Cytoskeletal Reorganization in Human Dental Pulp Stem Cells

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SUMMARY

Cytoskeletal f-actin reorganization is essential for many cellular processes. These processes include movement, cell division, sensing and transduction of mechanical environment, morphogenesis, and lineage commitment. It has been shown that as stem cells differentiate along various lineages they undergo a unique change in cytoskeletal organization. In dental pulp regeneration, dental pulp stem cells differentiate to replace lost or damaged odontoblasts. Odontoblasts are lineage committed cells found within the tooth whose responsibility is to maintain the mineralized tissue of the tooth. Dental pulp stem cells are precursor cells for these odontoblast cells. Upon bacterial infection or trauma, the odontoblast population may become damaged or lost and the dental pulp stem cells take their place and differentiate into new odontoblasts. Following infection or damage to the dental pulp the tissue may become inflamed. Inflammation is a complex process involving cellular microenvironment and mitogenic signaling. Exposure to inflammatory mediators induces several downstream cellular events mediating angiogenic signaling, a process crucial for pulpal inflammation and repair. Interestingly, inflammation has been shown to initiate certain changes in cytoskeletal organization. Also, it has been demonstrated that certain materials commonly used in dentistry may be play a role in the regeneration response of dental pulp stem cells. To date, several studies have addressed the effects of proinflammatory stimuli on the survival and differentiation of dental-pulp stem cells, in vitro. However, the mechanisms common to the inflammatory and angiogenic signaling involved in DPSC survival and differentiation remain unknown. Our studies observed that short-term exposure to TNF-α at 6 and 12 hours induced apoptosis with an upregulation of NF-κB signaling. In contrast, long-term, chronic, exposure for 10 days resulted in an increased proliferation with an associated telomere length shortening. Interestingly, DPSC pretreated with Nemo binding domain peptide, a cell permeable NF-κB inhibitor, significantly ameliorated TNF-α-induced increases in proliferation and the shortening of telomere length.

SUMMARY (continued)

Furthermore, NBD peptide pretreatment significantly ameliorated TNF-α-induced down regulation of proteins essential for differentiation, such as bone morphogenic proteins (BMP)-1 & 2, BMPR isoforms-1&2, tissue growth factor (TGF), osteoactivin and osteocalcin. Furthermore, inhibition of NF-κB signaling markedly increased the mineralization, abrogated by TNF-α signaling. Thus, our studies demonstrate that chronic inflammation mediates telomere shortening via NF-κB signaling in human DPSC. The resultant chromosomal instability leads to an emergence of angiogenic proliferation of DPSC and negative regulation of differentiation of DPSC, in vitro. Lastly, it was demonstrated that two root repair materials, mineral trioxide aggregate and Endosequence root repair material, and fluoride play a role in dental pulp regeneration. All three materials caused an increase in DPSC proliferation and had little observed effect on differentiation as measured by ALP activity. Further, there were observed similarities in the actin cytoskeletal structure between the groups cultured with the root repair materials and fluoride with the group with the DPSC cultured under mineralizing conditions. The addition of these common dental materials had no deleterious effects on the differentiation of DPSC.

ACKNOWLEDGMENTS

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Term, Definition
ALP	Alkaline phosphatase
ARS	Alizarin red s
BMP	Bone morphogenic protein
DAPI	4',6-diamidino-2-phenylindole
DPSC	Dental pulp stem cell
EDTA	Ethylenediaminetetraacetic acid
ERRM	Endosequence root repair material
MEM	Minimum essential medium
MSC	Mesenchymal stem cell
MTA	Mineral trioxide aggregate
NaF	Sodium fluoride
NBD	Nemo binding domain
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UV	Ultraviolet

CHAPTER 1: INTRODUCTION AND BACKGROUND

Regenerative approaches using cells endowed with stem cell properties in adult pulp, termed dental pulp stem cells, are considered an ideal tool for restoring lost dental tissues and regenerating the root canal system (Gronthos et al., 2000). DPSC have the potential to differentiate into osteoblasts and odontoblasts as well as into several other different cell types including adipocytes, neurons, chondrocytes, and endothelial cells (Cvek, 1992; Iohara et al., 2011; Gronthos et al., 2002). Dental caries or trauma with an ensuing inflammatory response in the pulp lead to an increase in the accumulation of inflammatory cells, which release host proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukins (Stashenko et al., 1998; Coil et al., 2004). Additionally, prolonged exposure to inflammatory environment leads to chronic hypoxia, causing altered metabolic shift-oriented cellular energy status and angiogenic switch.

It is well known that inflammatory mediators activate the crucial transcription factor NF-κB (Baldwin et al., 2001), which then is involved in regulating various other genes including major histocompatibility molecules, interleukins, IL-1, 2, 6, and 8, and intracellular adhesion molecule-1. In the unstimulated condition, NF-κB is retained in the cytoplasm in the most common form, p65 and p65/RelA heterodimers, by the inhibitory protein IκBα. Upon stimulation by TNF- α or other inflammatory stimuli, IKK-α and IKK-β are activated following IKK-γ ubiquitination (Yang et al., 2003; Baldwin et al., 2001). The activated IKK complex then phosphorylates IκB-α at the serine resides in the N-terminal region. The phosphorylated IκB-α is subsequently ubiquitinated and degraded by the 26S proteasome machinery. The degradation of IκB-α then activates NF-κB signaling (Wang et al., 1996, 1998; Baldwin, 2001). In this study, we tested whether TNF-α activated the NF-κB signaling pathway in order to better understand the role of inflammation and host response in DPSC.

The formation of new blood vessels from pre-existing blood vessels, or angiogenesis, has a crucial role in a number of pathological and physiological processes such as chronic inflammation, wound healing, and tissue regeneration. In dental pulp, a highly vascularized tissue, angiogenesis is an indeterminate phase for physiological tooth development and for healing following pulpal injury (Mullane et al., 2008; Tan-Hung et al., 2008; Kim et al., 2014). Additionally, inflamed tissues enhance the expression of several angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) in human pulp and gingival fibroblasts (Karaoz et al., 2011; Tran-Hung et al., 2006; Tan-Hung et al., 2008). Studies have demonstrated that angiogenic factors contribute to the destruction of peripacial and pulpal tissues by expanding the vascular network coincident to progression of the inflammation. Furthermore, these studies demonstrate that the angiogenic factors, especially VEGF, produced by the human pulp cells act directly on themselves in an autocrine manner to promote their proliferation and differentiation (Matsushita et al., 2000). VEGF alone or in combination with recombinant human macrophage-colony-stimulating factor produced a significant increase in the number of mature odonotoblasts or osteoblasts (Kohno et al., 2003). These findings cumulatively suggest that upregulation of angiogenic signaling during inflammatory processes significantly contributes to the pathogenesis associated with DPSC survival and differentiation into mature odontoblast-like cells. Therefore, when studying the effects of inflammation, it is not only required to identify the angiogenic molecules secreted, but it is also important to investigate the combined effects of inflammatory mediators and angiogenic molecules in arbitrating DPSC differentiation and proliferation. Since, inflammatory cytokines in conjunction with angiogenic signaling are essential for reparative dentinogenesis, the aim of this study was to examine the effect of TNF-α and angiogenic factors in mediating the proliferation and differentiation potentials of DPSC.

