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The Molecular Regulation of Nerve Growth Factor (NGF)-Mediated Osteogenic Differentiation

of Mesenchymal Stem Cells

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in Oral Biology

by

Sahlee Grace F. Peyton

2017

ABSTRACT OF THE THESIS

The Molecular Regulation of Nerve Growth Factor (NGF)-Mediated Osteogenic Differentiation

of Mesenchymal Stem Cells

by

Sahlee Grace F. Peyton

Master of Science in Oral Biology

University of California, Los Angeles, 2017

Professor Cun-Yu Wang, Co-chair

Professor Christine Hong, Co-chair

In the search for novel therapies in regenerative medicine, there has been an increasing interest in a small subset of cells that originate from the mesoderm known as mesenchymal stem cells (MSCs). MSCs prove to be a promising tool due to their potential to repair or regenerate damaged tissues. They are also capable of self-renewal and differentiating into mesoderm lineages, such as chondrocytes, osteocytes and adipocytes, but can also differentiate into ectodermic and endodermic cells. MSCs are extensively distributed in a wide range of postnatal tissue types and have been successfully isolated from orofacial tissues. MSCs derived from craniofacial tissues including dental mesenchymal stem cells (DMSCs) can be isolated and have

the potential for use in tissue engineering, including dental tissue, nerve and bone regeneration. Dental pulp stem cells, also known as DPSCs, can differentiate into cell types such as odontoblasts, osteoblasts, chondrocytes, cardiomyocytes, adipocytes, neuron cells, corneal epithelial cells, melanoma cells and insulin-secreting Beta cells; differentiation can be modulated with growth factors, transcriptional factors, extracellular matrix proteins and receptor molecules in the local microenvironment. The neurotrophin nerve growth factor (NGF) is important in the development and maintenance of sympathetic and sensory neurons, and its ability to promote mineralization also makes it an appealing candidate in bone and tissue regeneration. In this study, we wanted to investigate the molecular regulation of NGF-mediated osteogenic differentiation of MSCs from dental pulp. We found that exogenous treatment with NGF led to an increase in alkaline phosphatase (ALP) expression and activity; there was also an increase in alizarin red staining (ARS) and its quantification of calcium mineral deposition. We also observed a strong induction of mRNA expression of osteogenic genes: *BSP, DLX5, OCN, OPN,* and *RUNX2*. This treatment also led to the upregulation of the JNK and c-Jun signaling pathways in a time-dependent manner. Furthermore, we used a JNK inhibitor to evaluate the role of JNK in the osteogenic pathway and observed a decrease in osteogenic differentiation. The present study aims to better understand the molecular pathways involved in regulating NGF-mediated osteogenic differentiation of MSCs and may be useful in the development of an effective application in regenerative medicine.

The thesis of Sahlee Grace F. Peyton is approved.

Reuben Kim

Christine Hong, Committee Co-Chair

Cun-Yu Wang, Committee Co-Chair

University of California, Los Angeles

2017

This thesis is dedicated to

my parents, Edwin and Maria, and my sister, Sheena.

Thank you for your love, patience and support as I pursue my dreams.

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ACKNOWLEDGEMENTS

To the Grace that guides my path, and to all those who have provided inspiration

along the way—*I am forever grateful.*

………………………………..…………………

To my family, *I love you all*. I am grateful for the infinite support and motivation that you have

given me from an early age. I cherish every moment that we share together.

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and support on this journey—*thank you.*

1   INTRODUCTION

1.1 Mesenchymal Stem Cells

1.1.1   Definition of Mesenchymal Stem Cells

Stem cells are unspecialized cells that are capable of self-renewal and differentiation into specialized cell types, making them valuable in tissue regeneration and implementation in regenerative medicine [1, 2]. Stem cells can be categorized on the basis of origin: embryonic stem cells, induced pluripotent stem cells, and mesenchymal stem cells [3]. Embryonic stem cells (ESCs) isolated from early embryos are the most promising tool in regenerative medicine because they are pluripotent, capable of self-renewal, and can give rise to all cell lineages [3, 4]. Induced pluripotent stem cells (iPSCs) are similar to ESCs at the cellular level and are generated from adult cells by overexpressing the following transcription factors: Oct4/3, Sox2, Klf4, and c-Myc [3]. Ethical concerns regarding the isolation of ESCs as well as the potential for iPSCs to cause tumorigenesis have compromised their utilization in a clinical setting [4]. In the search for novel therapies in regenerative medicine, there has been an increasing interest in a small subset of cells that originate from the mesoderm known as mesenchymal stem cells (MSCs) which prove to be a promising tool [4-6]. MSCs have the potential to repair or regenerate damaged tissues [4, 5]. They are also capable of self-renewal and differentiating into mesoderm lineages, such as chondrocytes, osteocytes and adipocytes, but can also differentiate into ectodermic and endodermic cells [4]. MSCs exist in almost all adult tissue; they are easily isolated from the bone marrow, adipose tissue, the umbilical cord, fetal liver, muscle, and lung [4]. Additionally, MSCs can be expanded *in vitro* while maintaining their growth and multilineage potential [4, 7]. With few ethical concerns and exceptional genomic stability, the use of MSCs plays an important role in cell therapy, regenerative medicine and tissue repair [3].

1.1.2   Definition of Dental Mesenchymal Stem Cells

In the search for various sources of MSCs, the most common method to obtain them is through bone marrow aspiration, although it is painful and invasive [1, 8]. Using bone marrow stem cells (BMSCs) to stimulate bone formation has proved to be a promising tool in both animal studies and human trials [9, 10]. However, due to site morbidity, low cell number, and loss of phenotypic behavior during culturing, other potent sources of osteogenic stem cells have been explored [11]. MSCs are extensively distributed in a wide range of postnatal tissue types and have been successfully isolated from orofacial tissues [12]. MSCs derived from craniofacial tissues including dental mesenchymal stem cells (DMSCs) can be isolated and have the potential for use in tissue engineering, including dental tissue, nerves and bone regeneration [13]. Like BMSCs, DMSCs are: (1) capable of self-renewal, (2) able to differentiate into various cell types, and (3) are non-immunogenic [14]. Human DMSCs that have been isolated and characterized are: dental follicle stem cells (DFSCs), stem cells from human primary exfoliated deciduous teeth (SHEDs), dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), and stem cells from apical papilla (SCAPs) [15].

1.2 Dental Pulp

1.2.1   Definition of Dental Pulp

A tooth is composed of two layers of mineral, the enamel and dentin, that enclose the dental pulp [16]. The dental pulp is highly vascularized; blood vessels and nerve bundles enter the dental pulp through the apical foramen situated at the apex of the tooth's root that is surrounded by the rigid dentin walls and provide nutrition and a responsive sensory nervous system [17, 18]. Unlike bone, most hard tissues in the tooth do not undergo renewal after its formation; however, dentin can regenerate itself upon injury, which suggests that stem cell populations exist within

the tooth pulp [19]. Together, dental pulp and dentin act as a complex to maintain the integrity of tooth shape and function [13].

