The Expression of Periostin in Dental Pulp Cells

By

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Abstract

Introduction: The proper regulation of the dentin-pulp complex is intimately related through crucial cell-matrix interactions and important bioactive proteins. The proteins modulating these interactions are highly expressed during development and implicated in tissue repair and regeneration. Within this context, periostin is essential for ECM stability, collagen fibrillogenesis, and tissue healing. Periostin is regulated by TGF-β1 in response to biomechanical challenges in the PDL. In the scope of the dental pulp, periostin expression is reported during development and active dentinogenesis, but has yet to be evaluated in dental pulp cells specifically. We hypothesize that periostin is expressed by DPCs in response to TGF-β1 and biomechanical stimulation, which has implications in dental pulp tissue healing and regeneration. **Aims**: 1) To determine if periostin is expressed by DPCs and to analyze the effect in response to TGF-β1 2) To analyze the influence of biomechanical stimulation on the expression of periostin in DPCs, 3) To analyze the influence of periostin on the expression of collagen in DPCs. **Methods**: Human dental pulp stem cells (DPSC), human dental pulp fibroblasts (DPF), and murine odontoblast-like cells (MDPC-23) were treated with different concentrations of TGF-β1 or different regimens of biomechanical stimulation to evaluate periostin expression. Cells were also treated with periostin to evaluate the effect on collagen. Western blot and ELISA were used to evaluate protein expression. RNA analysis was performed by qRT-PCR and a Total Collagen Assay was utilized to evaluate collagen. Statistical analysis was performed by Student T-test and ANOVA with Fisher's LSD test. **Results**: Each cell line expressed periostin protein and mRNA. TGF-β1 supplementation resulted in significant changes of periostin expression. Biomechanical stimulation acts to

induce changes in periostin expression. No statistically significant differences were found in total collagen expression. **Conclusions**: Expression of periostin was identified in each of the dental pulp cell lines, which can be regulated by TGF-β1. DPSC are the most responsive cells to stimulation. Continued research and evaluation is needed to determine the potential therapeutic ability of periostin within the dental pulp.

Chapter 1 - Introduction

The term "Regenerative Endodontics" is defined as a "biologically-based procedure designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as the dentin-pulp complex" (1). The success of this procedure varies, and applying a clinical treatment protocol can be challenging (2). In most cases, this procedure is used to manage immature permanent teeth diagnosed with pulpal necrosis (2). However, current research is leading to a better understanding of the underlying mechanisms of pulpal regeneration and the potential exists to develop a reliable clinical protocol that may lead to a consistently successful outcome. Recently, it has been proposed that progenitor cell lines in combination with extracellular matrix (ECM) bioactive molecules (3), appropriate 3D scaffolds (4, 5), and biomaterials (6) can potentially achieve a state of pulpal regeneration (1, 3, 7, 8).

The structure and properties of the dentin-pulp complex are intimately related through crucial cell-matrix interactions (9). The proper regulation of these interactions determines the adaptive dentin-pulp response by orchestrating the function of important bioactive proteins such as growth factors, cytokines, chemokines, and neuropeptides (9-15). The proteins modulating these interactions are collectively known as matricellular molecules. These molecules act during the repair process by providing a range of signals to the constituent cell populations and modulating their phenotype. These molecules are expressed at different stages of the healing process with chemo-attractive properties that may signal endogenous cells from the pulp proper into the healing zone with the expectation of generating tertiary dentin to seal the injury, allowing for soft tissue remodeling and repair, maintaining the pulp vitality, or promoting regeneration of pulp-like tissue (16-20).

In this context, periostin, a matricellular protein has to be considered. Periostin is a 835-amino acid secreted protein; located on chromosome 13 in humans at map position 13q13.3 (21). It is a disulfide linked 90-kDa heparin-binding N terminus-glycosylated protein (21). It was originally named osteoblast-specific factor-2 (OSF-2), because it was discovered in mouse osteoblasts (22), but recently it has been renamed 'periostin' due to

its heavy localization to periodontal ligament (PDL) fibroblasts and the periosteum of mice during development (23). It has been reported in tissues throughout the human body which include bone, periosteum, skin and heart tissue; as well as being up-regulated in events needing repair, such as in vascular injury, myocardial infarction, muscle injury, epithelial ovarian cancer, colorectal cancer, and pulmonary vascular remodeling (21).