Along with understanding the interactions between inflammation and angiogenesis, knowing the influence of materials commonly used in dentistry on the process of pulpal regeneration is essential for better understanding how the pulp repairs itself following insult. It has been suggested that inflammation can lead to abnormal actin reorganization (Du et al., 2012; Thomas et al., 2006). Actin reorganization is an essential step in many cellular processes including stem cell lineage commitment (Titushkin and Cho, 2007). Given the importance of the role of actin cytoskeleton reorganization in stem cell differentiation and the implications of its dysfunction during inflammatory states it is important to study the relationship between these two phenomena. It is of further interest that inflammation is thought to reduce the differentiation potential of stem cells. Studying the effects of inflammation on the cytoskeleton may lead to important insights in stem cell biology (Kohno et al., 2003). Often times in vivo these processes, such as inflammation and differentiation, occur in the presence of dental materials such as root repair materials and fluoride. This is because these materials are used in situations where there may have been damage to the tooth or infection. MTA and Endosequence are used to replace native mineralized tissue following trauma or infection and fluoride is used to stop or prevent demineralization of the teeth. Further, fluoride is commonly found in tooth paste and drinking water (Chang et al., 2001). The pulp tissue may come in contact with these materials during repair and regeneration or during the inflammatory processes (Torabinejad and Chivian, 1999). Therefore, it is also important to study the processes of differentiation and proliferation with respect to these materials and also determine their possible effects on actin cytoskeleton reorganization.

CHAPTER 2: CYTOSKELTAL REORGANIZATION IN DENTAL PULP STEM CELLS

2.1 <u>**Methods**</u>

    2.1.1   <u>Culture of human DPSC</u>

DPSC obtained from healthy permanent premolars extracted during orthodontic treatment, were generously donated by Dr. Songtao Shi, University of Southern California. The single cell suspensions were cultured in αMEM (Gibco) supplemented with 20% FBS (Hyclone, UT, USA) and 1% Antibiotic-antimycotic (Gibco). Odontogenic medium was supplemented with 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 100 nM dexamethasone. DPSC were incubated at 37 °C with 5% CO2. DPSC between 3rd and 5th passages were used throughout the study. DPSC were also cultured in conditioned media with elutes from various materials used in dentistry. Mineral trioxide aggregate and Endosequence root end repair material were aged in media to create conditioned media at concentrations of 100 mg/mL. Sodium fluoride was supplemented at a concentration of 500 μM. Latrunculin A was supplemented to cells at a concentration of 100 nM to serve as a positive control for cytoskeletal reorganization.

    2.1.2   <u>Real Time PCR analysis</u>

Total RNA from DPSC was extracted using TRIzol reagent. RT was performed using oligo (dT) primers and superscript RT (Invitrogen) following the manufacturer's instructions. Human p65, BCL2, Survivin, BMP, BMPR, TGF-β1, TGF-β2, VEGF, EGF, FGF-1, FGF-2, osteocalcin, osteoactivin, RUNX2, and GAPDH were amplified using the primer sets. RT product (2 μl) was amplified in a 10-μl volume with iQTM SYBR Green supermix (Bio-Rad laboratories). Reactions were performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA).

2.1.3 CFSE staining and Flow cytometry analysis

After appropriate treatment conditions and the respective time points, DPSC were labeled for 10 minutes at 37°C with 2 µM CFSE (Invitrogen) in Dulbecco's PBS (D-PBS; Invitrogen) supplemented with 2% FBS (Invitrogen). The same volume of ice-cold D-PBS with 10% FBS was then added to stop the reaction. After washing with Mg2+/Ca2+-free PBS, 1 x 105 CFSE-labeled DPSC were then subjected to flow cytometry analysis. Each single division was determined as follows: a gate for zero division was set on the CFSE peak of the undivided naive cells, and subsequent divisions were determined according to reduced fluorescence intensity of peaks in respective histograms. The percentage of cells in different generations was plotted accordingly. Cells treated with concovalin A were used as a positive control, whereas media alone served as a negative control.

2.1.4 BrdU incorporation assay

For proliferation studies, DPSC were cultured to approximately 50% confluence in 96-well plates (BD Bioscience). At the end of treatment period, cells were starved overnight in low-serum media, followed by an 18-hour pulse with 10 µM 5-bromo-2'-deoxyuridine (BrdU) in EB-CM from different time points as well as control media. After the 18-hour pulse, cells were rinsed with PBS and fixed in 70% ethanol with 2M HCl for 10 minutes at room temperature then rinsed in PBS at least three times. The cell lysates were then measured at excitation 450 nm and emission 595 nm using ELISA plate reader (Thermo Scientific, USA).

2.1.5 MTT assay

DPSC cultured on 96-well plate at concentration 1 x 10^3 cells/well were subjected to appropriate treatment conditions, while grown in odontogenic medium. At appropriate time points the formation of formazan products was measured spectrophotometrically using methylthiazolyldiphenyl-tetrazolium

bromide (MTT) assay kit. The culture medium was replaced with 5 mg/mL MTT solution in PBS and the plates were incubated for 6 h at 37 °C. The precipitate was extracted with DMSO and optical density was measured at wavelength 550 nm.

2.1.6 Alizarin red s staining

DPSC seeded onto 12-well plates ($1x\ 10^4$ cells per well) were subjected to alizarin red s staining at day 14. Briefly, the cells were fixed in 4% paraformaldehyde for 20 min, then stained using alizarin red s (Sigma-Aldrich). The phase contrast images were then captured for analysis using EVOS® FL Cell Imaging System.

2.1.7 Alkaline phosphatase activity

DPSC were grown in odontogenic media for 14 days, at 37 °C. Cells were then fixed with 4% paraformaldehyde and ALP detection assay was performed according to the manufacturer's protocol (Sigma-Aldrich), and ALP activities were normalized based on protein concentrations.

2.1.8 Western blot

DPSC lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 10% separating gel under reducing conditions and transferred to Duralose membrane. Membranes were blocked with 5% dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 for 1 h. Membranes were incubated with indicated primary antibody diluted in blocking buffer overnight. After three washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Protein bands were detected by enhanced chemiluminescence.

2.1.9 Telomere length

Average telomere length was measured from total genomic DNA of DPSC using a sequence-independent multiplex qPCR technique using a SYBR Green master mix with 0.625U AmpliTaq Gold 360 DNA polymerase (Life Technologies) (Cawthon et al). Each reaction included 10 µL 2x SYBR Green

mix (Bio-Rad), 0.5 µL each of 10 µM forward and reverse primers, 4 µL water and 5 µL genomic DNA (10 ng/µL) to yield a 20-µL reaction. DNA samples were placed in adjacent three wells of a 96-well plate for telomere primers and reference gene primers, respectively. A Bio-Rad thermocycler (CFX96 system) was used with reaction conditions of 95 °C for 10 min followed by 40 cycles of data collection at 95 °C for 15 s, 60 °C anneal for 30 s, and 72 °C extend for 30 s along with 80 cycles of melting curve from 60 °C to 95 °C. CFX manager software was used to generate standard curves and Ct values for telomere signals and reference gene signals.

2.1.10 Statistical Analysis

Comparisons were made with a two-tailed Student's t test. Experimental values were reported as mean ± S.E. Differences in mean values between two or more groups were determined by one-way analysis of variance. A p value <0.05 was considered statistically significant.

2.1.11 F-Actin Staining

DPSC were cultured on glass cover slips and fixed with 10% formaldehyde. They were subsequently stained with rhodamine phalloidin. The fixed and stained samples were then imaged using fluorescent microscopy.

2.2 **Results**

Short-term exposure of TNF-α induces apoptosis via NF-κB signaling pathway in DPSC. To examine the reparative response of DPSC to short-term exposure with proinflammatory stimuli, we challenged cells with TNF-α for varying time points (0, 1, 2, 4, and 6h). In the first set of experiments, we examined the viability analysis using MTT assay (Fig. 1A). Cells exposed for both 4 and 6h exhibited a decrease in the number of viable cells, as measured. Also, propidium iodide (PI) staining in DPSC showed a marked increase in the number of apoptotic cells, at 4 hours following TNF-α treatment (Fig 1B). Additionally,

immunoblot analysis for caspase-3 showed approximately two-fold increase (Fig. 1C). These findings suggest that short exposure of proinflammatory stimuli (TNF-α) induce apoptotic signaling in DPSC, in vitro.