1.2.2 *Dental Mesenchymal Stem Cells from Dental Pulp*

DMSCs isolated from adult human tooth pulp were the first dental stem cells to be identified [13]. DPSCs are typically isolated from impacted third molars that are extracted during routine dental procedures; hence, they are a convenient, noninvasive and valuable source of DPSCs [1]. DPSCs can be cryopreserved for future use and revived when needed for regenerative therapies [1]. The natural function of DPSCs is to produce odontoblasts to create reparative dentin support and aid in tooth structure regeneration; however, DPSCs are also being investigated for tissue repair outside of the tooth [18]. Because DPSC differentiation is more restricted than that of BMSC *in vivo*, studying MSCs from dental pulp is ideal also because of the ease of accessibility and defined structure [16]. The extent to which DPSC biology overlaps that of skeletal stem cells is yet to be determined [16]. It has been shown that DPSC differentiation into cell types such as odontoblasts, osteoblasts, chondrocytes, cardiomyocytes, adipocytes, neuron cells, corneal epithelial cells, melanoma cells and insulin-secreting Beta cells can be modulated with growth factors, transcriptional factors, extracellular matrix proteins and receptor molecules in the local microenvironment [1, 13, 20]. Characteristics of dental pulp are also responsive to injuries or inflammation [21]. DPSCs are characterized by: their positive expression of stromal-associated markers such as CD90, CD29, CD73, CD105, CD44; their negative expression of hematopoietic antigens such as CD45, CD34 and CD14; and expression of multipotent marker STRO-1 and extracellular matrix proteins such as collagen, vimentin, laminin, and fibronectin [20]. The following pluripotent stem cell markers, Oct4, Nanog, Sox2, Klf4, SSEA4 & c-Myc, have also been reported to express on DPSCs [20]. DPSCs also express bone markers such as bone

sialoprotein, osteocalcin, alkaline phosphates, and type I collagen, which indicates their

commitment to differentiate into bone tissue [20].

1.3 Growth Factors

1.3.1 Definition of Growth Factors

Growth factors are water-soluble proteins mainly produced by osteoblasts and are

embedded in the extracellular matrix [22, 23]. Growth factors function by binding to specific cell

membrane-bound receptors that trigger intracellular signaling; this leads to signal transduction

into the nucleus where transcription factors are activated, and eventually affect the expression of

genes that encode for metabolic functions such as cell division and protein synthesis [23, 24].

Cytokines, which are typically referred to as immunomodulatory proteins or polypeptides, are

often used interchangeably with growth factors because of similarities in their actions [25].

Cytokines and growth factors tend to act locally on target cells, while hormones have more of

systemic effects [25]. Growth factors have been found in all tissue types and are known to regulate

cell-to-cell metabolism and mediate cellular effects of different hormones [23]. Growth factors play

a role in regulating multiple cellular functions including migration, proliferation, differentiation

and apoptosis of several cell types [25].

1.3.2 Effect of Growth Factors in Bone Regeneration

Studies have demonstrated that growth factors are of major importance for bone tissue

physiology making them candidates for use in regenerative medicine [23]. There is a large reservoir

of several growth factors in the bone matrix suggesting their role as regulators in bone

remodeling and initiators of bone healing [23]. Bone growth factors only affect the local cell

environment, which then stimulates neighboring bone cells to proliferate and increase matrix

protein synthesis [23]. Osteoblasts can also stimulate themselves to produce growth factors and

cause an increase in metabolic activity [23]. Studies have revealed that bone growth factors have numerous regulating effects on bone cells *in vitro*, while *in vivo* studies in animals have shown that some growth factors can stimulate the bone healing process [23]. These findings are promising for future possibilities for the clinical use of growth factor-stimulated bone formation and bone healing [23].

1.3.3   Definition of Nerve Growth Factor

Nerve growth factor (NGF) is the first neurotrophin, or neurotrophic factor, to be identified [26]. NGF is a glycoprotein of 118 amino acids, consisting of three sub-units (α_2, β, γ_2), with a molecular weight of 130 kD [27]. The active form of NGF is derived by the cleavage of its precursor form, pro-NGF, which has both pro-apoptotic and neurotrophic properties that play important roles during development and in adult life [28]. NGF is produced in and released by target tissues; specific receptors expressed on nerve terminals capture it and retrogradely transport it to the neuron body where it exerts its neurotrophic activity [28]. Since its discovery, NGF has been characterized for its physiological role first in the sensory and autonomic nervous system, then in the central nervous system, endocrine and immune systems [28]. NGF production and utilization have been found in several non-nervous cell types such as immune inflammatory cells, epithelial cells, keratocytes, and smooth muscle cells, which indicate that NGF may have roles outside the nervous system [27].

1.3.4   Nerve Growth Factor Signaling

NGF initiates its biological action through binding to its two cell surface receptors: tropomyosin kinase receptor A (TrkA) and p75 neurotrophin receptor (p75NTR) [25, 28]. The presence of two distinct receptors with specific/independent cellular activities represents a unique property for NGF in comparison to other growth factors and cytokines [27]. The affinity of

NGF binding to TrkA is higher than its binding to p75NTR; p75NTR binds all neurotrophins

with equal affinity (1 nM Kd) while TrkA binding is more specific (10 pM Kd) [27]. NGF binds to

both TrkA and p75NTR when they are co-expressed on the outer cell membrane, resulting in

enhanced NGF responsiveness, and selective binding, in which duration and magnitude depend

on the ratio of p75NTR and TrkA on the cell surface [27, 29].

The receptor TrkA is a transmembrane glycoprotein that belongs to the family of receptor

tyrosine kinase, encoded by a gene located on chromosome 1 [27]. The TrkA cascade involves the

MAPK-RAS-ERK pathway, phospholipase Cy1, P3I kinase and SNT proteins (suc-associated

neurotrophic factor-induced tyrosine-phosphorylated target) [27]. Most NGF-mediated biological

activities such as differentiation/activation to proliferation/survival are due to ligand-dependent

TrkA auto-phosphorylation and subsequent activation of several signal transduction cascades [27].

In comparison, p75NTR is a type-I transmembrane protein with an extracellular domain

that contains four repeated segments of six cysteines, which is a typical structure of the TNFR

superfamily (Fas/TNF receptor superfamily), and is devoid of intrinsic catalytic activity [27]. Its

role in NGF signaling is more complex and less clearly defined; p75NTR signaling involves

activation of NF-kB (Rel/NF-kB transcription factors), the phosphorylation of the transcription

factor c-Jun N-terminal kinase (JNK), as well as increased production of ceramide, which leads

to gene transcription or cell death [27, 29].