Within the oral cavity, periostin is implicated in the maintenance of proper periodontium function, integrity and tissue strength (21, 24). It regulates the structural and functional characteristics of the PDL (21, 24) in order to withstand the forces of mastication (25) and is regulated by TGF-β1 (22, 26) in response to biomechanical challenges (24, 27). Periostin is important for ECM homeostasis, remodeling and repair (21, 24) and plays a direct role in controlling tissue healing (21, 24, 25, 27). It is considered to be an extracellular modulator of cell function via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptor interactions with growth factors and matrix components such as collagen type 1 (26, 28). These matricellular interactions have been reported to influence cell behavior (28) as well as collagen fibrillogenesis (26, 29, 30).

Although periostin has been identified in numerous tissues throughout the body and localized to the PDL, its identification in the dental pulp has been controversial. Early evidence, using immunohistochemistry sections of mandibles from mice show heavy localized expression of periostin in the PDL only (23, 31). However, more recent studies using *in situ* hybridization of 4 & 10-week old mice mandibles show periostin expression throughout the dental pulp, specifically localized at the "pre-odontoblast layer" during tooth development (27). Additionally, periostin expression was recently observed throughout the pulp proper of fully developed molars in mice one day following cavity preparation without pulp exposure using immunohistochemistry sections (32). This experimental model suggests that the mechanical stress of drilling acts in the same way as mechanical stress of mastication in order to induce periostin expression. Interestingly, periostin was identified near the center of the coronal pulp, in proximity to the pulp chamber floor and as magnification was increased periostin localization was observed to be extracellular. Periostin expression peaked at 24 hours but was detected up to 7 days

following the cavity preparation. However, periostin was not identified in the odontoblast layer, but there was heavy periostin expression throughout the PDL (32).

The dental pulp is an appropriate tissue to investigate periostin expression since it is a loose connective tissue that is highly cellular, vascular and innervated (8). It contains multiple cell populations including odontoblasts, fibroblasts and dental pulp stem cells, as well as immunocompetent cells including dendritic cells, macrophages, lymphocytes, and mast cells (8). The presence of multiple cell populations creates ambiguity as to which, if any of these cells express periostin. Odontoblasts are a specialized post-mitotic cell responsible for the secretion of dentin (8, 9, 33). They line the inner wall of dentin around the periphery of the pulp proper. They have cell bodies that are highly polarized, with odontoblastic processes that extend into predentin and dentinal tubules and are able to secrete collagen, glycoproteins and calcium salts (8, 9) prior to mineralization of dentin. Conversely, dental pulp fibroblasts are located throughout the pulp proper and are responsible for the formation and maintenance of the fibrous components and ground substances of the connective tissue (8). Fibroblasts synthesize and secrete elements of the ECM including collagen, elastin, proteoglycans, glycoproteins, cytokines, growth factors and proteinases (8). Fibroblasts are involved in remodeling the connective tissue through degradation of collagen and can synthesize molecules for its replacement (8). The main difference in collagen between the cell types is the type of collagen secreted by odontoblasts normally becomes mineralized (8, 9). Dental pulp stem cells are adult stem cells localized to the perivascular region and the cell-rich zone near the odontoblastic layer in the pulp proper. These cells are unspecialized with the ability for self-renewal by mitosis while in an undifferentiated state. They are multipotent, have the capacity to continuously divide, and produce progeny cells that can migrate, differentiate and proliferate into a well-differentiated cell line (34). The pulp also contains an ECM, which is a structure-less mass that makes up the bulk of loose connective tissue. It is mainly composed of collagen I, and III, but contains small amounts of collagen V, VII and XII. It also contains other ground substances such as proteoglycan, glycosaminoglycan, non-collagenous protein, hyaluronic acid, chondroitin sulfate and elastic fibers (8, 9). The ECM functions in cell-matrix adhesion and signaling, regulates nutrient diffusion, waste

products, and soluble signaling in order to maintain pulpal homeostasis. The ECM also contains cytokines, growth factors and inflammatory mediators (8).