To address whether TNF-α-induced apoptosis occurs via NF-κB signaling pathway, we first examined the activation of p65 using Western blot analysis. Interestingly, we observed a time dependent TNF-α-induced phosphorylation of p65 (phospho-p65) (Fig 1D). Quantification of the analysis demonstrated an increase of approximately 1.5 fold when compared to the total p65 levels. We next investigated the role of TNF-α in mediating the activation of IκB-α on the basis of the proposal that NF-κB signaling is required for IκB-α expression and that IκB-α inhibits NF-κB activation in a negative feedback manner. We observed that the basal expression levels of phospho-IκB-α were markedly lower in DPSC; however, upon exposure to TNF- α at varying time points (0, 4, 6, and 12h) the levels of phospho-IκB-α significantly increased (Fig. 1F). Next, to determine whether short-term exposure to TNF-α influenced the expression of VEGF we performed western blot analysis. DPSC treated TNF- α for 0, 4, 6 and 12 h showed an increase in the levels of VEGF at both 6 and 12 h (Fig. 1G). These observations suggest that short-term exposure to proinflammatory cytokine TNF- α mediates DPSC apoptosis via NF-κB signaling pathway, with a concomitant increase in the levels of angiogenic signaling proteins.

Prolonged exposure of TNF- α induces phenotypic alterations and angiogenic proliferation of DPSC, in vitro. Dental-pulp and DPSC are often exposed to inflammatory and resultant hypoxic environments but are well equipped to survive for short time periods. Several studies have demonstrated the signaling and clinical aspects of short-term exposures; however, the effect of chronic exposure to proinflammatory mediators on DPSC phenotypic alterations, proliferation, and differentiation remain unknown. Hence, our studies were tailored to address the effect of DPSC on prolonged exposure to TNF-α, in vitro.

To examine the proliferation potential of DPSC, we performed a nonisotopic BrdU incorporation assay. In order to do this, DPSC primed with TNF-α for 2 days were challenged with VEGF for 5 and 10 days. As shown in Fig. 2A, cells challenged with TNF-α priming + VEGF showed a significant increase in proliferation when compared to cells treated with VEGF, TNF-α alone, or untreated. In parallel, qPCR analysis showed an upregulation of cell survival genes, namely BCL2 and Survivin, in TNF-α-treated cells on day 10 (Fig. 2B). These findings suggest that prolonged exposure to TNF-α may instigate phenotypic alterations with an anti-apoptotic resistant property.

To further corroborate our observations, we performed flow cytometry analysis in DPSC stained with CFSE dye at appropriate time points following TNF-α and VEGF treatment, as described above. As shown in Fig. 2C, cells exposed to TNF-α for 7 and 10 days displayed an increase in the proliferation profiles, with DPSC proliferation clearly being dependent on TNF-α treatment for varying time periods. An increase in the expansion of new generation cells, in day 7 and 10, unambiguously explicate the role of TNF-α in mediating angiogenic proliferation, with an anti-apoptotic resistant property. It is interesting to note the proliferation is evident only after 5 days of culture, a coherent feature of DPSC responding to angiogenic signaling. The findings were compared with the analysis performed in cells cultured in media containing 1% serum and concavalin A for 3 and 5 days. We next determined whether TNF-α perturbs proteins essential for dental-pulp longevity and mineralization. To determine that, we performed qPCR analysis to evaluate the levels of BMP, BMPR, and TGF family of proteins in cells treated with TNF-α for 5 and 10 days. As anticipated, we observed a significant decrease in the levels of BMP, BMPR, TGF-β1 and TGF- β2 in cells exposed to TNF-α (Fig. 2D). These findings clearly suggest that prolonged exposure to proinflammatory stimuli contribute significantly to an emerging angiogenic potential of DPSC.

Inhibition of NF-κB signaling restores TNF-α-induced angiogenic signaling in DPSC. Since our findings showed an initial induction of apoptosis, with an associated increase in NF-κB expression and signaling (activation of p65 and IκB-α), we examined whether inhibition of NF-κB signaling influenced TNF-□-induced angiogenesis and phenotypic alterations in DPSC. In order to do that, we first performed proliferation analysis using MTT assay. DPSC were primed with TNF-α for 48h in the presence of NEMO-binding domain (NBD) peptide, a NF-κB blocker which inhibits the IKK complex. Treatment with NBD peptide at 5 and 10 uM resulted in an approximately 20% reduction in TNF-α-induced increase in proliferation at day 5. However, proliferation analysis performed on day 7 and day 10 showed a dose-dependent decrease in proliferation (40–80% respectively), which was consistently observed at NBD doses of 5 and 10 uM (Fig. 3A). These results suggest a potential role for NF-κB inhibition in restoring a TNF-α-induced increase in proliferation of DPSC, in vitro. Furthermore, to determine whether NF-κB inhibition will reinstate the angiogenic signaling, we examined the expression levels of VEGF, EGF, and FGF family of growth factors. DPSC grown in 1% serum containing medium treated with TNF-α in combination with varying doses of NBD peptide were employed for qPCR analysis. Cells treated with TNF-α or NBD peptide alone served as control. As shown in Fig. 3B, the levels of VEGF, EGF and FGF-2 were significantly decreased or restored in cells pretreated with NBD peptide at 10 uM. The lower dose (5 uM) showed no significant changes in the expression levels.

It is fairly evident from our studies that prolonged exposure to proinflammatory stimuli may drive DPSC into senescence or a cancer cell-like state, a condition in which dysregulation of telomere binding proteins occurs leading to telomere shortening. Therefore, we moved to determine whether prolonged exposure of TNF-α influenced telomere shortening. In order to do that, DPSC treated with TNF-α for 10 days in the presence or absence of NBD peptide were used for sequence-independent multiplex qPCR analysis. It is interesting to note from the observations that cells treated with TNF-α for 10 days exhibited

a significant decrease in telomere length, which was eventually restored when treated with NBD peptide at 5 and 10 uM (Fig. 3C). These findings further corroborate our hypothesis that TNF-α-induced initial apoptosis causes the emergence of an angiogenic phenotype in DPSC.

Early inhibition of NF-κB potentiates DPSC mineralization and differentiation. Since DPSC exhibited phenotypic alterations and upregulated angiogenic signaling, we investigated whether prolonged exposure of TNF- α impeded odontogenesis in DPSC. Cells cultured in odontogenic media and challenged with TNF- α and VEGF were subjected to alizarin red s staining on day 14. Compared with untreated DPSC, the number of mineralized nodules was significantly increased in odontogenic medium; however, when the cells were treated with TNF- α the number of nodules were observed to be diminished, significantly (Fig 4A; i-iii). This indicates that prolonged exposure to TNF- α impedes the mineralization potential of DPSC.

Next, to identify the possible involvement of NF-κB in this effect of TNF-α, we cultured DPSC in odontogenic media in the presence of NBD peptide (10 uM), for 14 days. NBD peptide was replaced every 4 days. Interestingly, our studies showed a marked increase in the appearance of mineralization nodules in cells co-treated with NBD peptide (Fig. 4A: vi). Cells treated with NBD peptide in the absence of; TNF-α (Fig. 4A: v) or odontogenic medium served as a control (Fig. 4A: iv). Furthermore, cells either challenged in the absence or presence of TNF-α or NBD peptide were used for ALP activity assay. As shown in Fig. 4B and in consistence with the observations (Fig. 4A), NBD peptide treatment in combination with TNF-α significantly restored ALP activity, at day 14.

To investigate whether inhibition of NF-κB induces the expression of genes associated with mineralization, the expression of osteocalcin, osteoactivin, and RUNX2 mRNA was determined after DPSC had been treated with TNF-α in the absence or presence of NBD peptide. Quantitative PCR analysis showed that compared with untreated control, TNF-α-treated DPSC exhibited significant suppression of

the expression of osteocalcin, osteoactivin, and RUNX2 mRNA (Fig. 4C). Nevertheless, cells treated with the combinations of TNF-α and NBD peptide showed a significant increase in the expression of osteocalcin and osteoactivin, when compared to TNF-α or NBD peptide treatment alone. RUNX2 showed a trend to increase in its level; however, it failed to show significance statistically.