1.3.5   *Role of NGF on Osteogenic Differentiation*

NGF-triggered cellular activities involve migration, proliferation, differentiation, survival

and even apoptosis [27-29]. Findings on the potential pro-healing capacity of NGF may provide new

possibilities for its use in modulating the healing processes in several pathological conditions [27].

In vitro studies have shown that NGF can stimulate differentiation and inhibit apoptosis of an

osteoblastic cell line [30]. In a rabbit inferior alveolar nerve defect model, an incidental observation revealed that NGF administration stimulated bone formation around the induced regenerating axons [31]. Local administration of NGF in a rat fracture model also demonstrated an increase in the quality and rate of fracture repair [32]. It has also been shown that NGF induces the differentiation of immortalized dental papilla cells into odontoblasts *in vitro*, suggesting that NGF acts as a stimulant for mineralization [25]. These findings reveal the possibility of using NGF in a wide range of bone and tissue repair. To further understand the therapeutic potential of NGF, it is crucial to comprehend the molecular mechanisms through which NGF activates osteogenic differentiation.

1.3.6   *Signaling Pathways Implicated in Osteogenic Differentiation*

It is important to identify the molecular mechanisms involved in osteogenic differentiation for the development of regenerative therapies using growth factors such as NGF. The downstream signaling implicated in the osteogenic differentiation of MSCs is not well defined. Previous studies have shown that Runt-related transcription factor 2 (RUNX2) is a master transcription factor that regulates the expression of several osteogenic genes, including alkaline phosphatase (ALP), osteocalcin (OCN), and bone sialoprotein (BSP) [33]. The MAPK pathway has been shown to be involved in osteoblast development; in particular, the ERK and P38 MAPKs are essential for RUNX2 activation, whereas JNK is important for late-stage osteoblast differentiation [33]. Zhao *et al.* recorded that BMP9, a bone morphogenetic protein belonging to the transforming growth factors β (TGFβ), stimulated the activation of JNK in MSCs [34]. Past studies provide a foundation for understanding the signaling pathways that occur during osteogenic differentiation of DPSCs and how proteins in the local microenvironment, such as NGF, play a role in mineralization.

2 MATERIALS AND METHODS

2.1 Cell Isolation and Culture

Primary dental pulp cells were isolated from extracted adult third molars (IRB#13-000241-CR-00004) under approved guidelines set by the University of California Los Angeles Institutional Review Board. Extracted teeth were stored in alpha modified Eagle's medium (α-MEM, Invitrogen, USA) with antibiotics. Dental pulp was removed, and tissue was enzymatically digested with collagenase and dispase. DPSCs were grown in α-MEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL non-essential amino acids, 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen, USA) and stored in a humidified 5% CO_2 incubator at 37°C. Media was changed every 2-3 days, and cells were passaged at 80-90% confluency. DPSCs used in this study were from passages 8-12.

2.2 Induction of Osteogenic Differentiation

DPSCs were plated into 12-well plates at 1×10^5 cells/well. To induce osteogenic differentiation into osteogenic lineages, DPSCs were grown in osteogenic induction medium (OIM) composed of α-MEM (Invitrogen, USA), 10% FBS (Invitrogen, USA), 50 μg/mL ascorbic acid, 5 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma-Aldrich, USA). OIM was replaced every 2-3 days. For experiments with human recombinant β-nerve growth factor (NGF) (R&D Systems, USA), either 2 ng/mL or 10 ng/mL was supplemented into media and replaced every 2-3 days, respectively. For experiments with a JNK inhibitor (JNKi) (SP600125, Santa Cruz Biotechnology, USA), cells were pre-treated with either 5 uM or 10 uM for one hour before NGF was added; media was replaced every 2-3 days, respectively.

ALP staining and quantification:

After seven days of osteogenic induction, DPSCs 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, USA) dissolved in 0.1 M Tris buffer (pH 9.3), and ALP staining was evaluated. ALP activity assays were performed using an ALP kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. All results were normalized based on protein concentrations. Alizarin red staining (ARS) and quantification:

After fourteen days of osteogenic induction, DPSCs were fixed with 4% paraformaldehyde and then stained with 2% Alizarin Red (Sigma-Aldrich, USA) to detect mineralization potential. Alizarin Red was then de-stained with 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate for 30 minutes at room temperature to quantify calcium mineral deposition. Optical absorbance was measured at 562 nm on a multi-plate reader with a standard calcium curve in the same solution. The final calcium level in each experimental group was normalized based on protein concentrations measured from a duplicate plate.

2.3 Quantitative RT-PCR

Total RNA was isolated from DPSCs using Trizol reagents (Life Technologies, USA). In order to synthesize cDNA, 2-μg aliquots of RNA were used along with random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen, USA). Real-time PCR reactions were performed using the iQ SYBR Green Supermix (Bio-Rad) and the Icycler iQ Multi-color Real-time PCR Detection System (Bio-Rad). The human gene primer sequences used in the following experiments are listed in Table 1.

Genes	Forward (5'-3')	Reverse (5'-3')
GAPDH	TCATTGACCTCAACTACATG	GCTCCTGGAAGATGGTGAT
BSP	GAATGGCCTGTGCTTTCTCAA	TCGGATGAGTCACTACTGCCC
DLX5	GCTCTCAACCCCTACCAGTAT	CTTTGGTTTGCCATTCACCATTC
OCN	AGCAAAGGTGCAGCCTTTGT	GCGCCTGGGTCTCTTCACT
OPN	TTGCAGCCTTCTCAGCCAA	CAAAAGCAAATCACTGCAATTCTC
RUNX2	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA

Table 1. Human Gene Primer Sequences Used for Quantitative RT-PCR.

2.4 Western Blot Analysis

Cells cultured in 15-mm dishes were washed with PBS and were then collected using a scraper. The cells were lysed using radioimmunoprecipitation (RIPA) assay buffer (10mM Tris-HCl, 1 mM EDTA, 1% SDS, 1% NP-40, 50 mM β-glycerophosphate, 50 mM sodium fluoride) supplemented with protease inhibitor cocktail (Promega, USA) for one hour on ice. Samples were centrifuged at 4 °C, and supernatants were then collected and stored at -80 °C. The protein concentration of each sample was measured colorimetrically using Bio-Rad reagents. 40-μg aliquots of protein were separated on a 10% SDS-polyacrylamide (PAGE) gel and transferred to a polyvinylidene difluoride (PDVF) membrane using the Bio-Rad semidry transfer system. The membranes were then incubated with a blocking solution that contained 5% dry-milk in TBS/Tween 20 buffer for one hour at room temperature followed by the addition of primary antibodies at 4 °C overnight. The primary antibodies used were: phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb, SAPK/JNK Rabbit mAb, phospho-c-Jun (Ser73) (D47G9) Rabbit mAb, c-Jun (60A8) Rabbit mAb, (all from Cell Signaling Technology, USA) and mouse α-tubulin (Sigma-Aldrich, USA). Luminol/Enhancer Solution and Super Signal West Stable Peroxide Solution (Thermo) were used to perform blot detection.