The characteristics of the pulp allow for a unique environment as it is enclosed entirely by dentin, which forms the dentin-pulp complex. Specifically, dentin is a calcified connective tissue that is made up of 10% water, 20% organic material and 70% inorganic material (9, 33). The inorganic matrix is composed of hydroxyapatite (9), while the organic material consists of about 90% collagen, the majority of which is collagen type I, with small traces of collagen type V (9, 33). Collagen fibrils are important to dentinogenesis as they provide an organized scaffold for mineralization (33). The non-collagenous components include dentin phosphoproteins (DPP), dentin matrix protein (DMP1), dentin sialoprotein (DSP), Osteopontin (OPN), Osteocalcin, and bone sialoprotein (BSP), as well as other proteoglycans, Gla proteins, glycoproteins, growth factors and lipids (6). The growth factors included are bone morphogenetic proteins (BMP), insulin-like growth factors (IGF), and transforming growth factor beta (TGF-β). These non-collagenous components and growth factors play a crucial role in dentinogenesis as signaling molecules and regulators of mineralization (8).

The primary functions of the dentin-pulp complex are sensory, nutritional, and protective. The dentin-pulp complex is similar to other organs throughout the body, in that it possesses innate and adaptive immunity to defend against acute and/or chronic, physiologic or pathologic challenges. It responds as a defensive organ and is regulated by its ability for pulpal homeostasis and dentinogenesis (6). Generally, any irritation or stimulation to the pulp that causes the local release of inflammatory mediators, may result in arteriolar vasodilation, increased capillary hydrostatic pressure, leading to increased tissue pressure and the potential for localized areas of inflammation (35). In a clinical scenario, this sequence of events could be triggered by restorative procedures, dental trauma, uncontrolled orthodontics, para-functional forces, abrasion, or dental caries (36). The dental pulp's ability to protect itself is based on the nature and degree of the stress, the severity and proximity to the pulp as well as the amount of force placed on the entire tooth (15). In general, the potential outcomes from such events include repair and

healing, canal obliteration, or complete pulpal necrosis (36). In the event of repair and healing, the pathologic challenge must be removed and the neurovascular supply must remain intact. This allows for reactionary dentin, a sub-set of tertiary dentin, to be formed (9). It is characterized as a response by the original odontoblasts from a mild to moderate stress (8, 9). Specifically, an up-regulation of molecular events, initiated by the stimulatory effects that release growth factors (TGF-β1, BMP, FGF) from dentin (20, 37, 38) leads to collagen secretion from existing post-mitotic odontoblasts and subsequent mineralization. Alternatively, reparative dentin may be formed when the pathological challenge to the pulp is harmful enough to cause cell death to the original odontoblasts (20). It is proposed that progenitor cells within the pulp are then signaled to migrate, proliferate and differentiate into odontoblast-like cells at the site of injury leading to reparative dentin formation (20). Lastly, when a pathological challenge is strong enough to cause all cells within the pulp to die, definitive root canal treatment must be performed. Necrosis occurs as a result of unmanageable inflammation leading to irreversible damage of the pulp.

Under normal conditions, the pulp and PDL are maintained by appropriate mechanical loading (27, 39, 40) regulated by TGF-β1 (22-25, 27). It is a regulator of cell growth and differentiation, matrix biosynthesis (41, 42), acts as a chemo-attractant and promotes dentinogenesis (9). TGF-β1 has been implicated in odontoblast differentiation and dentin formation, as well as in the initiation of collagen synthesis (41). TGF-β1 is also known to regulate periostin expression and studies have shown there is a dose-dependent increase of periostin mRNA by recombinant TGF-β1, in both PDL fibroblasts (24, 43) and pre-odontoblasts *in vitro* (27). In this context, there was a 3-fold increase of periostin mRNA levels in comparison to the age-matched control mice when stimulated with TGF-β1. Furthermore, TGF-β1-null mice displayed no apparent tooth phenotype during normal early tooth development. The periostin mRNA level in the TGF-β1-null incisor was reduced to 75% of the age-matched control (27).