Lastly, to study the effects of dental materials on the regeneration potential of dental pulp tissue DPSC were cultured with media conditioned with mineral trioxide aggregate, Endosequence root repair material, and sodium fluoride. The DPSC exhibited statistically significant increased proliferation in the presence of each of the three dental materials after 24 hours (Figure 5A). Further, the DPSC did not show a decrease in differentiation potential as shown by ALP activity (Figure 5B). To investigate the effects of these dental materials on the essential process of actin cytoskeleton reorganization in DPSC actin staining was performed at one and two weeks during odontogenic differentiation. The DPSC culture in each dental material expressed less stress fiber formation as compared to the DPSC cultured in only growth medium (Figure 6).

2.3 <u>Discussion</u>

Dental pulp tissue and DPSC are often exposed to an inflammatory milieu. Although, DPSC are equipped to survive in these environments for short time periods, the effect of long-term exposure is unclear. Several cytokines have been detected in inflamed dental pulp, including TNF-α, interleukins, interferon-γ, and TGF family of proteins. These cytokines play a predominant role during inflammatory responses and are thereby believed to play a central role in the pathogenesis of pulpitis. Elegant studies by Rutherford and Gu (2000) demonstrated that local dentin-pulp inflammation interfered with odontoblast differentiation and dentin repair. Other studies attempting to investigate the effect of proinflammatory mediators showed perplexing outcomes on dental pulp longevity and differentiation

potential. The present study demonstrated that, in contrast to the effects observed by TNF-ɑ signaling alone, TNF-ɑ in combination with VEGF alters the proliferation and differentiation potential of DPSC.

Dental pulp tissue has several functions including nutrition, initiation, protection, formation, repair, and promotion of tooth vitality. During inflammatory episodes, dental pulp is more sensitive to changes in tissue pressure and requires an active drainage system to eliminate excess fluid and macromolecular substances. This system plays a crucial role in the repair and wound healing processes following inflammatory states. In particular, the enhanced cellular differentiation and neovascularization of the dental pulp are important events for pulp healing (Arfuso, 2006). The pulpal healing potential is associated with the ability of dental pulp cells to secrete growth factors, including angiogenic factors (Tran-Hung et al., 2006; 2008). Various reports have demonstrated the role of inflammatory cytokines on dental pulp longevity and differentiation potential. However, this has been a matter of continuing controversy because of the fact that inflammatory stimulus is always paired with the angiogenic signaling. Therefore, studies to investigate the effect of inflammatory mediators in combination with the angiogenic signaling is highly crucial to further the current understanding of the pathogenesis associated with dental pulp regeneration. In the present study, we demonstrated that TNF-□ along with VEGF increased the proliferation of DPSC with a concomitant decrease in the length of telomeres. This finding is indicative of an angiogenic proliferation of DPSC in human dental pulp during inflammation.

The present study also demonstrated that TNF-ɑ activated the NF-κB signaling pathway in DPSC. NF-κB signaling has been considered the master transcription factor that regulates a variety of pro-inflammatory cytokines and angiogenic factors in dental pulp fibroblasts and oral epithelial cells (Nakane et al., 1995; Martin et al., 2001; Ogawa et al., 2002; Nociti et al., 2004). Furthermore, several studies have proposed that NF-κB may be an important target for inhibiting oral and dental inflammation. However, the mechanisms by which NF-κB signaling might be responsible for the angiogenic signaling and its

resultant effects on the phenotypic alterations and differentiation potential of DPSC have not been explored. We found that TNF-ɑ treatment of DPSC significantly induced p65 phosphorylation, a transactivation domain critical for NF-κB transcription (Baldwin et al., 2001). In parallel, we also observed an increase in the phosphorylation of IκB-ɑ, a process essential for NF-κB activation (Wang et al, 1996, 1998, Baldwin et al., 2001).

Our studies have revealed an interesting finding that short-term exposure to TNF-ɑ induced apoptosis; whereas chronic exposure (10 days) significantly altered the proliferation potential of DPSC into an apoptotic resistant state, with an increase in cell survival genes BCL2 and Survivin. It is interesting to note that cells primed with TNF-ɑ exhibited an increased proliferation at 5 and 10 days, while cells treated with TNF-ɑ alone showed no difference in proliferation. These findings reveal an intriguing possibility that chronic exposure to TNF-ɑ leads to an increased proliferation of DPSC attaining a cancer cell-like phenotype. Simultaneous increase in the angiogenic signaling proteins such as VEGF, EGF, FGF-1, FGF-2, BMP, and TGF and a decrease in the telomere length may possibly explain pulp neovascularization.

To investigate whether TNF-ɑ-induced NF-κB signaling contributes to the angiogenic proliferation and differentiation, we blocked NF-κB using NBD peptides. It was observed that nearly 40% to 50% of the increase in TNF-ɑ/VEGF-induced proliferation was significantly decreased in the DPSC population treated with NBD peptide. Additionally, it is interesting to note from our findings that TNF-ɑ-induced abrogation of DPSC mineralization and differentiation was significantly ameliorated in cells treated with NBD peptide. Our results are the first demonstration that TNF-ɑ-induced NF-κB signaling and the ensuing upregulation of angiogenic signaling contribute significantly to the proliferation and differentiation potential of DPSC.

DPSC were cultured in conditioned media to investigate the effects of various dental materials on the proliferation and differentiation of DPSC in vitro. These materials, mineral trioxide aggregate, Endosequence root repair material, and sodium fluoride, are often present when dental pulp is undergoing the regeneration process. DPSC cultured in conditioned media with each of the materials exhibited an increase in proliferation as measured by MTT assay. Since there was an observed increase in proliferation there may be a potential therapeutic use of these materials to counteract the deleterious effects of inflammation on the proliferation and differentiation on DPSC. To investigate the effects of these materials on differentiation of DPSC ALP activity was measured and actin cytoskeleton was stained and imaged. There was no observed difference in ALP activity between DPSC cultured in differentiation media and those cultured in conditioned media. Further the DPSC cultured in conditioned media had fewer stress fibers than DPSC cultured in growth media alone similar to DPSC cultured in differentiation media. This indicates that these materials had little effect on the differentiation potential of DPSC. Since there was an observed increase in proliferation and minimal effect on the differentiation potential of DPSC when cultured with conditioned media with MTA, ERRM, or sodium fluoride this indicates that they are acceptable materials to use with dental pulp tissue that is undergoing repair and regeneration. Further these materials may have a therapeutic effect as indicated by the increased proliferation.

APPENDIX : DENTAL PULP STEM CELL CULTURE PROTOCOL

Reference: (Ravindran, Gao, Kotecha, Magin, Karol, Bedran-Russo, George. 2012)

Reagents
- Dental pulp stem cell growth media (500 mL); store at 4°C
    - 395 mL MEM Alpha (1X) + GlutaMAX (Life Technologies 32561-037); store at 4°C
    - 100 mL fetal bovine serum (Life Technologies 16000-044); store at -20°C
    - 5 mL 100X Antibiotic-antimycotic (Life Technologies 15240-062); store at -20°C
    - Filter before use
- Trypsin-EDTA (Corning cellgro 25-053-CI); store at -20°C
- Trypan blue (Sigma-Aldrich T8154)
- DMSO (Fisher D128-1)
- Sterile PBS without Ca^{2+} or Mg^{2+} (Thermo Scientific SH3025601)

Supplies and equipment
- Hausser phase contrast hemacytometer (Fisher 02-671-5)
- Benchtop Centrifuge (Thermo Scientific 75007210)
- Optical microscope
- Water bath (37°C, Polyscience WA05A11B)
- Sterile disposable bottle top filters (Thermo Scientific 295-4545)
- 5, 10, 25 mL Sterile polystyrene disposable serological pipets (Fischer 13-678-11D)
- T25, T75 Sterile tissue culture flasks, vented (Thermo Scientific 130189, 130190)
- 15 mL Sterile centrifuge tubes (Fischer 05-539-800)

*Note: All steps should be performed in the tissue culture hood unless otherwise noted. All items should be wiped dry and sprayed with 70% ethanol before being placed in the tissue culture hood.

