 days of odontogenic induction in CD271+ DMSCs with NGF treatment. (A) DLX5 **(B)** RUNX2 and **(C)** BGLAP **(D)** DMP1 **(E)** DSPP. Values were normalized to day 0 with 0 ng/mL of NGF
* $p<0.05$, ** $p<0.001$.

5.2 NGF has no effect on the migration of CD271+ DMSCs

Since NGF increased the odotonogenic potential of DMSCs we wanted to see if it had the capacity to promote migration of CD271+ DMSCs from DP. The effect of 2ng/mL and 10ng/ML of NGF were examined by Matrigel invasion assay. We found no difference between NGF and FBS control. (Fig 5.3)

5.3 NGF activates signaling through the MAPK, AKT, MTOR, and NF-κB pathways in CD271+ DMSCS

Based on previous studies that have shown the significance of MAPK, AKT, MTOR, and NF-κB in both odontogenic differentiation and NGF signaling we first decided to investigate if NGF activates through similar signaling pathways in DMSCs. To determine if intracellular MAPK, AKT, MTOR, and NF-κB were activated by the presence of extracellular NGF, DMSCs were treated with 2ng/mL of human recombinant β-NGF for 0, 30min, 1hr, 2hrs, and 4hrs; and then analyzed on western blot. As shown in Fig 5.4 all were activated by NGF in a time dependent matter, but with varying degrees. MAPK activity increased significantly compared with the AKT, MTOR, and NF-κB at 4hrs. In addition, we also observed a decrease in p38 with NGF treatment.

5.4 TrkA and p75 promotes odontogenic differentiation of DMSCs

To evaluate whether TrkA and p75 play a role in the fate determination of DMSCs we knocked down both TrkA and p75 with siRNA. When DMSCs were induced to undergo odontogenic differentiation, siRNA of p75 cells lost their capacity to differentiate as demonstrated by ALP staining on the seventh day more so than TrkA siRNA (Fig 5.5A, B). Furthermore, the formation of mineralized nodules with prolonged treatment with odontogenic

media for 14 days was suppressed with p75 siRNA (Fig 5.5A, B). We further confirmed that p75 siRNA inhibited odontogenic potential by examining mRNA expression of several odontogenic marker genes at different time points after induction. In particular, p75 siRNA significantly suppressed the expression of marker genes *DLX5*, *RUNX2*, *BGLAP* during odontogenic differentiation indicating that p75 is required for odontogenic differentiation of MSCs (Fig 5.6A, B, C).

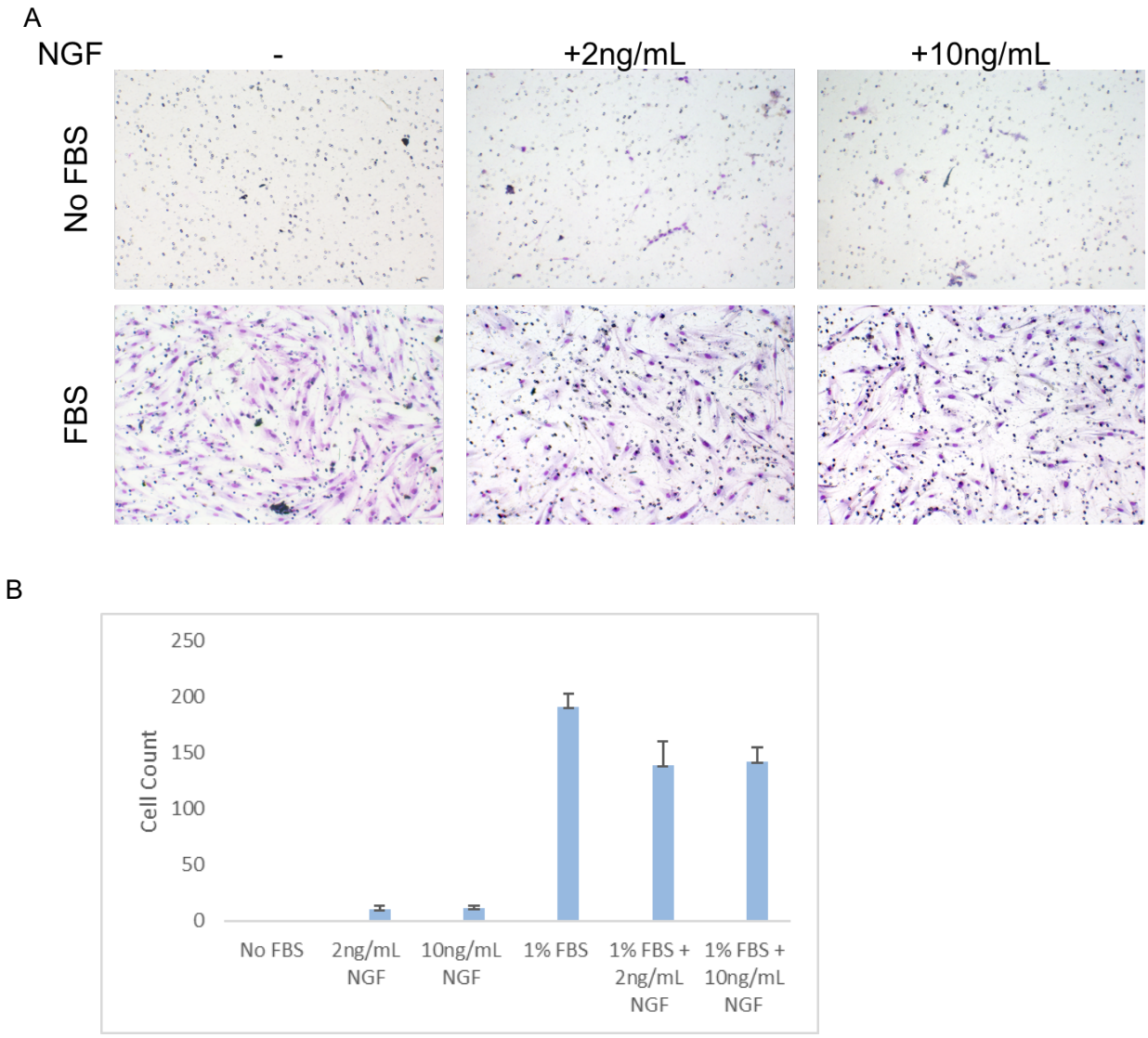

Figure 5.3 NGF has no effect on migration ability of CD271+ DMSCs. (A) Light microscopy images of CD271+ DMSCs with 2ng/mL and 10ng/mL of NGF alone, and with 1% FBS after 24 hours. Magnification was 10x. **(B)** Quantification of Matrigel Invasion Assay