Application of biomechanical stimulation through cyclic strain has been used to evaluate dental pulp cells, and how it effects proliferation (44), production of inflammatory

cytokines and collagen (45). It has also been shown to stimulate odontoblastic differentiation of dental pulp stem cells (40). The application of cyclic strain has also been shown to significantly increase periostin expression in PDL cells (27, 30). In PDL cells, periostin mRNA expression is increased by the application of uniaxial cyclic strain and the addition of exogenous TGF-β1 (24, 30). The effects of biomechanical stimulation on periostin have not been evaluated in dental pulp cells. In general, periostin-null dentin in knockout mice show increased mineralization and decreased pulp space under mechanical loading. Periostin-null dentin maintains integrity of pulp space under loading-free conditions (27), but mechanical loading of teeth during mastication produce thick secondary dentin, whereas impacted teeth rarely produce secondary dentin (39). Additionally, during periostin-null mice knock out experiments the dentin in the incisor became thicker and the pulp space gradually became narrower under occlusal loading. There was approximately 80% reduction of pulp space compared with that of the age-matched control. The contralateral loaded-free incisor maintained the integrity of the pulp tissue and space. Interestingly, deletion of periostin also lowers the overall non-collagen protein levels and affects the group of small integrin-binding ligand, N-linked glycoproteins (SIBLING) in dentin (27). NCP with the ECM are believed to be essential for initiation and control of mineralization (9, 41, 46). In periostin-null mice, DSP levels were increased, while DMP-1 levels were decreased, suggesting that deletion of periostin leads to dramatic changes of SIBLING protein expression profiles, affecting the mineralization of dentin and further suggesting that periostin plays a role in regulating these proteins (27, 41).

Recruitment of appropriate/adequate cells to the area, stabilization of the matrix, and cell proliferation are critical characteristics that influence tissue healing responses and homeostasis. An effect on collagen matrix content and quality translate into altered biomechanical properties and diminish its capacity to respond to stress. Periostin not only influences cell proliferation and migration but has a considerable effect on collagen fibrillogenesis during wound healing with a clear effect on tissue strength (29, 30). The extracellular matrix, mainly composed of collagen, serves as a scaffold for cellular organization (29). The matricellular molecules, such as periostin, serve as mediators

and/or signaling molecules to modulate cell activities. Furthermore, periostin's ability to bind collagen and interact with cell surface integrin receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) highlights its potential role in different cell functions (29). These properties could enhance its potential role as a mediator of collagen metabolism by different DPC populations relevant in dental pulp homeostasis, repair and regenerative response.

Periostin expression has been reported within the dental pulp at different developmental stages (27) and in response to cavity preparation (32). In this system, it has been proposed to play a role in collagen synthesis, dentinogenesis and the overall integrity of the dental pulp. Collectively this evidence suggests a critical role of periostin, serving as a potential modulator of important tissue and cellular functions, regulated by TGF-β1 in response to biomechanical challenges. However, it is currently unknown if periostin's role is exclusive to a specific cell type, its expression is induced as a reaction to biomechanical stimulation to the pulp and what affect this may have on collagen fibrillogenesis within the pulp.

Therefore, our goal is to identify the expression of periostin in different dental pulp cell populations, evaluate its ability to be induced by TGF-β1 and biomechanical stimulation and to investigate its effect on collagen synthesis.

Hypothesis: Dental pulp cells express periostin, which can be induced by TGF-β1 and biomechanical stimulation, leading to increased expression of periostin and changes in collagen expression.

Null Hypothesis: Dental pulp cells do not express periostin, nor can periostin expression be induced by TGF-β1 or biomechanical stimulation, and does not effect collagen expression or dental pulp healing.

The following specific aims will address the hypothesis:

1) To determine if periostin is expressed by Dental Pulp Cells *in vitro* and the effect of TGF-β1 on its expression

2) To analyze the influence of biomechanical stimulation on the expression of periostin in different dental pulp cells *in vitro*
3) To analyze the effects of periostin on collagen expression by different dental pulp cell populations *in vitro*