DPSC thawing procedure

1) Place DPSC growth media in water bath and warm to 37°C or for at least 20 minutes.

2) Simultaneously, place a T25 flask containing 5 mL DPSC growth media in incubator at 37°C.

3) Rapidly thaw frozen cell vial in 37°C water bath for about 2 min.

4) Transfer 500 uL of warmed DPSC growth media into the thawed cell vial aspirating up and down gently to homogenize the solution.

5) Removed T25 flask from incubator and drop-wise transfer the homogenized cell solution from the vial to the T25.

6)   Gently and carefully rock flask back and forth to ensure cells are uniformly distributed across its surface.

7) Place the flask(s) in the incubator. Check the confluency every 24 h and change media every 3-4 days.

DPSC media changing procedure

1) Warm DPSC growth media and sterile PBS in water bath to 37°C for approximately 20 minutes.

2) Aspirate all old media from each flask, carefully so as to not scrape the cell growth surface.

3) Add enough PBS to each flask to cover its surface and gently rock to rinse cells.

4) Remove the PBS and add 3-5 mL DPSC growth media to a T25 flask or 8-12 mL for a T75 flask. Return flasks to incubator and change media every 3-4 days.

DPSC passaging procedure

1) Warm DPSC growth media and sterile PBS in water bath to 37°C for approximately 20 minutes.

2) Aspirate all old media from each flask, carefully so as to not scrape the cell growth surface.

3) Add enough PBS to each flask to cover its surface and gently rock to rinse cells.

4) Remove the PBS and add enough trypsin to cover the surface of the flask. Return the flasks to the incubator for 2 minutes to allow for the cells to detach from the flask. You can allow the cells to sit for additional time in the incubator if they do not detach or gently tap flasks a few times to detach cells.

5) Add 5 mL of DPSC growth media to each flask to neutralize the trypsin and to flush cells off of the tissue culture plastic.

6) Aspirate the cell suspension from each flask and add to a 15 mL centrifuge tube. Remove a 10 uL cell suspension aliquot for counting. Centrifuge the cells at 1200 rpm for 5 minutes.

7) While cells are spinning down, mix the 10 µL cell suspension aliquot with 10 µL of Trypan blue. Pipette several times to mix the stain and cell suspension.

8) Place a cover slip on the hemocytometer and pipette 10 µL of the stain/cell suspension into the hemocytometer.

9) Cell counts are performed in as many of the nine separate regions of the hemocytometer as is feasible. Average number of cells per region is used to calculate the total cell population. For this calculation, the dilution factor is typically 2 (1:1 ratio of cell suspension to Trypan blue).

Total Cell Population = (Mean Cells per Region) * Dilution * 10,000 * (Cell Suspension Volume)

10) Aspirate off the media supernatant while carefully avoiding the cell pellet and add new media to dilute cells to desired concentration.

11) Seed the cells at the required density (usually 5,000 cells/cm^2).

12) Place the flask(s) into the incubator. Check the confluence every 24 h and change media every 3-4 days.

DPSC freezing procedure

1) Grow cells to 100% confluence and change media the day before freezing.

2) Warm DPSC growth media and sterile PBS in water bath to 37°C for approximately 20 minutes.

3) Aspirate all old media from each flask, carefully so as to not scrape the cell growth surface.

4) Add enough PBS to each flask to cover its surface and gently rock to rinse cells.

5) Remove the PBS and add enough trypsin to cover the surface of the flask. Return the flasks to the incubator for 2 minutes to allow for the cells to detach from the flask. You can allow the cells to sit for additional time in the incubator if they do not detach or gently tap flasks a few times to detach cells.

6) Add 5 mL of DPSC growth media to each flask to neutralize the trypsin and to flush cells off of the tissue culture plastic.

7) Aspirate the cell suspension from each flask and add to a 15 mL centrifuge tube then centrifuge the cells at 1200 rpm for 5 minutes.

8) Aspirate off the media supernatant and add 10 mL freezing media (70% DMEM, 20% FBS, 10% DMSO).

9) Aliquot 1 mL cell solution per cryogenic tubes and snap freeze in liquid nitrogen.

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Ogawa, T., Asai, Y., Hashimoto, M., Takeuchi, O., Kurita, T., Yoshikai, Y., et al. Cell activation by Porphyromonas gingivalis lipid A molecule through Toll-like receptor 4- and myeloid differentiation factor 88-dependent signaling pathway. Infect Immun 14:1325-1332:2002.

Ravindran, S., Gao, Q., Kotecha, M., Magin, R.L., Karol, S., Bedran-Russo, A., and George, A. Biomimetic extracellular matrix-incorporated scaffold induces osteogenic gene expression in human marrow stromal cells. Tissue Engineering Part A 18:295-309:2012.

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Thomas, T.H., Advani, A. Inflammation in cardiovascular disease and regulation of the actin cytoskeleton in inflammatory cells: the actin cytoskeleton as a target. Cardiovasc Hematol Agents Med Chem 4:165-82:2006.

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Wang, C.Y., Mayo, M.W., Korneluk, R.C., Goeddel, D.V., Baldwin, A.S. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281:1680-1683:1998.

Yang, F., Yamashita, J., Tang, E., Wang, H., Guan, G., Wang, C.Y. The zinc finger mutation C417R of I-κB kinase g impairs lipopolysaccharide- and TNF-mediated NF-κB activation through inhibiting phosphorylation of the I-κB kinase B activation loop. J Immunol 172:2446-2452:2004.

Michael Boyle

EDUCATION

August 2014-Present, anticipated May 2018
University of Illinois at Chicago
Doctor of Dental Medicine

August 2012-Present, anticipated December 2014
University of Illinois at Chicago
Master of Science, Bioengineering

January 2010-May 2012
University of Illinois at Chicago
Bachelor of Science, Bioengineering
Concentration in Cell and Tissue Engineering

June 2006-August 2006
Ecole d'Ingénieurs de Purpan, Toulouse, France
Study Abroad

August 2003-May 2008
University of Illinois at Urbana-Champaign
Bachelor of Science, Animal Sciences
Concentration in Science, Biotechnology, & Pre-Veterinary Medicine
Minor in Chemistry

PRESENTATIONS AND POSTERS

Michael Boyle, Premanand Sundivakkam, Michael Cho, Satish Alapati. Cytoskeletal reorganization during odontogenic differentiation of dental pulp stem cells. AADR/CADR Annual Meeting and Exhibition, Charlotte, NC, 2014.

Crystal Chun, Chelsee Strojny, **Michael Boyle**, Amelia Bartholomew, Premanand Sundivakkam, Satish Alapati. Inflammation-induces initial apoptosis impedes dental pulp stem cell differentiation. AADR/CADR Annual Meeting and Exhibition, Charlotte, NC, 2014.

Crystal Chun, **Michael Boyle**, Chelsee Strojny, Amelia Bartholomew, Premanand Sundivakkam, Satish Alapati. Acute inflammation induces apoptosis via NF-κB-mediated signaling axis in human dental pulp stem cells. University of Illinois College of Dentistry Clinic and Research Day, Chicago, IL, 2014.

Michael Boyle, Chelsee Strojny, Crystal Chun, Amelia Bartholomew, Premanand Sundivakkam, Satish Alapati. Inflammation-mediated angiocrine effect of endothelial cells impedes dental pulp tissue regeneration. University of Illinois College of Dentistry Clinic and Research Day, Chicago, IL, 2014.