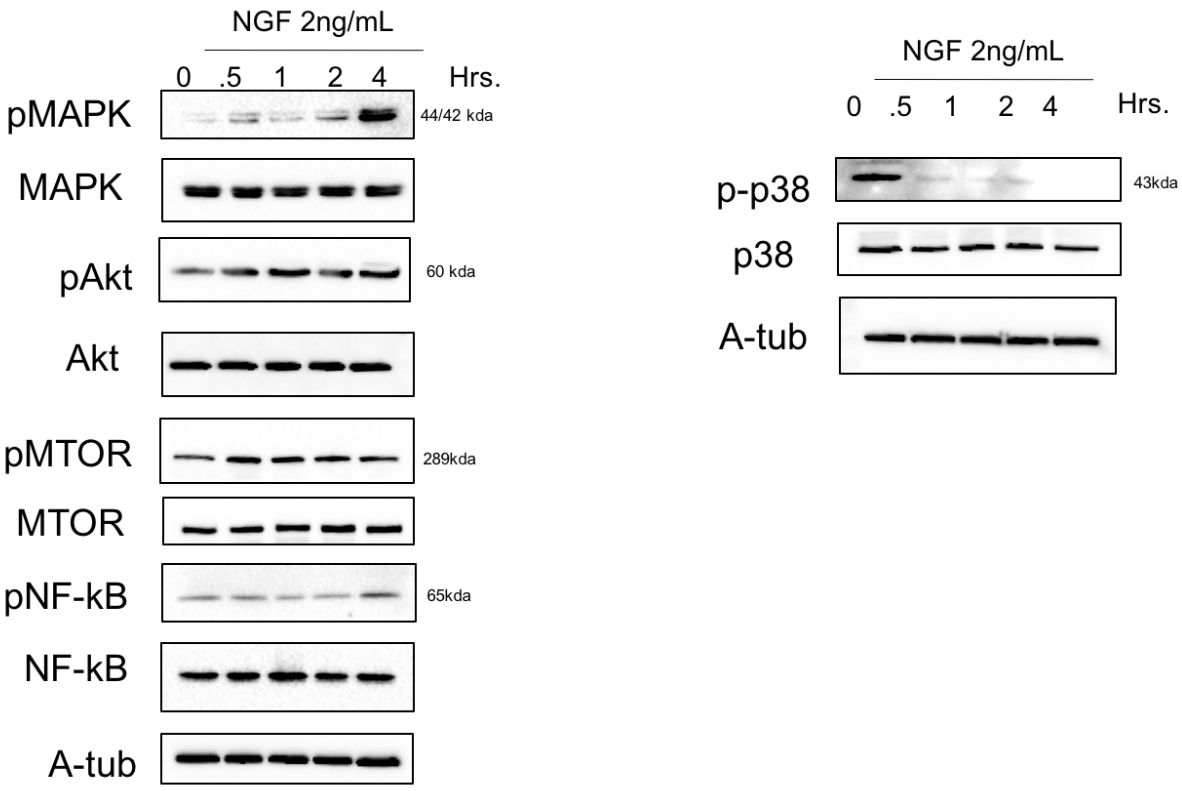

Figure 5.4 NGF activates MAPK, Akt, MTOR, and NF-κB signaling pathways in CD271+ DMSCs. Western blot was performed to evaluate the expression of MAPK, Akt, MTOR, NF-κB, and p38 with 2ng/mL of NGF at 0, 30min, 1hr, 2hrs, and 4hrs.

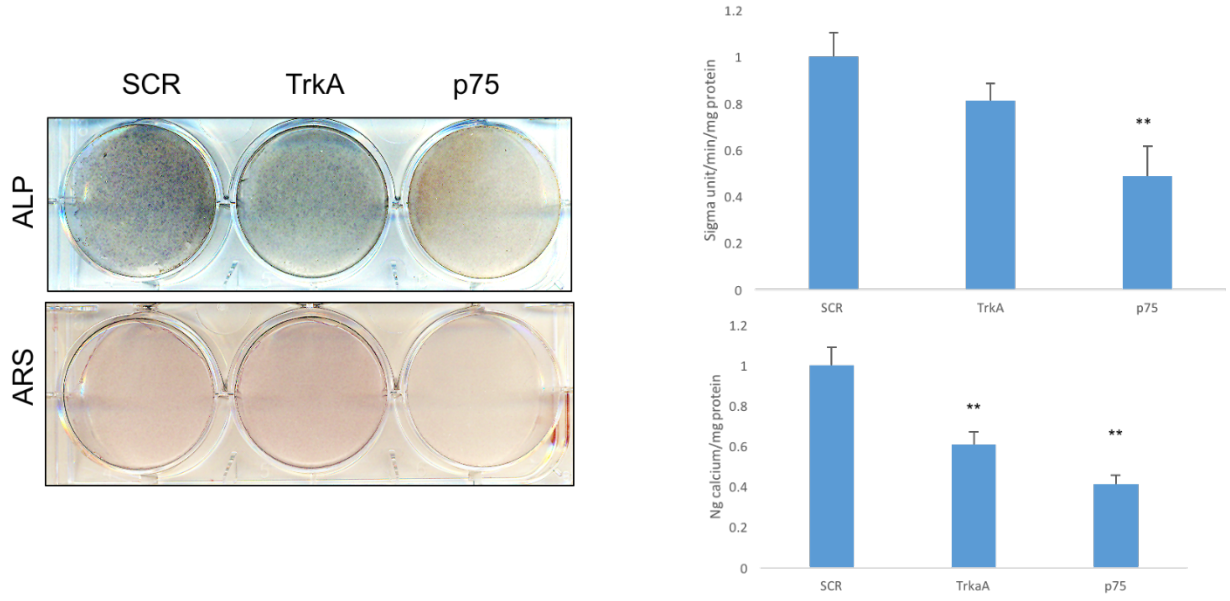

Figure 5.5 TrkA and p75^{NTR} promotes odontogenic differentiation of DMSCs (A) ALP staining and Alizarin Red staining after 24hr siRNA knockdown and cells were induced to differentiate into odontogenic lineages for 7 days and 14 days respectively. **(B)** Quantification of ALP activity **(C)** Quantification of ARS. Values were normalized to SCR * $p<0.05$, ** $p<0.001$.