3 RESULTS

3.1 Exogenous NGF Promotes Osteogenic Differentiation of DPSCs

To evaluate the effect of NGF on differentiation capacity, isolated DMSCs from dental pulp were induced to undergo osteogenic differentiation. OIM supplemented with 2 ng/mL and 10 ng/mL of NGF, respectively, both led to an increase in the capacity of DPSCs to differentiate into the osteogenic lineage compared to OIM alone as demonstrated by ALP staining on the seventh day (Figure 1). ALP activity demonstrated a significant increase of approximately 4 to 6-fold in DPSCs treated with either 2 ng/mL or 10 ng/mL of NGF, respectively, compared to OIM alone (Figure 2).

Furthermore, isolated DPSCs treated with NGF showed an increase in calcium mineral deposition after treatment with OIM for 14 days as demonstrated by ARS (Figure 3). Quantification of ARS revealed significant mineralization potential in NGF-treated DPSCs with an approximate 2 to 3-fold increase compared to induction with OIM alone (Figure 4).

3.2 Exogenous NGF Upregulates the mRNA Expression of Osteogenic Genes: *BSP, DLX5, OCN, OPN,* and *RUNX2*

We further confirmed the osteogenic potential of NGF-treated DPSCs by examining the mRNA expression of several osteogenic genes: *BSP, DLX5, OCN, OPN,* and *RUNX2,* at different time points: 0, 1, and 3 days after osteogenic induction with OIM and either 2 ng/mL or 10 ng/mL of NGF, respectively. Osteogenic gene expression was significantly upregulated for NGF-treated DPSCs, which supports our ALP and ARS results. In particular, there was a marked increase of *BSP, DLX5, OCN, OPN,* and *RUNX2* expressions of approximately 3 to 4-fold, 2 to 4.5-fold, 3.5 to 4-fold, 3.5 to 4-fold, and 3 to 4-fold, respectively, at day 3 of induction with OIM and NGF in a dose-dependent manner in comparison to induction with OIM alone (Figure 5).

3.3 Exogenous NGF Activates Signaling through the JNK and c-Jun Pathways

Next, we decided to further investigate if NGF activates the JNK and c-Jun signaling pathways in DPSCs based on previous studies that have shown their significance in both osteogenic differentiation and NGF signaling. To determine if intracellular JNK and c-Jun were activated by the presence of extracellular NGF, DPSCs were treated with 2 ng/mL of NGF over the times periods: 0, 15 minutes, 30 minutes, and 60 minutes, respectively; next, they were examined through western blot analysis. The JNK and c-Jun signaling pathways were activated by NGF in a time-dependent manner, but with varying degrees (Figure 6).

3.4 Exogenous NGF Supplemented with the JNK Inhibitor Decreases Osteogenic Differentiation of DPSCs

To evaluate whether the activation of the JNK pathway plays a role in determining the fate of DPSCs, OIM treated with NGF was further supplemented with an extracellular JNK inhibitor (JNKi). Cells were pre-treated with either 5 uM or 10 uM of the JNKi, respectively, for one hour prior to the addition of 2 ng/mL of NGF to the OIM. The addition of the JNKi led to a significant reduction in the capacity of DPSCs to differentiate into the osteogenic lineage in a concentration-dependent manner compared to OIM supplemented with NGF only as demonstrated by ALP staining on the seventh day (Figure 7). Quantification of ALP activity demonstrated an approximate 7 and 10.5-fold decrease in DPSCs treated with either 5 uM or 10 uM of the JNKi, respectively, compared to OIM treated with NGF only (Figure 8).

Furthermore, isolated DPSCs treated with the JNKi showed a reduction in calcium mineral deposition after treatment with OIM supplemented with 2 ng/mL of NGF for 14 days as demonstrated by ARS (Figure 9). Quantification of ARS revealed a decrease in mineralization potential for DPSCs pre-treated with either 5 uM or 10 uM of the JNKi, respectively. Both JNKi

groups experienced an approximate 2-fold decrease compared to induction with OIM treated with NGF alone (Figure 10).

3.5 Exogenous NGF Supplemented with the JNK Inhibitor Suppresses the JNK and c-Jun Signaling Pathways

Furthermore, we decided to investigate the effects of using the JNKi on the JNK and c-Jun signaling pathways in DPSCs. To determine if intracellular JNK and c-Jun were deactivated by the presence of extracellular JNKi, DPSCs were pre-treated for one hour with either 5 uM or 10 uM of the JNKi, respectively, before 2 ng/mL of NGF was added for 30 minutes; next, they were examined by western blot analysis. The JNK and c-Jun signaling pathways were both downregulated by the JNKi in a concentration-dependent manner, but with varying degrees (Figure 11).

4   DISCUSSION

In the search for novel therapies in regenerative medicine, there has been an increasing interest in MSCs, which prove to be a promising candidate due to their potential use in tissue engineering, including tissues, nerves and bone regeneration [13]. Differentiation can be modulated with growth factors, transcriptional factors, extracellular matrix proteins and receptor molecules in the local microenvironment [1, 13, 20]. NGF is well-known for its physiological role in neurobiology, yet it is also produced and utilized by several non-nervous cell types [27]. NGF, which exerts its effects by the TrkA and p75NTR receptors, may promote mineralization making it an appealing candidate in bone and tissue regeneration [25]. In this study, we decided to explore the role of NGF in the osteogenic differentiation of MSCs isolated from dental pulp. Our results revealed that treatment with exogenous NGF led to an increase in osteogenic potential of DPSCs in a concentration-dependent manner since there was an increase of ALP, ARS, and mRNA expression of osteogenic genes. Previous studies have shown that local administration of NGF induces differentiation of immortalized dental papilla cells into odontoblast-like cells *in vivo*, which corresponds to our findings suggesting that NGF induces mineralization [25]. Since our results indicated that NGF increases osteogenic differentiation, we decided to further assess the role of JNK in this signaling cascade.

NGF activity is mediated through binding to its two cell surface receptors: TrkA and p75NTR [25, 28]. While it is known that the TrkA signaling cascade involves the MAPK-RAS-ERK pathway, phospholipase Cy1, P3I kinase and SNT proteins, NGF signaling via p75NTR is not well-defined [27]. Studies have shown that p75NTR signaling involves activation of NF-kB, the phosphorylation of transcription factor JNK, as well as increased production of ceramide, which leads to gene transcription or cell death [27, 29]. The MAPK pathway has been shown to be

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involved in osteoblast development; in particular, the ERK and P38 MAPKs are essential for RUNX2 activation, whereas JNK is important for late-stage osteoblast differentiation [33]. Previous studies have shown that RUNX2 is a master transcription factor that regulates the expression of several osteogenic genes, including ALP, OCN, and BSP [33]. In order to elucidate the role of NGF signaling in osteogenic differentiation of DPSCs, we selected a few candidates (i.e., JNK and c-Jun) based on previous studies and supplemented 2ng/mL of NGF to the media for one hour. Our findings demonstrated that JNK and c-Jun activity increased in a time-dependent manner. Our results are consistent with previous studies and contribute to a better understanding of NGF and MSC signaling.