Chapter 2 – Materials and Methods

General Cell Culture

All cell types used in this experiment have been well characterized and were donated by Dr. Tatiana Botero (University of Michigan, Ann Arbor, MI, USA). All cell culture tasks were carried out under a laminar flow hood. Human dental pulp stem cells (DPSC, passage 2) as seen in Figure 1 (47); human dental pulp fibroblasts (DPF, passage 2) as seen in Figure 2 (47); and murine MDPC-23 odontoblast-like cells (passage 52) as seen in Figure 3 (48), were stored in recovery cell culture freezing medium (Gibco #12648-010) in liquid nitrogen cryosystem. Cells were thawed in 37°C warm water bath and the contents transferred to a T-75 flask (Corning Life Sciences) with 10ml of Dulbecco's Modified Eagle's (DMEM (High glucose 1x, Gibco #11995-065)) medium supplemented with heat inactivated 10% fetal bovine serum (FBS, Gibco #10082-147), 1% penicillin/streptomycin (100 IU/ml penicillin and 100µg/ml streptomycin, Gibco #15140-122), and 1:1000 fungizone (Gibco #15290-018). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 hours, the media was removed by vacuum pipetting and 10ml of fresh media was added. All cells were cultured to reach a minimum of 75% confluence. At which point the media was removed by vacuum pipetting, the cell layer was washed with 5ml of sterile PBS, and the cells were passed by adding 1ml of 0.25% trypsin and 0.05% EDTA to the flask and incubating for 5 minutes. When the cells were visibly detached and floating in the media, 5ml of DMEM was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1500 RPM. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 10ml of DMEM. Two 5ml aliquots of re-suspended cells were then placed in two T-75 flasks each containing 10ml DMEM and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Specific Aim 1: To determine if periostin is expressed by Dental Pulp Cells *in vitro* and the effect on TGF-β1 on its expression

We analyzed periostin expression levels of DPSC, DPF and MDPC-23 by qRT-PCR, Western blotting and ELISA. DPSC and DPF are primary cell lines and were used between passages 4 and 7, while MDPC-23 cells are an immortalized cell line. Cells were cultured as previously described until a level of 75% confluence was reached. At which point the media was removed by vacuum pipetting, the cell layer was washed with 5ml of sterile PBS, and the cells were passed by adding 1ml of 0.25% trypsin and 0.05% EDTA to the flask and incubating for 5 minutes. When the cells were visibly detached and floating in the media, 5ml of DMEM was added to inactivate the Trypsin-EDTA. The contents of the flask were transferred to a 15ml conical tube (BD Falcon, San Jose, CA) and centrifuged for 5 minutes at 1500 RPM. The media was removed via vacuum pipetting and the pellet of cells was re-suspended in 10ml of DMEM. A volume of 15ul of re-suspended cells was removed and placed on a Weber hemocytometer (chamber depth 0.1mm x 1/400mm) and counted. Total cell count was made to ensure at least 100,000 cells were placed in 2ml of DMEM in each well of a 6-well plate and sub-cultured with media changes every 48 hours to reach 75% confluence. At which point, cell cultures were divided into groups defined as Group 1: control, cells in DMEM only (no TGF-β1 treatment); Group 2: cells treated with 10ng/ml TGF-β1 at time point 0 and after 24 hours; and Group 3: cells treated with 10ng/ml TGF-β1 at time point 0 and after 24 hours. All treatment groups had a total volume of 2ml DMEM at the start and changed once at 24 hours. Cell collection occurred at 48 hours. We utilized 10ng/ml and 20ng/ml TGF-β1 concentrations due to the reports that periostin expression in PDL cells increases with increasing TGF-β1 concentration (23). It is also known that odontoblast cells can be stimulated by TGF-β1 (38) and all DPCs express TGF-β1 (49). A pilot study was completed using 0ng/ml, 5ng/ml, 10ng/ml, and 20ng/ml TGF-β1 concentrations (41) to determine the effects of TGF-β1 on periostin expression. No relevant results were identified for the 5ng/ml TGF-β1 groups, and were eliminated from the experimental design.

RNA Extraction

RNA isolation was performed using the PureLink® RNA Mini Kit (Invitrogen). Total RNA was isolated from cells by washing the cells twice with 2ml of PBS, and collected