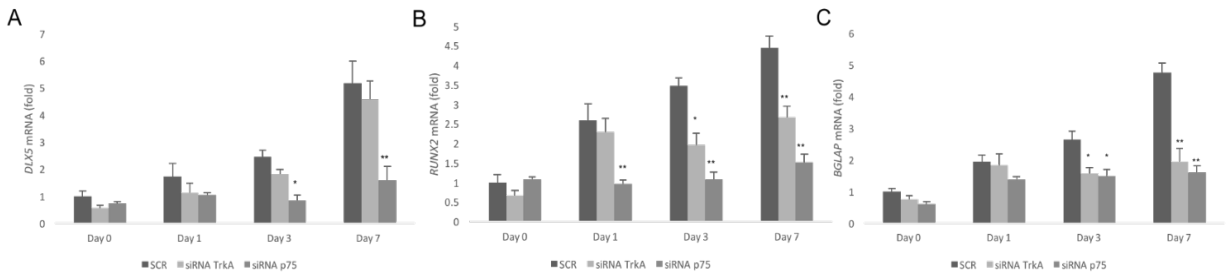

Figure 5.6 mRNA expression of odontogenic genes after 0, 3, 7, and 10 days of odontogenic induction after siRNA knockdown of TrkA and $p75^{NTR}$ receptors in DMSCs. **(A)** DLX5 **(B)** RUNX2 and **(C)** BGLAP. Values were normalized to day 0 SCR. * $p<0.05$, ** $p<0.001$.

5.5 NGF activation of MAPK, AKT, MTOR, and NF-κB pathways is mediated via the TrkA and p75^{NTR} receptors

As our results demonstrate, both TrkA and p75 promote odontogenic differentiation; with p75 having a greater effect. To test whether NGF signaling through MAPK, AKT, MTOR, and NF-κB is mediated by TrkA and p75 receptors we assessed the activation of these pathways after knockdown of TrkA and p75 in DMSCs. After 24hours of siRNA knockdown, we treated cells with 2ng/mL of NGF for 4hrs. Compared to the SCR control, we saw that MAPK, AKT, MTOR, and NF-κB signaling were significantly affected with TrkA knockdown (Fig 5.7). MAPK and AKT pathways were affected with p75NTR siRNA, but not as dramatic as with the TrkA knockdown (Figure 5.7). Although these results indicate that TrkA has a greater effect on signaling pathways compared to p75 after 4hr treatment, longer time course assessment would be important since odontogenic differentiation is assessed at 7 and 14 days with ALP, ARS, and mRNA expression of marker genes.

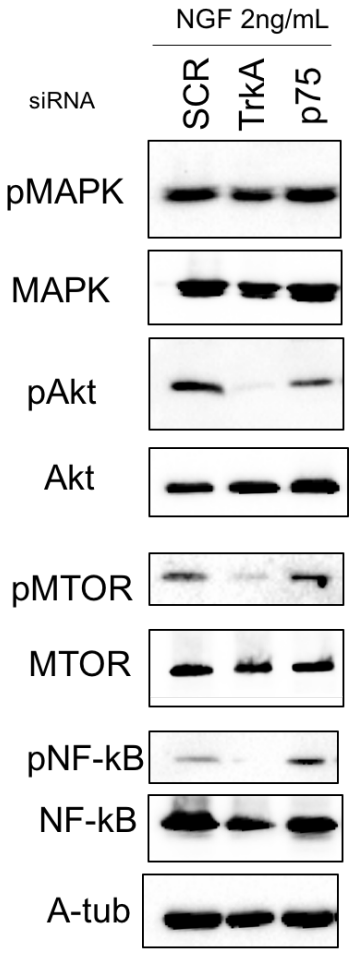

Figure 5.7 MAPK, Akt, MTOR, and NF-κB after TrkA and P75NTR siRNA knockdown in DMSCs. Western blot was performed to evaluate the expression of MAPK, Akt, MTOR, NF-κB after 36hrs of siRNA knockdown and 4hr of 2ng/mL of NGF