Furthermore, we used the JNKi to evaluate the effect of inhibiting JNK in the osteogenic pathway. We observed a significant decrease in osteogenic differentiation compared to the control in a dose-dependent manner. We also pre-treated cells with the JNKi for one hour prior to adding NGF for thirty minutes. Through western blot analysis, we evaluated the JNK and c-Jun pathways. Our results demonstrated that JNK inhibition led to the downregulation of both the JNK and c-Jun signaling pathways.

In conclusion, our findings from this study have demonstrated that NGF promotes osteogenic differentiation of MSCs from dental pulp. Our findings may demonstrate that when NGF binds to the p75NTR receptor, the intracellular JNK and c-Jun signaling pathways are triggered, which then leads to the signal transduction into the nucleus where the activated transcription factor c-Jun can directly bind to DNA and promote the expression of osteogenic genes (Figure 12). Our results may also indicate that the downregulation of these pathways would lead to the deactivation of c-Jun within the nucleus and consequently suppress the expression of osteogenic genes. As a whole, this study improves our understanding of the

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mechanisms involved in the molecular regulation of NGF-mediated osteogenic differentiation of

MSCs from dental pulp. Our findings on the interactions between MSCs and NGF may be useful

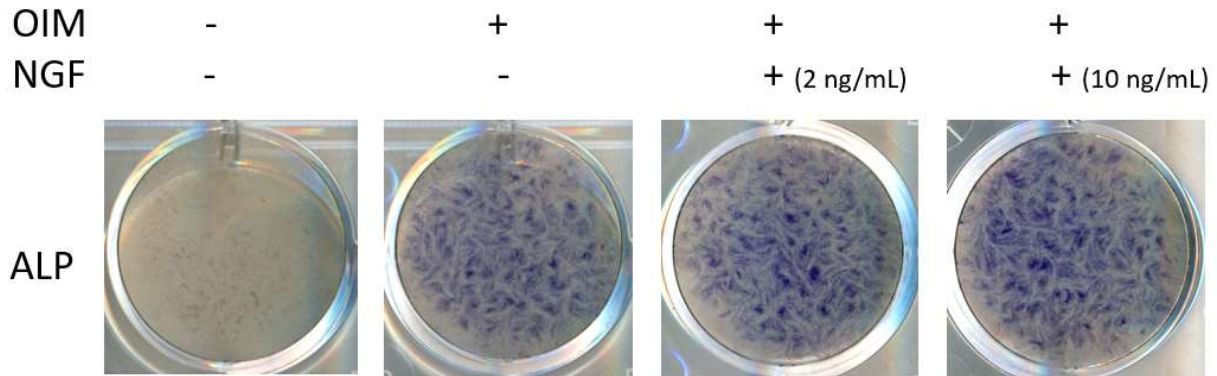in the development of an effective application in regenerative medicine.

Figure 1. ALP Staining of DPSCs with NGF Treatment. Alkaline Phosphatase (ALP) staining after cells were induced to differentiate into osteogenic lineages for 7 days with OIM showed that the addition of exogenous NGF promoted osteogenic differentiation of DPSCs.
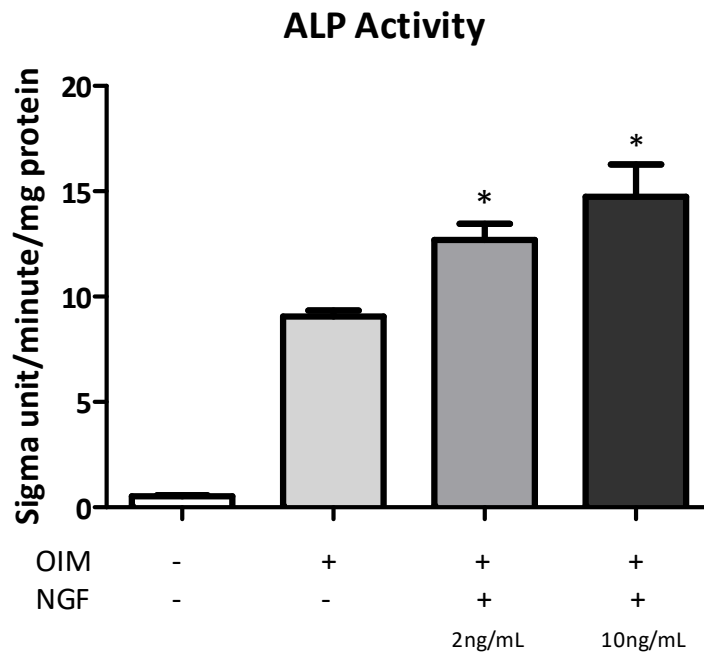

Figure 2. Quantification of ALP Activity of DPSCs with NGF Treatment. ALP activity quantification confirmed that exogenous NGF supplemented to OIM for 7 days increased osteogenic differentiation of DPSCs. Values were normalized to non-induced DPSCs. *$p<0.05$.
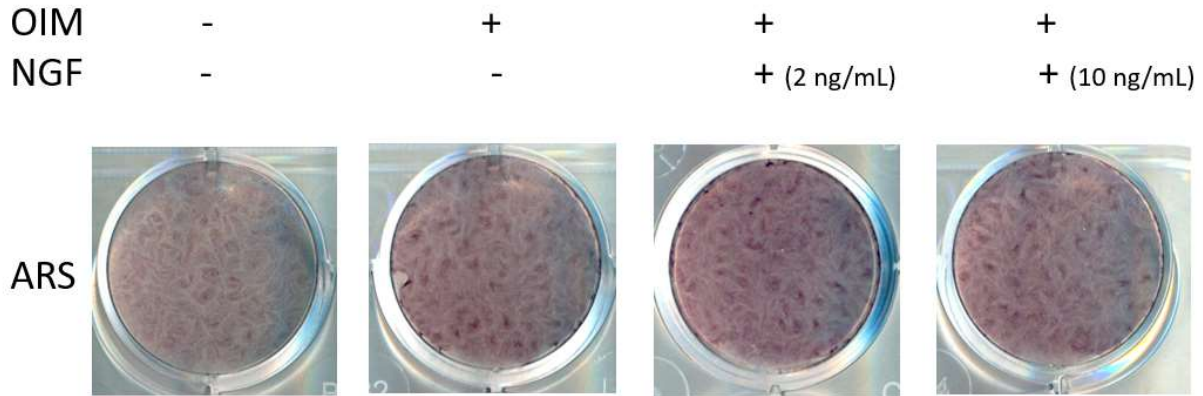
| | OIM | - | + | + | + |
| | NGF | - | - | + (2 ng/mL) | + (10 ng/mL) |