in 500ml lysis buffer (Trizol®) by scraping the cells into 1.5mL microcentrifuge tubes. The cells collected in Trizol® were incubated for 5 minutes, followed by addition of 0.1ml chloroform to each microcentrifuge tube. The tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2–3 minutes. The tubes were then centrifuged at 12,000 Å~ g for 15 minutes at 4^{o}C. After centrifugation, 400µL of the upper colorless aqueous phase (containing RNA) was transferred to a fresh RNase–free tube. At this point an equal volume 70% ethanol was added and vortexed to obtain a final ethanol concentration of 35%. The tubes were then inverted to disperse any visible precipitate. A volume of 700µL of each sample was then transferred to a spin cartridge and centrifuged at 12,000 Å~ g for 15 seconds at room temperature. The flow-through volume was discarded and the spin cartridge was re-inserted into the same collection tube (this was repeated until the entire sample was collected). Next, 350µL wash buffer I was added to the spin cartridge containing the bound RNA. It was centrifuge at 12,000 Å~ g for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was inserted into a new collection tube. At this point the DNase step was performed by PureLink® DNase treatment protocol. A total volume of 80µL PureLink® DNase mixture (10X DNase I reaction buffer (8µL), re-suspended DNase (3U/µL) (10µL), RNase-Free water (62µL) was added directly onto the surface of the spin cartridge membrane and incubated at room temperature for 15 minutes. Following this, 350µL wash buffer I was added to the spin cartridge and centrifuged at 12,000 Å~ g for 15 seconds at room temperature. The flow-through was discarded and a new collection tube was inserted into the spin cartridge. A volume of 500µL wash buffer II with ethanol was then added to the spin cartridge. It was centrifuged at 12,000 Å~ g for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was reinserted into the same collection tube (repeated once). The spin cartridge was centrifuged at 12,000 Å~ g for 1 minute to dry the membrane with bound RNA. The collection tube was discarded and the spin cartridge was inserted into a recovery tube. At this point, 50µL RNase-Free water was added to the center of the spin cartridge and incubated at room temperature for 1 minute. The spin cartridge and recovery tube were centrifuged for 1 minute at ≥12,000 Å~ g at room temperature. Samples were stored as purified RNA in RNase-Free water in Eppendorf tubes. To measure the purity of RNA, a 1:25 dilution was

made using 4µL of RNA and 96µL of RNase-Free water, placed in a cuvette compartment and the absorbance ratio of 260/280 was measured using a DU-640 Spectrophotometer (Beckman Coulter). A total of 81 samples were stored in -80^{o}C freezer until further processing.

Reverse Transcription

In order to perform qRT-PCR, the RNA samples required reverse transcription (RT) to produce complementary DNA (cDNA). Calculations were performed to allow for reverse transcription reactions to be performed on an equivalent amount of mRNA for each sample (1-2ug). A 50µl RT reaction with 1.0µg total RNA was performed using Taqman Reverse Transcription Reagents (Applied Biosystems). The RT reaction was composed of a volume of 30.75ul RT Mix (10x Taqman RT Buffer, 25mM MgCl, dNTPs, Hexamers, RNase Inhibitor, Multiscribe RT (50U/ul) plus a volume of 19.25ul mix RNase-Free water and sample of RNA. The kit included all the necessary components for the transcription process. The thermal condition for RT was 25°C for 10 minutes, 48°C for 60 minutes and 95°C for 5 minutes and kept at 4°C until the samples were removed from the machine. Samples were then stored in -80^{o}C freezer until further processing.

Quantitative Real-Time Polymerase Chain Reaction

A real-time polymerase chain reaction (qRT-PCR) protocol was performed in triplicate on the cDNA samples from each cell line. The qRT-PCR probes for GAPDH, Periostin, and Collagen I were performed on a DNA sequence detector, using 20ng cDNA per reaction. The conditions for PCR were as follows: 2 minutes at 50°C and 10 minutes at 95°C; then, 40 cycles each of 15 seconds at 95°C and 1 minute at 60°C on optical 96-well plates covered with optical film. Each plate contained triplicates of the test cDNA templates and no-template controls for each reaction mix. The $2^{\Delta\Delta Ct}$ method was used to calculate gene expression levels relative to GAPDH (50). The Taqman Gene Expression Assay IDs (human) are as follow: GAPDH – Hs02758991_g1; POSTN – Hs01566748_m1; COL1 – Hs00164004_m1. The Taqman Gene Expression Assay IDs (mouse) are as follows: GAPDH – Mm99999915_g1; POSTN – Mm00450111_m1; COL1 – Mm01302043_g1.

Baseline Messenger RNA Expression

Baseline expression was evaluated and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using qRT-PCR with TaqMan primer/probes.