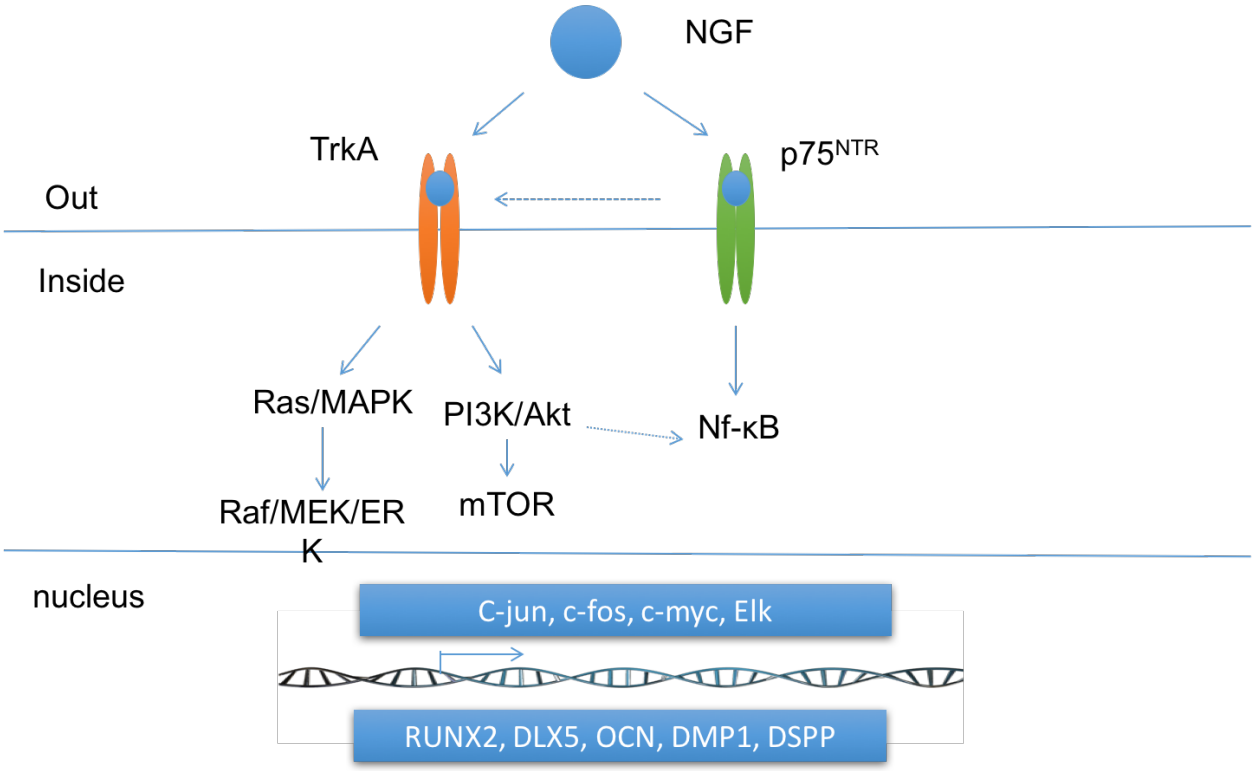

Figure 5.8 Proposed model of NGF signaling in DMSCs

5.6 Discussion

While numerous stem cell surface markers have been routinely used to identify putative MSCs, specific markers for successful isolation of DMSCs that lead to potent differentiation into multiple lineages are lacking.[4,5] In this study, we systematically used three different sets of surface markers (CD51/CD140α, CD271 and STRO-1/CD146) to isolate putative stem cell populations from primary DP and PDL cultures and compared their differentiation capacity into odonto/osteogenic, chondrogenic, and adipogenic lineages. CD51/CD140α and CD271 have been previously used to isolate neural crest cell progenitors while STRO-1/CD146 combination has been used to isolate MSCs localized to blood vessels.[6,8,32,67] In addition, the utilization of CD51/CD140α combination for DMSC isolation is not known. Our results demonstrated that although all three combinations of surface markers were able to isolate DMSCs from DP and PDL with varying magnitudes of differentiation capabilities, CD271 was the best single marker for isolating the DMSC progenitor population with greatest differentiation potential.

Mabuchi *et al*. first utilized CD271/CD90/CD106 combination of surface markers to selectively isolate a distinct subpopulation of highly potent MSCs *in-vivo*.[7] Therefore, we initially decided to explore the use of all three markers in DPCs and PDLCs. Our data showed unreasonably high proportion of both DPCs and PDLCs expressed CD90 (Fig 3.1D) suggesting that CD90 may cause non-specific isolation of DMSCs. Conversely, FACS analysis revealed that0,1% of DPCs and 0% of PDLCs expressed both CD271 and CD106 (Fig 3.1E). As such, CD271 alone was used in this study to identify DMSCs from DPCs and PDLCs. CD271, or p75 neurotrophin receptor ($p75^{NTR}$ or low affinity nerve growth factor receptor /LNGFR), is a common receptor for all neurotrophins that stimulate neuronal cells to survive and differentiate.[68,69] Of particular use with BMSCs, it has been demonstrated that CD271 consistently isolates highly multipotent BMSCs and has been described as the most selective marker for the purification and isolation of BMSCs.[7,70,71,72,73,74]

10.6% of primary culture DPCs were positive for CD271 (Figure 3.1). On the contrary to our studies, CD271+ cells from CD44+/CD90+ MSCs of human deciduous dental pulp tissues could not differentiate into osteoblasts and adipocytes.[67] Currently, the reasons for these conflicting results are unknown. It could be due to the discrepancy in tissue sources for MSC isolation. Our study showed that CD271+ DMSCs exhibited the most significant odontogenic differentiation potential with high ALP activity, mineralization capacity, and upregulated expression of *DLX5*, *RUNX2*, and *BGLAP* (Figure 3.3,3.4). Similarly, during chondrogenic lineage differentiation, CD271+ DMSCs exhibited the greatest chondrogenic potential demonstrated by increased Alcian Blue Staining and *SOX9* expression levels. Therefore, CD271 can be identified as a single specific surface marker for isolating putative DMSCs that are highly multipotent.

We successfully isolated DMSCs from PDL using CD271 but the expression was minimal at just 0.8% of PDLCs. This low percentage of CD271+ cells is consistent with the results in BMSCs, which reported less than 1% are CD271+.[7,70,71,72,73,74] Although CD271 isolated only 0.8% of DMSCs, CD271+ cells had highly significant osteogenic potential and produced the greatest upregulation of osteogenic and periodontal marker genes during induction. Alexander *et al.* previously reported that CD271 is an early surface marker of osteogenic capacity *in vitro* in human jaw periosteum-derived progenitor cells (JPCs) as CD271 was able to distinguish between mineralizing JPCs and non-mineralizing JPCs using FACS analysis.[75]

The use of CD51/CD140α to isolate DMSCs from DP or PDL has not been previously studied. The intermediate filament protein, Nestin, identifies a distinct population of highly multipotent MSCs from mouse bone marrow.[6] However, because of the intracellular location of Nestin, its utilization to identify BMSCs *in-vivo* would require cell permeabilization preventing the isolation of live cells.[6] In order to address this shortcoming, Pinho *et al.* used CD51/ CD140α cell marker combination to isolate a stromal population that is Nestin+ and highly enriched in

MSCs in the mouse and human bone marrow.[6] Clonally expanded CD51+/ CD140α+ cells exhibited robust differentiation potential into osteoblastic, adipocytic, and chondrocytic lineages *in vivo*.[6] Our results demonstrated that CD51 and CD140α markers are also useful for isolating DMSCs that exhibit multilineage potential. (Figures 3.3,3.4,3.5) On average, 27.3% of DPCs were positive for both CD51 and CD140α, providing the largest proportion of DPCs among the three surface marker combinations in this study (Figure 3.1). Consistent with these findings, a high proportion (24%) of PDLCs were positive for CD51 and CD140α(Figure 4.1). This large yield of CD51+/CD140α+ DMSCs from the PDL is consistent with high Nestin expression in the majority of adult neural crest stem cells.[76,77]