ARS

Figure 3. ARS of DPSCs with NGF Treatment. Alizarin red staining (ARS) after cells were induced to differentiate into osteogenic lineages for 14 days with OIM showed that the addition of exogenous NGF promoted calcium mineral deposition in DPSCs.
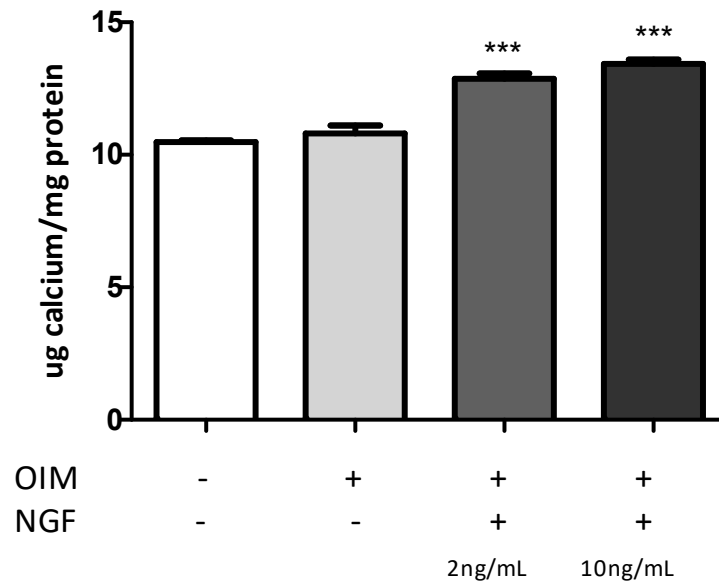
ARS Quantification

Figure 4. ARS Quantification of DPSCs with NGF Treatment. Quantification of ARS confirmed that exogenous NGF supplemented to OIM for 14 days increased calcium mineral deposition in DPSCs. Values were normalized to non-induced DPSCs. ***$p<0.001$.
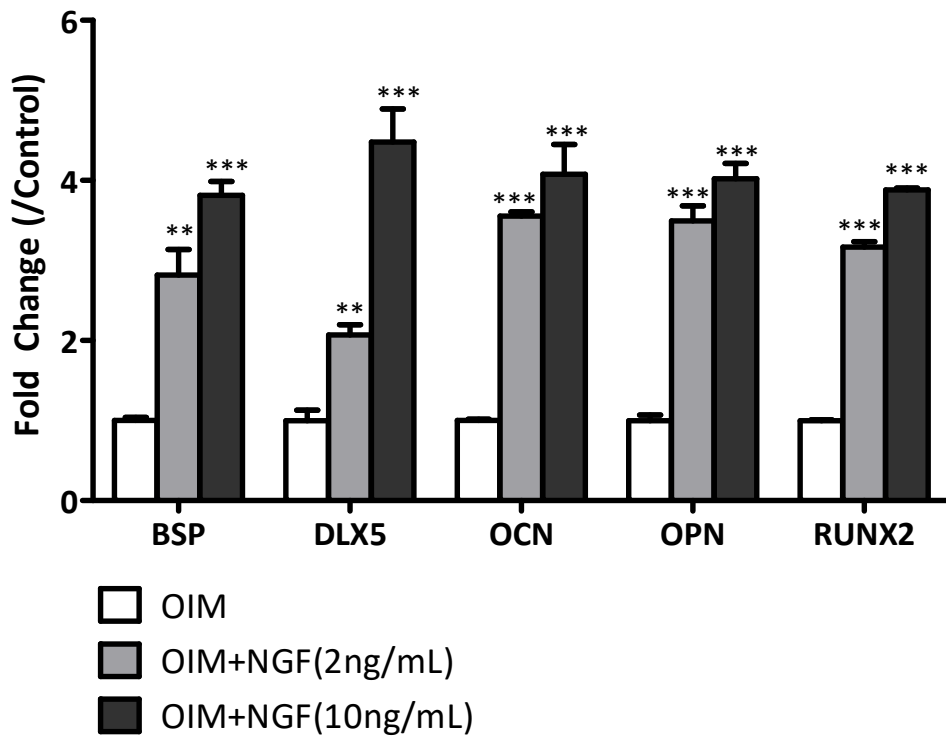
Figure 5. mRNA Expression of Osteogenic Genes After 3 Days of Osteogenic Induction in DPSCs with NGF Treatment. Quantitative RT-PCR analysis revealed an increase in *BSP, DLX5, OCN, OPN,* and *RUNX2* relative mRNA levels, respectively, after supplementing OIM with exogenous NGF. Values were normalized to DPSCs with OIM and 0 ng/mL of NGF. **$p<0.01$, ***$p<0.001$.
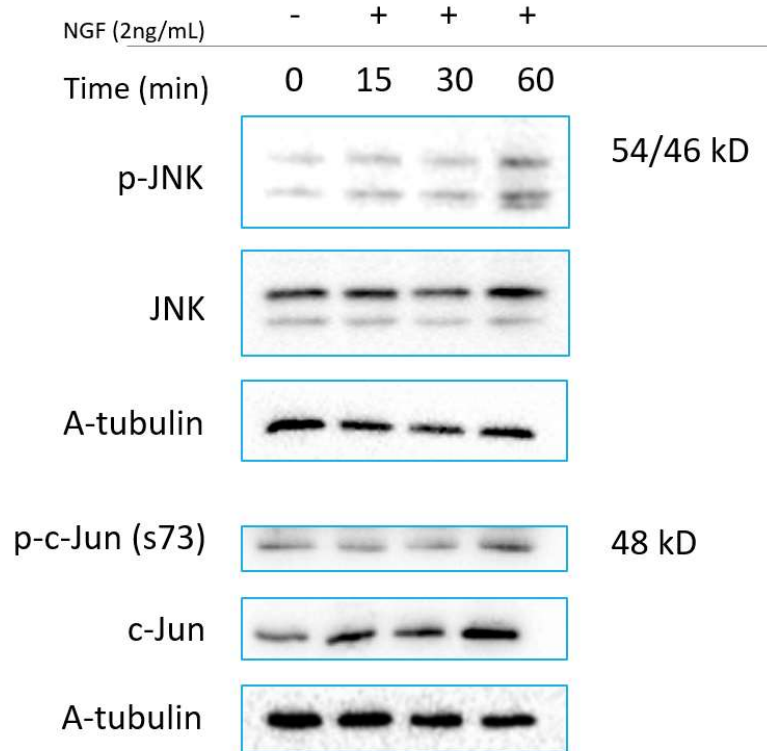
Figure 6. NGF Activates the JNK and c-Jun Signaling Pathways in DPSCs. Western blot analysis was used to evaluate the expression of JNK and c-Jun in DPSCs treated with 2 ng/mL of exogenous NGF at times: 0, 15 minutes, 30 minutes, and 60 minutes.
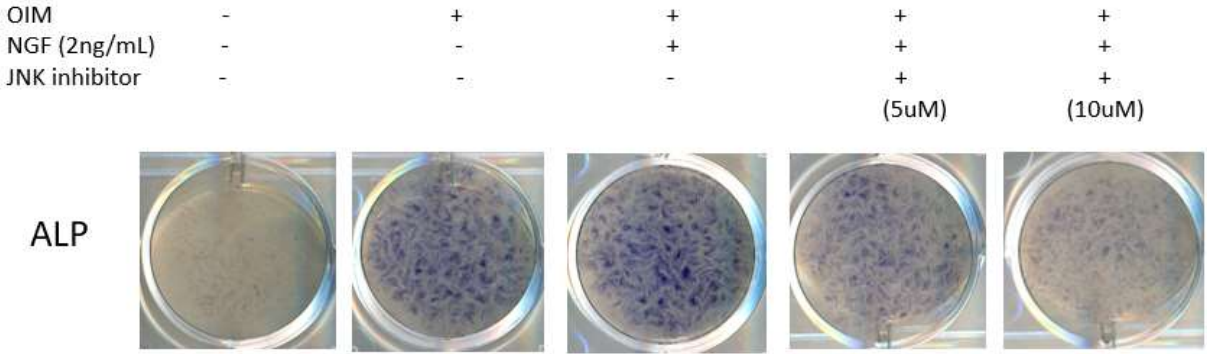
OIM	-	+	+	+	+
NGF (2ng/mL)	-	-	+	+	+
JNK inhibitor	-	-	-	+	+
				(5uM)	(10uM)