Michael Boyle, Chelsee Strojny, Crystal Chun, Gabriella Szewczyk, Premanand Sundivakkam, Amelia Bartholomew, Satish Alapati. Inflammation-induces initial apoptosis followed by the emergence of dental pulp stem cell proliferation, impeding its differentiation potential. Annual Stem Cell and Regenerative Medicine Program, Chicago, IL, 2013.

Meghana Prabha, Khushbu Barot, Milena Bulic, **Michael Boyle**, Rachel Vorwaller, Aaron Vogt, Satish Alapati. The effect of hyaluronic acid on the osteogenic differentiation of human dental pulp stem cells. University of Illinois College of Dentistry Clinic and Research Day, Chicago, IL, 2013.

Khushbu Barot, Milena Bulic, **Michael Boyle**, Rachel Vorwaller, Aaron Vogt, Meghana Prabha, Satish Alapati. The effect of TNF-α/ASA on the odontogenic differentiation of hDPSCs – an *in vitro* study. University of Illinois College of Dentistry Clinic and Research Day, Chicago, IL, 2013.

Milena Bulic, Khushbu Barot, **Michael Boyle**, Rachel Vorwaller, Aaron Vogt, Meghana Prabha, Satish Alapati. Gene expression analysis: the effect of TNF-α/ASA on the odontogenic differentiation of hDPSCs. University of Illinois College of Dentistry Clinic and Research Day, Chicago, IL, 2013.

Melanie Borman, **Michael Boyle**, Madhuri Paravastu, Hamed Naimipour, Michael Cho, Satish Alapati. Regenerative tissue engineering of dental pulp. 23rd Annual Engineering Expo Senior Design Competition, Chicago, IL, 2012.

VOLUNTEER ACTIVITIES

September 2013-August 2014
The Craniofacial Center at the University of Illinois Hospital

August 2013-May 2014
Tutoring Chicago

August 2013-Present
Chicago Medical Reserve Corps

PROFESSIONAL EXPERIENCE

June 2012-Present
University of Illinois at Chicago Department of Endodontics
Research Assistant

November 2008-January 2010
Monsanto Genetic Quality Assurance Laboratory
Laboratory Technician

May 2007-October 2007
University of Illinois at Urbana-Champaign Department of Crop Sciences
Research Assistant

HONORS AND AWARDS

2014 Schour Scholar

2014 UIC Student Travel Presenter's Award

2014 Center for Clinical and Translational Science Multidisciplinary Team Science Award

2013 Illinois State Dental Society Best Basic Science Award

2013 UIC College of Dentistry Clinic and Research Day Predoctoral Student Best Basic Science

2010-2012 Dean's List

PROFESSIONAL MEMBERSHIPS

American Association of Endodontists

American Dental Education Association

Illinois Academy of General Dentistry

Illinois State Dental Society

International Association for Dental Research

American Association for Dental Research

AADR National Student Research Group

Chicago Dental Society

Engineering World Health

Biomedical Engineering Society

Figure 1
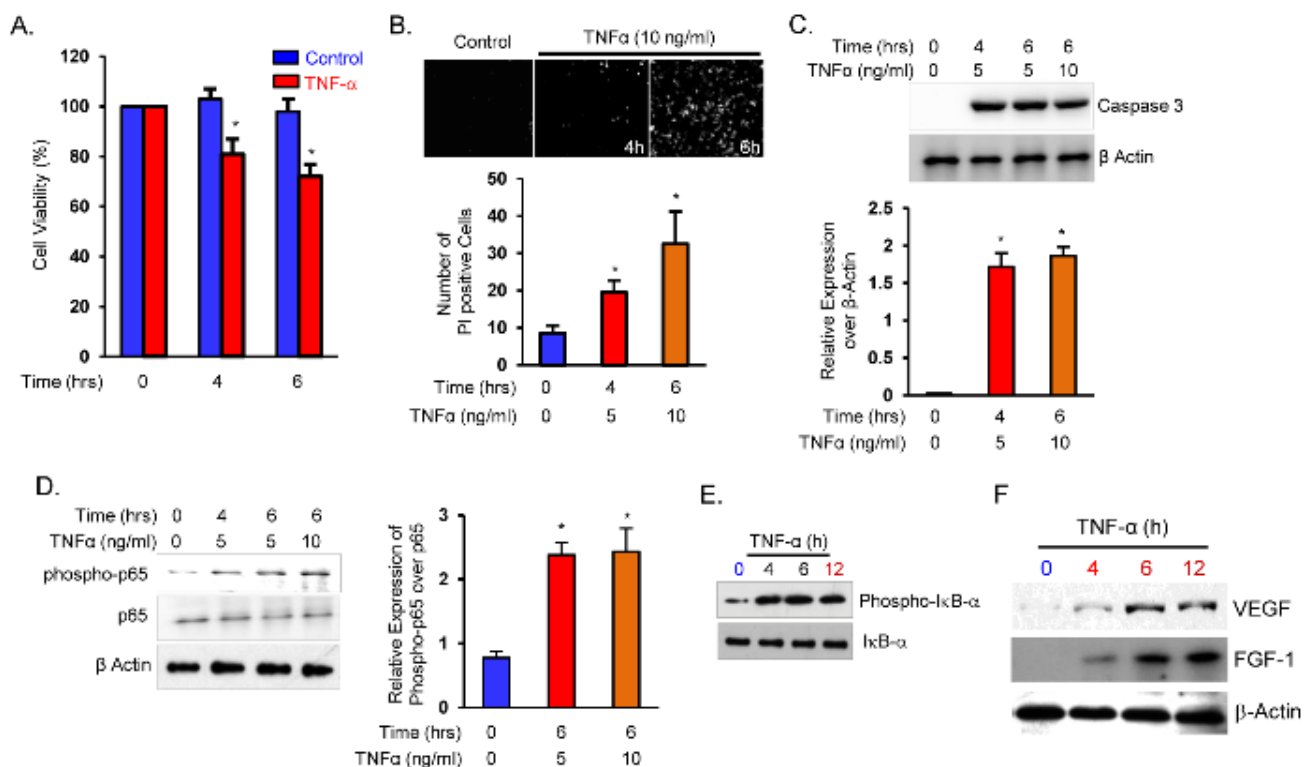
Figure 1A. Cell viability as measured by MTT B. Propidium iodide staining C. Western blot analysis of caspase 3 D. Western blot analysis of p65 and phospho-p65 along with quantification E. Western blost analysis of IκB-α
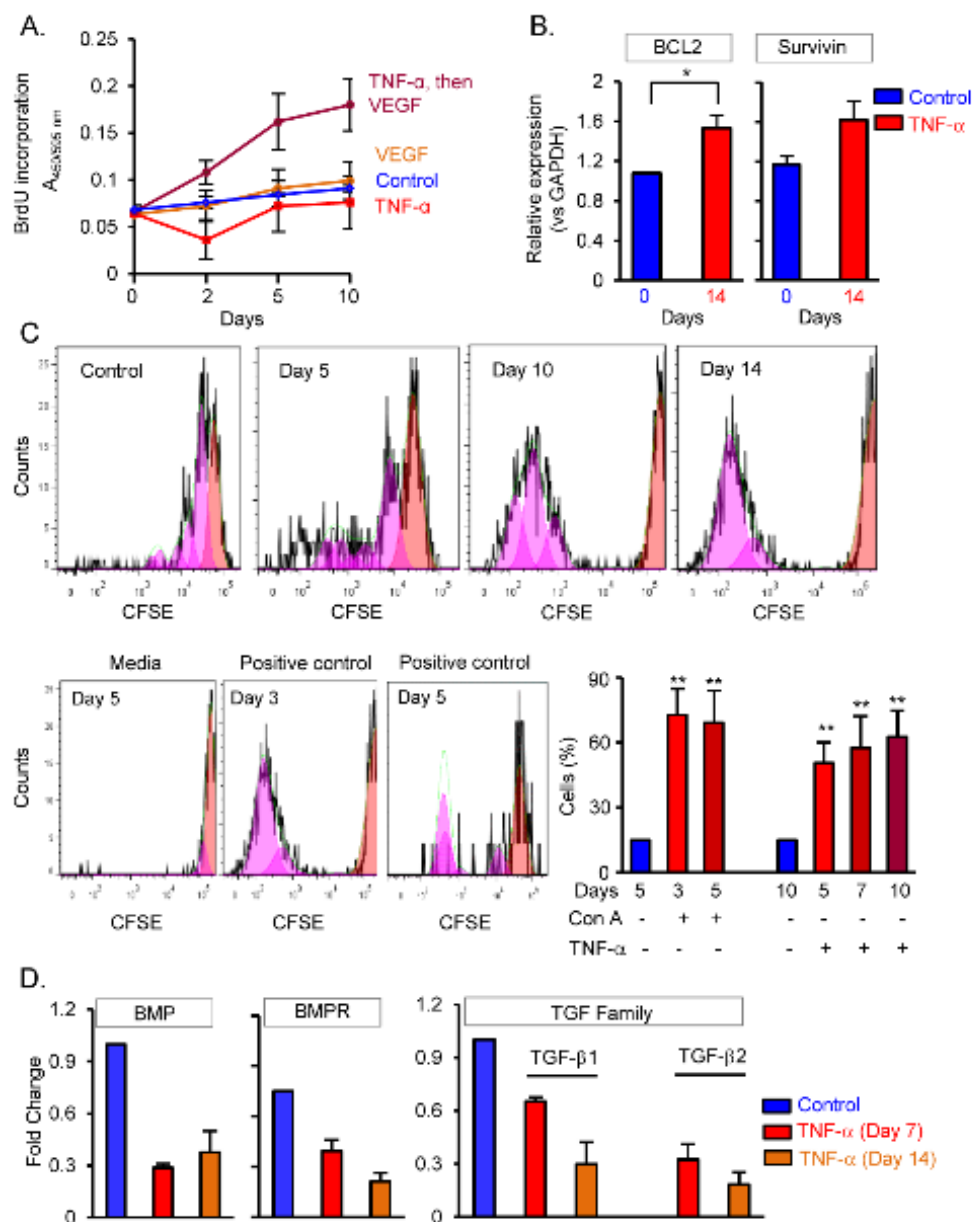
Figure 2