STRO-1 and CD146 are considered to be early MSC surface markers that have been identified in BMSCs and are derived from perivascular cells.[78] STRO-1 was initially associated with the identification of osteogenic precursors isolated from bone marrow and later described as a promising marker for MSCs.[19] However, it was suggested that the use of STRO-1 as a single marker for MSC isolation is limited; as it is not universally expressed in all types of MSCs and it can gradually decline with increasing culture passages.[14,79,80] In this study, CD146 was used with STRO-1 to address these STRO-1 related concerns. The lowest proportion of DPCs, 0.3%, was STRO-1+/CD146+ (Figure 3.1), making it challenging to expand the STRO-1+/CD146+ DMSCs for further use. The proportion of STRO-1+/CD146+ cells was significantly smaller in these DPCs compared to periodontal ligament cells (2.6%) and apical papilla cells (13.4%).[32,66] While ALP activity and mineralization capacity was increased in induced STRO-1+/CD146+ DMSCs compared to non-induced STRO-1+/CD146+ DMSCs, the enhancement was not as significant as CD271+ or CD51/CD140α DMSCs. Less significant odontogenic and chondrogenic potential was found in STRO-1+/CD146+ DMSCs compared to the other two combinations (Figures 3.3,3.4, 3.5). Although STRO-1 is a widely utilized MSC marker, it is not the best surface marker for isolating DMSCs with high differentiation capabilities from DPCs

even when combined with CD146.[8,19,80] Our study with FACS analysis using the STRO-1/CD146 combination isolated 2.4% of PDLCs and these sorted STRO-1+/CD146+ DMSCs demonstrated high odonto/osteogenic potential (Figure 4.1C), confirming the results of a previous study.[32] Consistent with the study by Xu et al, the adipogenic potential of STRO-1+/CD146+ DMSCs was insignificant confirming a preferential bias of STRO-1+/CD146+ DMSCs toward odonto/osteogenic and chondrogenic differentiation (Figure 4.7).[32]

It is noteworthy that DP and PDL are originally derived from neural crest cells that have both ectodermal and mesenchymal components and can provide a pure MSC population.[5,18,81,82] As some recent studies hypothesized that MSCs are neural crest derived, isolating DMSCs with surface markers of a neural crest origin such as CD51/CD140α and CD271 may be desirable.[83,84] In line with this notion, our study indicated that CD271+ and CD51+/CD140α+ DMSCs exhibited more significant multipotent populations from DP and PDL compared to STRO-1+/CD146+ DMSCs. Therefore, isolation of DMSCs using surface markers of neural crest origin other than CD271 and CD51/CD140α may be important to further evaluate their potency in differentiation potential.

In conclusion, our findings demonstrated the successful isolation of distinct subpopulations of DMSCs from human DP and PDL with the use of CD51/CD140α, CD271, and STRO-1/CD146 surface markers and demonstrated their capacity to undergo differentiation into odonto/osteogenic and chondrogenic lineages. Each marker yielded a different quantity of isolated mesenchymal progenitor cells with varying magnitude of multi-lineage differentiation potential. As CD51/CD140α produced isolation of significantly higher proportion of DPCs and PDLCs than the other two cell surface marker combinations, CD51/CD140α may be a sufficient marker combination to use with FACS analysis to obtain highly multipotent MSCs from the PDL. Further studies are needed to validate whether these isolated cells may differentiate into functionally different lineages *in-vivo*.

Based on these findings, we decided to investigate the role of CD271 in the odontogenic differentiation of DMSCs. Since CD271 was sufficient for the isolation of a strong odontogenic progenitor population of DMSCs from DP, we isolated these cells and studied the effect of NGF on odontogenic differentiation. We decided to use NGF, since it exerts its effect through the $trkA^{NGFR}$ and $p75^{NTR}$/CD271 receptors.[70] Our results demonstrate that exogenous treatment of NGF increased odonotogenic potential of CD271+ DMSCs from DP as demonstrated through the increase of ALP, ARS, and expression of odontogenic marker genes. Although it is well known for its physiological role in neurobiology, NGF has been found to be produced and utilized by several non-neuronal cell types. Previously, NGF has been found to be associated with tooth morphogenesis and induce the differentiation of immortalized dental papilla cells into odontoblast-like cells *in vitro*, suggesting that NGF acts as a stimulant for mineralization which is consistent with our findings.[68] Since our results indicated that NGF increases odontogenic activity, we decided to asses if NGF increases migration ability of DMSCs with a Matrigel invasion assay. 2ng/mL and 10ng/mL of NGF produced no effect on migration of DMSCs.

NGF is a glycoprotein consisting of 3 subunits (α_2,β,γ_2). This form of NGF is also known as proNGF.[58] The γ subunit of this complex acts as a serine protease, and cleaves the N-terminal of the β subunit, thereby activating the protein into a functional NGF. [58] Its effects are mediated by the TrkA and $p75^{NTR}$/CD271 receptors.[58] The cascade involved in TrkA signaling features MAPK-Ras-ERK pathway, phospholipase Cy1, P3I kinase and SNT proteins.[85] Less known NGF signaling is through $p75^{NTR}$. It involves activation of Nf-κB and phosphorylation for the transcription factors JNK as well as increased production of ceramide, leading to gene transcription or programmed cell death.[46] Previously, NGF has been found to activate PI3K/Akt and MAPK signaling during MSC differentiation into neurons.[85,86] Nf-κB activation is specific to $p75^{NTR}$, and dentin phosphoryin promoted odontoblastic survival and differentiation through activation of Akt and mTOR pathways leading to the activation of Nf-κB.[63,87] Yet, the role of NGF

signaling in the odontogenic differentiation of DMSCs has never been done. Based on this knowledge we decided to investigate NGF signaling in DMSCs. We selected a few candidates: MAPK, AKT, MTOR, and Nf-κB based on previous studies. 2ng/mL of NGF was used on DMSCs, since this concentration was sufficient in promoting odontogenic potential. We found that activity of all candidates increased in a time dependent manner, yet MAPK was significantly upregulated. Our findings are consistent with previous studies, and provide novel knowledge of the effect of NGF on DMSC signaling.

In order to understand the functional role of $TrkA^{NGFR}$ and $p75^{NTR}$/CD271 in the odontogenic differentiation of DMSCs, we used siRNA to knockdown both receptors and evaluate their effect. Decreased ALP and ARS activity compared to the SCR control was seen with both $TrkA^{NGFR}$ and $p75^{NTR}$/CD271 knockdown, but p75NTR produced a greater effect. This was further confirmed with decreased expression of odontogenic genes *DLX5*, *RUNX2*, *BGLAP*. The functional role of $p75^{NTR}$/CD271 has not been investigated in DMSCs. Since most NGF signaling has been associated with the TrkA receptor, our results are novel and provide insight into the role of $P75^{NTR}$/CD271 during odontogenic differentiation. To further investigate the role of both receptors in NGF signaling, we looked at the effects of TrkA and $P75^{NTR}$/CD271 knockdown on the protein level. After knockdown, we treated cells with 2ng/mL of NGF for 4 hours and evaluated MAPK, AKT, MTOR, and Nf-κB pathways. Our results show that TrkA knockdown provided a greater effect on NGF signaling compared to $P75^{NTR}$/CD271. This is interesting since $P75^{NTR}$/CD271 promotes odontogenic differentiation. Although TrkA appears to play an important role in NGF signaling, assessing longer time points would be necessary to further understand the role of $P75^{NTR}$/CD271 in NGF mediated odontogenic differentiation of DMSCs.