Figure 7. ALP Staining of DPSCs with NGF Treatment Supplemented with the JNK inhibitor (JNKi). ALP staining after cells were induced to differentiate into osteogenic lineages for 7 days with OIM showed that the addition of the JNKi and exogenous NGF reduced osteogenic differentiation of DPSCs.
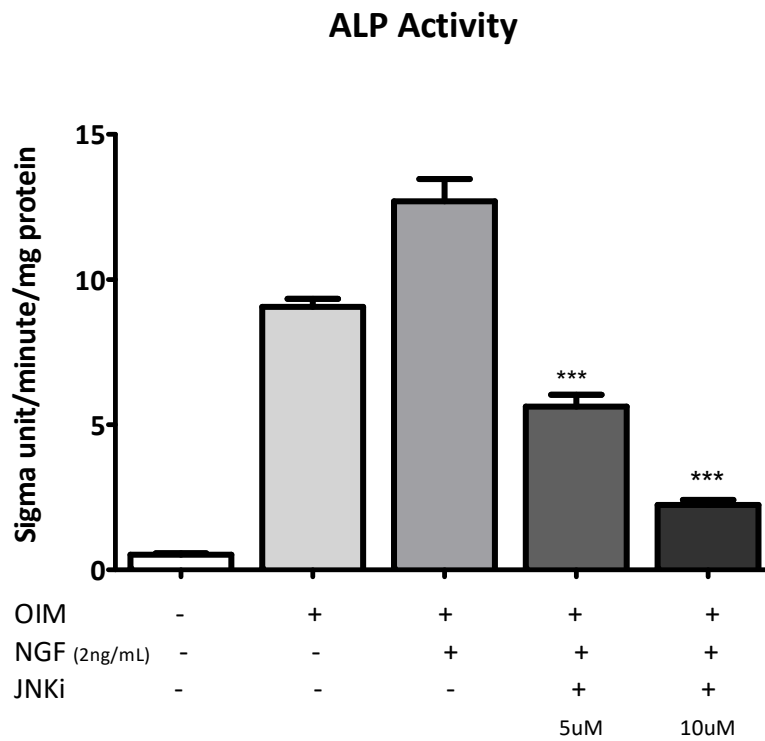
ALP Activity

OIM	-	+	+	+	+
NGF (2ng/mL)	-	-	+	+	+
JNKi	-	-	-	+	+
				5uM	10uM

Figure 8. Quantification of ALP Activity of DPSCs with NGF Treatment Supplemented with the JNKi. ALP activity quantification confirmed that the addition of the JNKi and exogenous NGF supplemented to OIM for 7 days decreased osteogenic differentiation of DPSCs. Values were normalized to non-induced DPSCs. ***$p<0.001$.
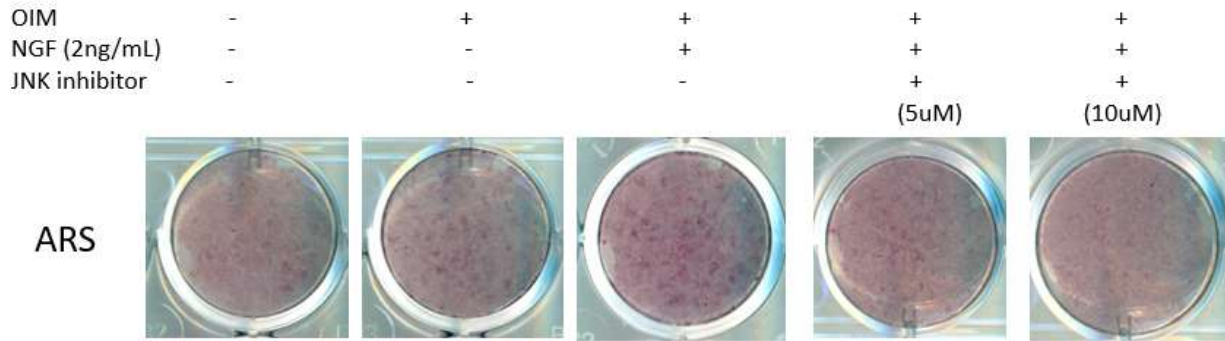
21

OIM	-	+	+	+	+
NGF (2ng/mL)	-	-	+	+	+
JNK inhibitor	-	-	-	+ (5uM)	+ (10uM)