Figure 2A. BrdU analysis with VEGF, TNF-α, AND both B. Survival related gene expression: BCL2 and Survivin C. CSFE staining and flow cytometry with TNF-α D. TGF Family gene expression
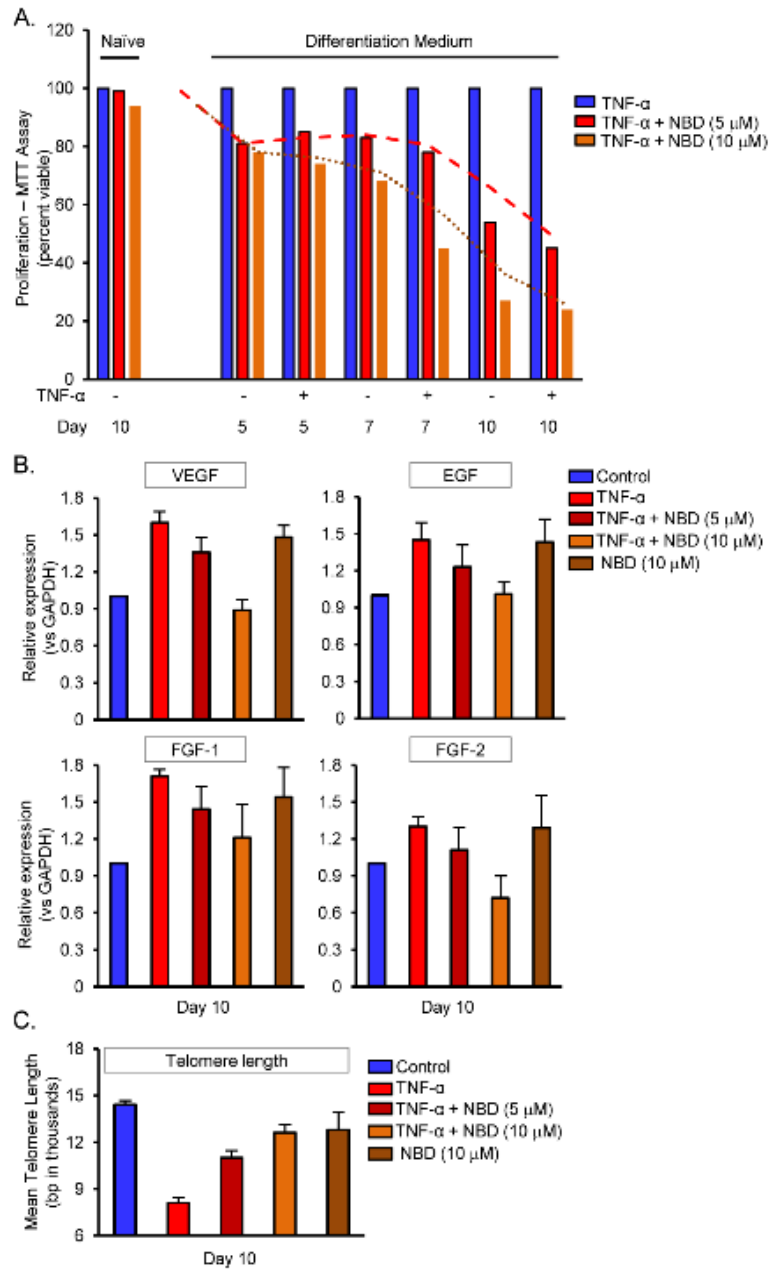
Figure 3

Figure 3A. MTT assay with TNF-α B. Mitogenic gene expression with Tnf-α C. Telomere shortening with Tnf-α
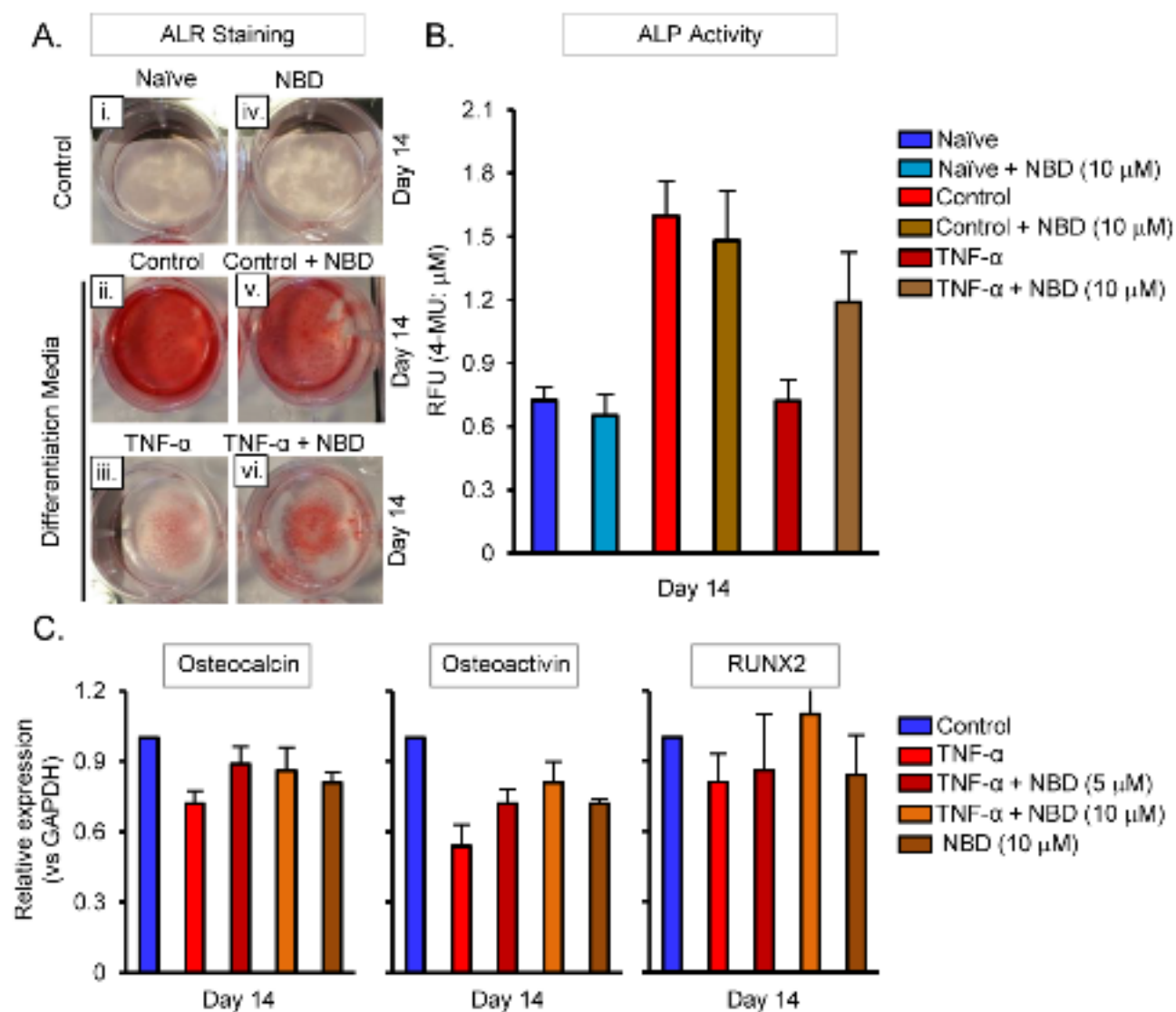
Figure 4