In conclusion, our findings demonstrated the successful isolation of homogenous populations of DMSCs from heterogenous DPCs and PDLCs with the use of specific neural

surface markers and showed their ability to differentiate into odontogenic and chondrogenic lineages. The most effective marker was CD271, which isolated DMSCs from DP and PDL and had the strongest odontogenic and chondrogenic potential. Further studies are needed to validate whether these isolated cells may differentiate into functionally different lineages *in-vivo*. Furthermore, NGF was found to promote odontogenic differentiation of CD271+ DMSCs from DP and activated MAPK, AKT, MTOR, and NF-κB pathways (Figure 5.8). Interestingly, the functional role of $P75^{NTR}$/CD271 was found to promote odontogenic differentiation in DMSCs as demonstrated through differentiation assays and expression of odontogenic genes. Although NGF signaling appears to be occur mostly through the TrkA receptor at early time points, further studies are need to evaluate signaling at later time points. Collectively, the findings of this study present the possibility of using the synergistic ability of DMSCs and NGF to promote odontogenic differentiation for tissue regeneration, but these results need to be further confirmed *in-vivo.*

6 SUMMARY AND CONCLUSION

Our findings provide important insight into using specific surface markers for identifying DMSCs in human DP and PDL for their improved utilization in regenerative medicine. Secondly, our findings will contribute to the use of growth factors such as NGF with DMSCs in developing novel therapeutics for pulpal regeneration that would benefit patients suffering of dental caries; a leading infectious disease in the United States.

In Chapters 4 and 5:

1. We used different combinations of surface markers CD51/CD140α, CD271, and STRO-1/CD146 to isolate homogenous populations of DMSCs from human heterogeneous DP and PDL.
2. We found that all three combinations (CD51/CD140α, CD271, and STRO-1/CD146) successfully isolated DMSCs with significant odont/osteogenic differentiation and chondrogenic potentail. Adipogenic potential was minimal.
3. CD271 was effective in isolating a significant multilineage population from both DP and PDL. Surprisingly, CD271 has not been used in the isolation of DMSCs.

In Chapter 6:

1. We found that exogenous treatment of NGF increased odonotogenic potential of CD271+ DMSCs from DP.
2. Furthermore, we investigated NGF signaling in DMSCs and found that MAPK, Akt, mTOR, and NF-κB increased with 2ng/mL of NGF treatment in a time dependent manner.
3. In order to fulling understand the role of CD271 in NGF signaling we used siRNA to knockdown both trkA^{NGFR} and p75^{NTR} receptors, and found that both decreased odontogenic potential of DMSCs, but p75^{NTR} affected differentiation of DMSCs. This was

confirmed with ALP and ARS assays, and decreased expression of odontogenic genes. Most NGF signaling has been studied with the trkA^{NGFR} receptor, our provides novel information about the importance of p75^{NTR} in mineralization and odontogenic differentiation.

4. Furthermore on the protein level, knockdown of trkA^{NGFR} and p75^{NTR} demonstrated that loss of trkA^{NGFR} produced a greater effect with NGF signaling. Yet, longer time points need to investigated to understand the role of p75^{NTR} in NGF signaling.

Our findings may provide an efficient method to use DMSCs and NGF in regenerative medicine, dental therapeutics for pulpal regeneration or craniofacial defects, and improve our understanding of the molecular mechanisms involved in odontogenic differentiation and NGF signaling.

7 REFERENCES

1. Dye BA, Thornton-Evans G, Li X, Iafolla Tj. Dental caries and tooth loss in adults in the United States, 2011-2012. NCHS Data Brief 2015; 197: 1-9.

2. Estrela C, Alencar AH, Kitten GT, Vencio EF, Gave E. Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. Braz Dent J 2011; 22(2):91-98.

3. Karamzadeh R & Eslaminejad MB. Dental-related stem cells and their potential in regenerative medicine. Razieh Karamzadeh and Mohamadreza Baghaban Eslaminejad (2013). Dental-Related Stem Cells and Their Potential in Regenerative Medicine, Regenerative Medicine and Tissue Engineering, Prof. Jose A. Andrades (Ed.), 2013. InTech.

4. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 2009; 88(9):792-806.

5. Mao JJ, Prockop DJ. Stem cells in the face: tooth regeneration and beyond. Cell Stem Cell 2012; 11(3):291-301.

6. Pinho S, Lacombe J, Hanoun M, Mizoguchi T, Bruns I, Kunisaki Y, Frenette PS. PDGFRα and CD51 mark human nestin^{+} sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. J Exp Med 2013 210(7):1351-1367.

7. Mabuchi Y, Morikawa S, Harada S, Niibe K, Suzuki S, Renault-Mihara F, Houlihan DD, Akazawa C, Okano H, Matsuzaki Y. LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. Stem Cell Rep 2013; 1(2):152-165.

8. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone and Miner Res 2003; 18(4):696-704.

9. Norsat CA, Fried K, Ebendal T, Olson L. NGF, BDNF, NT3, NT4 and GDNF in tooth development. Eur J Oral 1998; 106(1):94-99.

10. Wei X, Yang X, Han ZP, Qu FF, Shao L, Shao L, Shi YF. Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol Sin 2013; 34(6):747-754.

11. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007; 213(2):341-347.

12. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411):143-147.

13. Caplan AI, Correa D. The MSC: an injury drugstore. Cell Stem Cell 2011; 9(1):11-15.

14. Lv FJ, Tuan RS, Cheung KM, Leung VY. The surface markers and identity of human mesenchymal stem cells. Stem Cells 2014; 32(6):1408-1419.

15. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. J Dent Res 2002; 81(8):531-535.

16. Jo YY, Lee HJ, Kook SY, Choung HW, Park JY, Chung JH, Choung YH, Kim ES, Yang HC, Choung PH. Isolation and characterization of postnatal stem cells from human dental tissues. Tissue Eng 2007; 13(4):767-773.

17. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue Eng 2006; 12(10):2813-2823.