ARS

Figure 9. ARS of DPSCs with NGF Treatment Supplemented with the JNKi. ARS after cells were induced to differentiate into osteogenic lineages for 14 days with OIM showed that the addition of the JNKi and exogenous NGF reduced calcium mineral deposition in DPSCs.
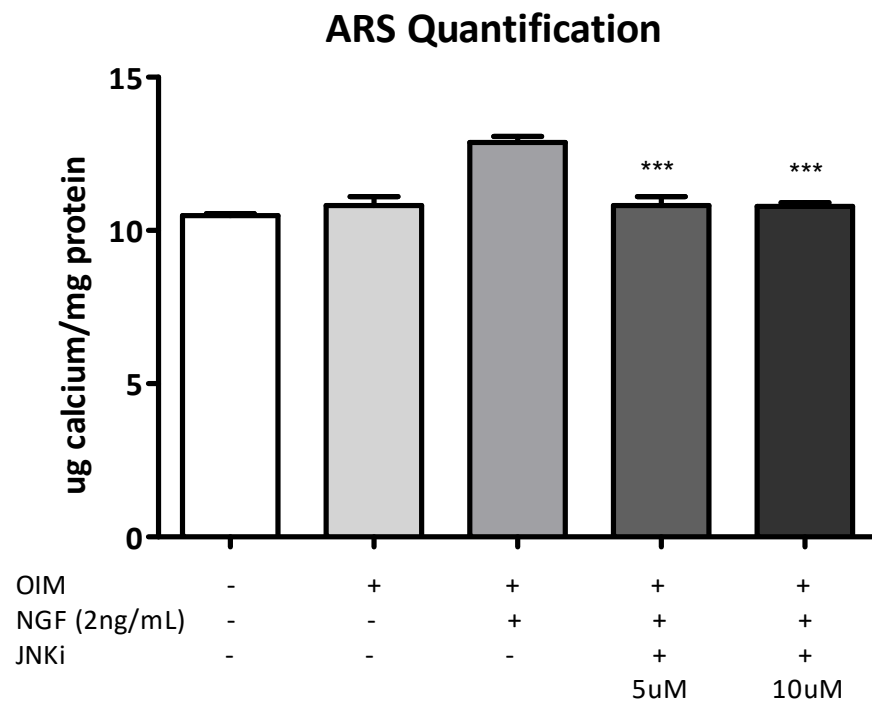

ARS Quantification

Figure 10. ARS Quantification of DPSCs with NGF Treatment Supplemented with the JNKi. Quantification of ARS confirmed that the JNKi and exogenous NGF supplemented to OIM for 14 days decreased calcium mineral deposition in DPSCs. Values were normalized to non-induced DPSCs. ***$p < 0.001$.
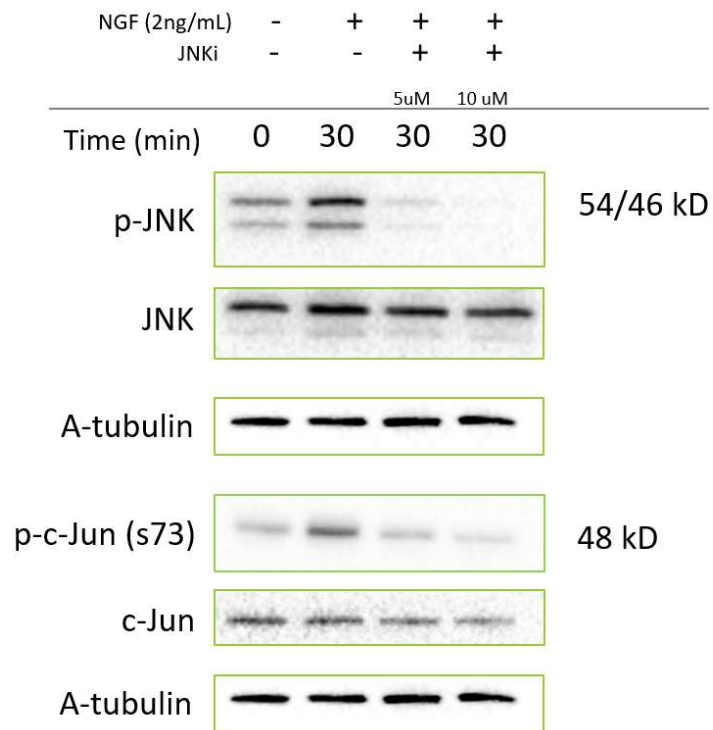
Figure 11. JNKi Deactivates the JNK and c-Jun Signaling Pathways in DPSCs. Western blot analysis was used to evaluate the expression of JNK and c-Jun in DPSCs pre-treated with either 5 uM or 10 uM of the JNKi, respectively, supplemented with 2 ng/mL of exogenous NGF at times: 0, and 30 minutes.
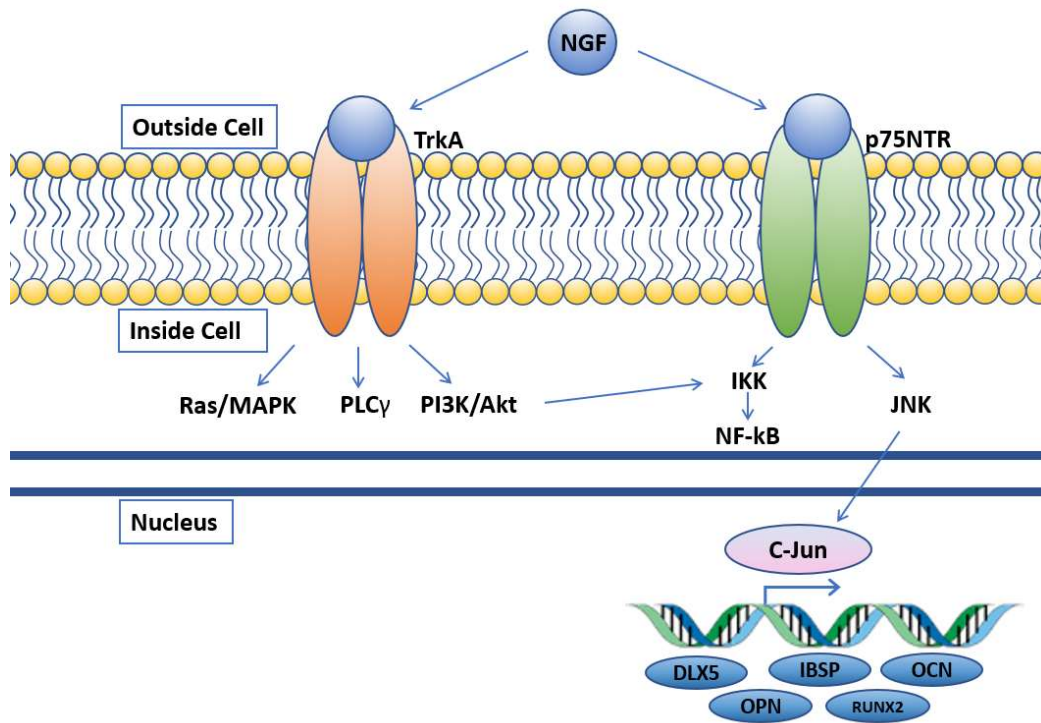
Figure 12. Proposed Model of NGF Signaling in DPSCs.

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