Figure 4A. Alizarin red s staining with Tnf-α B. Alkaline phosphatase activity with Tnf-α C. Osteogenic gene expression with Tnf-α
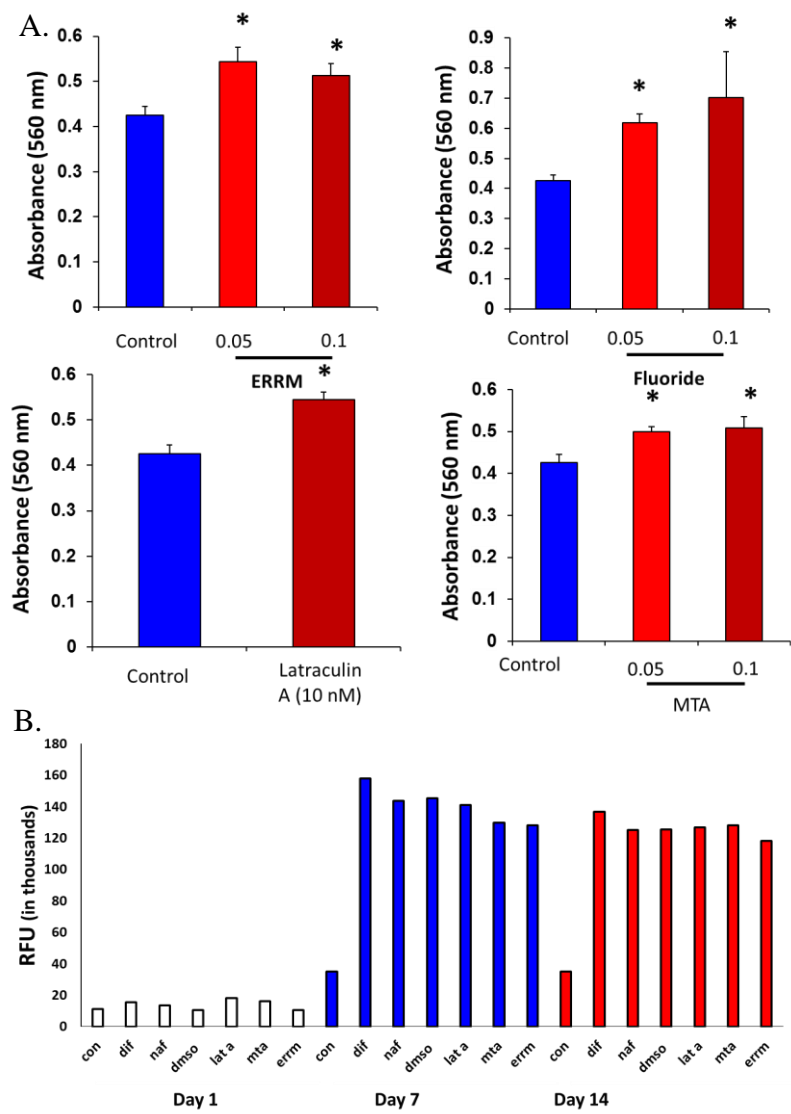
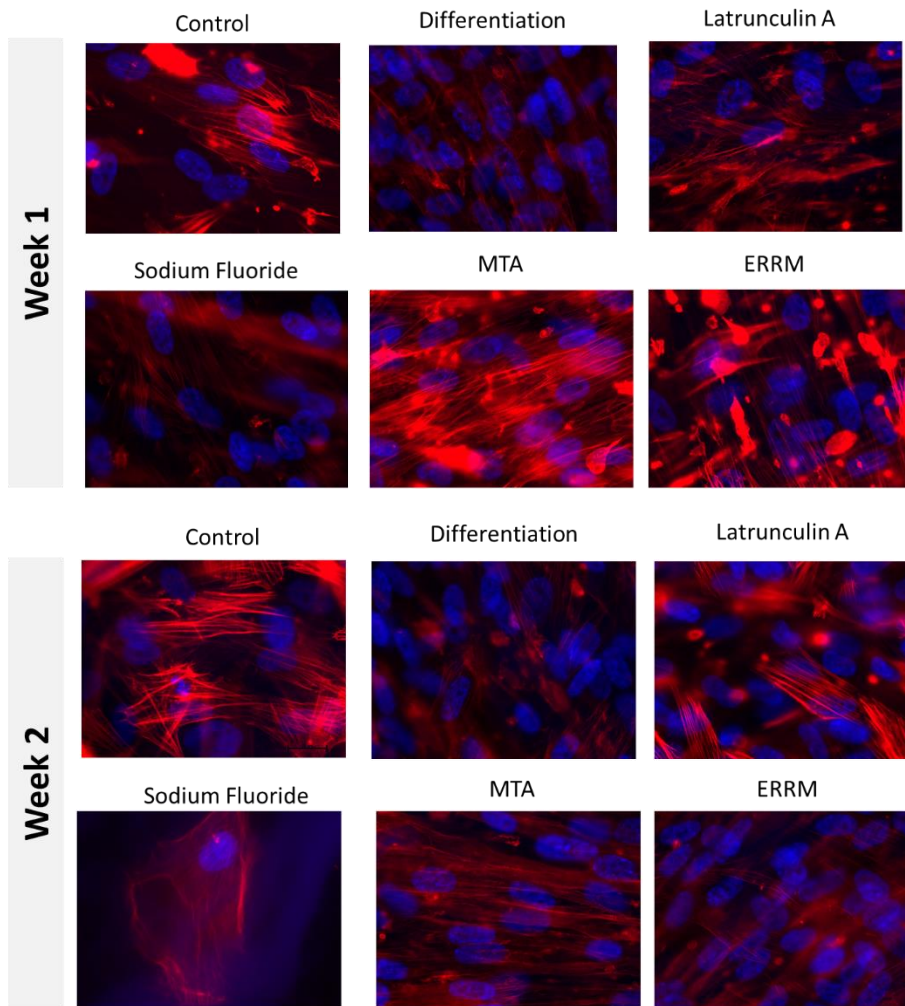Figure 5A. Proliferation as measured by MTT assay B. ALP activity

Figure 6. Actin staining at one and two weeks during odontogenic differentiation