18. Navabazam AR, Sadeghian-Nodoshan F, Sheikhha MH, Miresmaeili SM, Soleimani M, Fesahat F. Characterization of mesenchymal stem cells from human dental pulp, preapical follicle and periodontal ligament. Iran J Reprod Med 2012;11(3):235-242.

19. Coura GS, Garcez RC, De Aguiar CB et al. Human periodontal ligament: a niche of neural crest stem cells. *J Periodontal Res* 2008. 43(5): 531-536.

20. Miletich I, Sharpe PT. Neural crest contribution to mammalian tooth formation. *Birth Defects Res C Embryo Today* 2004.72(2): 200–212.

21. Lekic P, Rojas J, Birek C et al. Phenotypic comparison of periodontal ligament cells in vivo and in vitro. *J Periodontal Res* 2001. 36(2): 71–79.

22. Shimono M, Ishikawa T, Ishikawa H et al. Regulatory mechanisms of periodontal regeneration. *Microsc Res Tech* 2003. 60(5): 491–502.

23. Nagamoto K, Komaki M, Sekiya I et al. Stem cell properties of human periodontal ligament cells. *J Periodontal Res* 2006. 41(4): 303-310.

24. Reinhardt RA, Payne JB, Maze CA et al. Influence of estrogen and osteopenia/osteoporosis on clinical periodontitis in postmenopausal women. *J Periodontol* 1999. 70(8): 823-828.

25. Petersen PE, Ogawa H. The global burden of periodontal disease: towards integration with chronic disease prevention and control. *Periodontol 2000* 2012. 60(1): 15-39.

26. Kim SH, Seo BM, Chuong PH et al. Adult stem cell therapy for periodontal disease. I*nt J Stem Cells* 2010. 3(1): 16-21.

27. Lindhe J, Lang NP. *Clinical periodontology and implant dentistry*, 6th edn. Oxford: Wiley-Blackwell, 2015.

28. Bartold PM, Xiao Y, Lyngstaadas SP et al. Principles and applications of cell delivery systems for periodontal regeneration. *Periodontol 2000* 2006. 41(1): 123-135.

29. Park JC, Kim JM, Jung IH et al. Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from inflamed PDL tissue: *in vitro* and *in vivo* evaluations. *J Clin Periodontol* 2011. 38(8): 721-731.

30. Silvero KG, Rodrigues TL, Coletta RD et al., Mesenchymal stem cell properties of periodontal ligament cells from deciduous and permanent teeth. *J Periodontol* 2010. 81(8): 1207-1215.

31. Seo BM, Miura M, Gronthos S et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004. 364:149-55.

32. Xu J, Wang W, Kapila Y et al. Multiple differentiation capacity of STRO-1+/CD146+ PDL mesenchymal progenitor cells. *Stem Cells Dev* 2009. 18(3): 487-496.

33. Bueno C, Ramirez C, Rodriguez-Lozano FJ et al. Human adult periodontal ligament derived cells integrate and differentiate after implantation into the adult mammalian brain. *Cell Transplant* 2013. 22(11): 2017-2028.

34. Li X, Gong P, Liao D. *In vitro* neural/glial differentiation potential of periodontal ligament stem cells. *Arch Med Sci* 2010. 6(5): 678-685.

35. Wada N, Menicanin D, Shi S et al. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* 2009. 219(3):667-676.

36. Nagamoto K, Komaki M, Sekiya I et al. Stem cell properties of human periodontal ligament cells*. J Periodontal Res* 2006. 41(4): 303-310.

37. Trubiani O, Di Primio R, Traini T et al. Morphological and cytofluorimetric analysis of adult mesenchymal stem cells expanded ex-vivo from periodontal ligament. *Int J Immunopathol Pharmacol* 2005.18(2): 213-221.

38. Iwata T, Yamato M, Zhang Z et al. Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. *J Clin Periodontol* 2010. 37(12): 1088-1099.

39. Lin NH, Gronthos S, Bartold PM. Stem cells and future periodontal regeneration. *Periodontol 2000* 2009. 51:239-51.

40. Dangaria SJ, Ito Y, Luan X, et al. Successful periodontal ligament regeneration by periodontal progenitor preseeding on natural tooth root surfaces. *Stem Cells Dev* 2011. 20: 1659–1668.

41. Gault P, Black A, Romette JL et al. Tissue-engineered ligament: implant constructs for tooth replacement. *J Clin Periodontol* 2010. 37(8): 750-780.

42. Kim RH, Mehrazarin S, Kang MK. Therapeutic potential of mesenchymal stem cells for oral and systemic diseases. *Dent Clin North Am* 2012. 56(3): 651-675.

43. Abdallah BM, Kassem M. Human mesenchymal stem cells: from basic biology to clinical applications. Gene Ther 2008; 15(2):109-116.

44. Kim SG, Solomon C, Zheng Y, Suzuki T, Mo C, Song S, Jiang N, Cho S, Zhou J, Mao J. Effect of growth factors on dental stem/progenitor cells. Dent Clin North Am 2012; 56(3): 563-575.

45. Aloe L, Rocco ML, Bianchi P, Manni L. Nerve factor: from the early discoveries to the potential clinical use. J Transl Med 2012; 10(239):1-15.

46. Micera A, Lambiase A, Stampachiacchiere B, Bonini S, Bonini S, Levi-Schaffer F. Nerve growth factor and tissue repair remodeling : trkANGFR and p75NTR, two receptors one fate. Cytokine Growth Factor Rev 2007 ; 18(3-4) : 245-56.

47. Levi-Montalcini R. The nerve growth factor 35 years later. Science 1987; 237:1154-62.

48. Micera A, Puxeddu I, Aloe L, Bonini Se, Levi-Schaffer F. New Insigt on the involvement of nerve growth factor in allergic inflammation and fibrosis. Cytokine Growth Factor Rev 2003; 14:369-74.

49. Micera A, Lambiase A, Aloe L, Bonini Se, Levi-Schaffer F, Bonini St. Nerve growth factor involvement in the visual system: implications in allergic and neurodegenerative diseases. Cytokine Growth Factor Rev 2004; 15:411-7.

50. Lambiase A, Micera A, Sgrulleta R, Bonini Se, Bonini St. Nerve growth factor and the immune system: old and new concepts in the cross-talk between immune and resident cells during pathophysiologyical conditions. Cur Opin Allergy Clin Immunol 2004; 4:425-430.

51. Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 2001; 24:1217-1281.

52. Lad SP, Peterson Da, Bradshaw RA, Neet KE. Individual combined effects of TrkA and p75 NTR Nerve Growth Factor Receptors: a role for the high affinity receptor site. J Biol Chem 2003; 278:24808-24817.

53. Meakin So, Shooter EM. The nerve growth factor Receptors. Trends Neurosci 1992;15:323-31.

54. Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV. Competitive signaling between TrkA and p75 NTR Nerve growth factor receptors determines cell survival. J Neurosci 1998; 18:3273-3281.

55. Hempstead Bl. They many faces of p75(NTR). Curr Opin Nueorbiol 2002. 12:260-270.

56. Lutz M, Krieglstein K, Schmitt S, Dijke P, Sebald W, Wizenmann A, et al. Nerve growth factor mediates activation of the Smad pathway in PC12 cells. Eur J Biochel 2004.

57. Meakin So, Shooter EM. The nerve growth factor family of receptors. Trends Neurosci 1992; 15:323-31.

58. Aloe L, Bracci-Laudiero L, Bonini S , Manni L. The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. Allergy 1997; 52(9):883-94.

59. Mizuno N, Shiba H, Xu WP, Inui T, Fujita T, Kajiya M, Takeda K, Hasegawa N, Kawaguchi H, Kurihara H. Effect of neurotrophins on differentiation, calcification and proliferation in cultures of human pulp cells. Cell Biol Int. 2007; 31(12):1462-1469.

60. Arany S, Koyota S, Sugiyama T. Nerve growth factor promotes differentiation of odontoblast-like cells. J Cell Biochem 2009; 106(4): 539-545.

61. Bryers MR, Wheeler EF, Bothwell M. Altered expression of NGF and P75 NGF-receptor by fibroblasts of injured teeth precedes sensory nerve sprouting. Growth Factors. 1992; 6(1):41-52.
62. Luukko K, Moshnyakov M, Sainio K, et al. Expression of neutrotrophin receptors during rat tooth development is developmentally regulated, independent of innervation, and suggests functions in the regulation of morphogenesis and innervation. DevDyn. 1996; 206 (1): 87-99.

63. Eapen A, George A. Dentin phosphophoryn in the matrix activates AKT and mTOR signaling pathway to promote preodontoblast survival and differentiation. Front Physiol. 2015; 6(221): 1-9.

64. Wu H, Teng PN, Jayaraman T, Onishi S, Li J, Bannon L, Huang H, Close J, Sfeir C. Dentin Matrix Protein 1 (DMP1) signals via cells surface integrin. JBC. 2010; 286(34): 29462-29469.

65. Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 2013; 19(1):35-42.

66. Bakopoulou A, Leyhausen G, Volk J, Koidis P, Geurtsen W.. Comparative characterization of STRO-1(neg)/CD146(pos) and STRO-1(pos)/CD146(pos) apical papilla stem cells enriched with flow cytometry. Arch Oral Biol 2013; 58(10):1556-1568.

67. Mikami Y, Ishii Y, Watanabe N, Shirakawa T, Suzuki S, Irie S, Isokawa K, Honda MJ. CD271/p75NTR Inhibits the differentiation of Mesenchymal stem cells into osteogenic, adipogenic, chondrogenic, and myogenic lineages. Stem Cells and Dev 2011; 20(5):901-913.

68. Tomellini E, Lagadec C, Polakowska R et al. Role of p75 neurotrophin receptor in stem cell biology: more than just a marker. *Cell Mol Life Sci* 2014.71(13): 2467-2481.
69. He XL, Garcia KC. Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science* 2004. 304(5672): 870–875

70. Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA et al. LNGFR (CD271) as a marker to identify mesenchymal stem cells from different human sources: umbilical cord blood, wharton's jelly and bone marrow. *Bone Marrow Res* 2013. 1(132): 1-6.

71. Kuci S, Kuci Z, Kreyenberg H et al. CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. *Haematologica* 2010. 95(4): 651-659.

72. Jones EA, Kinsey SE, English A et al. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* 2002. 46(12): 3349-3360.

73. Quirici N, Soligo D, Bossolasco P et al. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 2002. 30(7): 783–791.

74. Flores-Torales E, Orozco-Barocio A, Gonzalez-Ramella OR et al. The CD271 expression could be alone for establisher phenotypic marker in Bone Marrow derived mesenchymal stem cells. *Folia Histochem Cytobiol* 2010. 48(4): 682–686.

75. Alexander D, Schafer F, Munz A et al. LNGFR induction during osteogenesis of human jaw periosteum-derived cells. *Cell Physiol Biochem* 2009. 24(3-4): 283-290.

76. Park D, Xiang AP, Mao FF et al. Nestin is required for the proper self-renewal of neural stem cells. *Stem Cells* 2010. 28(12): 2162–2171.

77. Kaltschmidt B, Kaltschmidt C, Widera D. Adult craniofacial stem cells: sources and relation to the neural crest. *Stem Cell Rev* 2012. 8(3): 858-671.

78. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration?. Arch Oral Biol 2012; 57(11):1439-1458.

79. Kolf CM, Cho E, Tuan RS. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther 2007; 9(1):204-214.

80. Yu J, He H, Tang C, Zhang G, Li Y, Wang R, Shi J, Jin Y. Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging. BMC Cell Bio 2010; 11(32):1-7.

81. d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G. Human dental pulp stem cells: from biology to clinical applications. J Exp Zool B Mol Dev Evol 2008; 312B(5):1-8.

82. Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, Formstecher P, Bailliez Y, Poakowska RR. Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev 2008; 17(6):1175-1184.

83. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 2003; 100(10):5807-5812.

84. Pierret C, Spears K, Maruniak JA, Kirk MD. Neural crest as the source of adult stem cells. Stem Cells Dev2006; 15(2):286-291.

85. Yuan J, Huan G, Xiao Z, Han T. Overexpression of β-NGF promotes differentiation of bone marrow mesenchymal stem cells into neurons through regulation of AKT and MAPK pathway. Mol Cell Biochem 2013; 383(1-2):201-11.

86. Wang W, Hu X, Xie X et al. Nerve growth factor induces cord formation of mesenchymal stem cell by promoting proliferation and activating the PI3K/Akt signaling pathway. Acta Pharmacol Sin. 2011; 32(12): 1483-90.

87. Carter BD, Katschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Mattaei R, Baeuerle Pa, Barde YA. Selective activation of Nf-κB by nerve growth factor through the neurotrophin receptor p75. Science 1996; 272(5261): 542-545.