Western Blot

Periostin protein expression was analyzed by western blot. Protein isolation and analysis was performed following cell collection. Cells were washed twice with 2ml of PBS, and collected in 1ml of PBS by scraping vigorously for 2 minutes into 1.5ml microcentrifuge tubes. Cells/PBS mixture was centrifuged at 3,000rpm for 10min at 4ºC to form a pellet. The PBS was aspirated by vacuum pipette and the cells were re-suspended in lysis buffer (0.1M Tris pH 6.8, 2% SDS, 1% ß-mercaptoethanol, 1:100 protease inhibitor cocktail), homogenized in the bullet blender (Next Advance) for 5 minutes, and incubated on ice for 30 minutes. The lysates were centrifuged again (12,000 rpm for 10 minutes at 4ºC) and protein supernatants collected. Total protein concentration was quantified using the micro assay Bradford method and read using the Multiskan Ascent at a wavelength of 595 nm. Calculations for protein concentration were made and 20µg of each sample was added to the appropriate volume of laemmli sample buffer and 2-mercaptoethanol. After the mix was prepared, each sample was boiled at 95^{o}C for 5 minutes and electrophoretically resolved using 10% SDSPAGE gels (100 V, 2 hours), then electroblotted onto polyvinylidene fluoride membranes (PVDF) (90 V, 90 minutes). Membranes were blocked (5% milk in TBST pH 7.4, 1 hour), and immuno-probed for periostin (0.25ug/ml in 5% milk, rabbit polyclonal to periostin overnight; 1:4,000 goat anti-rabbit IgG-HRP, 1 hour) and GAPDH (0.167ug/mL, in 5% milk, antihuman/mouse/rat GAPDH overnight; 1:4,000 donkey anti-goat IgG-HRP, 1 hour). Immunopositive bands were detected by enhanced chemiluminescence (ECL) and autoradiography. Positive controls for periostin included recombinant protein and human periodontal ligament cells (hPDL) on passages 4-7, cell culturing was identical to the methods previously described (43).

ELISA Protein Analysis

For periostin protein quantification the DuoSet ELISA Development System using sandwich ELISA was utilized to measure natural and recombinant human Periostin/OSF-2 for DPSC and DPF (R&D Systems, DY3548) and mouse Periostin/OSF-2 for MDPC-23 (R&D Systems, DY2955). Cell culturing was completed as previously described. Protein isolation and analysis was performed following cell collection. Cells were washed twice with 2.0ml PBS, and collected in 1.0ml PBS by scraping vigorously for 2 minutes into 1.5ml microcentrifuge tubes. Cells/PBS mixture was centrifuged at 3,000rpm for 10 minutes at 4ºC to form a pellet. The PBS was aspirated by vacuum pipette and the cells were re-suspended in an ELISA compatible lysis buffer (Invitrogen, FNN0071), homogenized in the bullet blender for 5 minutes, and incubated on ice for 30 minutes. The lysates were centrifuged again (12,000 RPM for 10 minutes at 4ºC) and protein supernatants collected. Specific concentration parameters for each kit were identified for the human kit (range: 187 - 12,000pg/ml with no sensitivity listed) and the mouse kit (range: 0.156 - 10ng/m with sensitivity: 0.065ng/ml). A 96-well micro-plate was coated with 100µL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature. After 24 hours, each well was aspirated and forcefully washed with 400µL of wash buffer from a squirt bottle; this process was repeated for a total of three washes. Following the last wash, aspirating the plate and blotting it against clean paper towels to remove all remaining wash buffer. Next, 300µL of blocking buffer was added to each well and incubated at room temperature for 1 hour. Following this, the aspiration/wash protocol was repeated. Next, 100µL of each sample or standards in reagent diluent was added to each well. The micro-plate was covered with an adhesive strip and incubated for 2 hours at room temperature. After incubation, the aspiration/wash step was repeated. Next, 100µL of biotinylated detection antibody diluted in reagent diluent was added to each well. The micro-plate was again covered with a new adhesive strip and incubated for 2 hours at room temperature. The aspiration/wash step was repeated. Next, 100µL of the working dilution of Streptavidin-HRP was added to each well. The micro-plate was again covered and incubated for 20 minutes at room temperature out of direct light. The aspiration/wash step was then repeated. Next, 100µL substrate solution was added to each well and incubated for 20 minutes at room

temperature out of direct light. Finally, 50µL of stop solution was added to each well and the micro-plate was gently tapped to ensure thorough mixing. A micro-plate reader set to 450nm was used to determine the optical density of each well immediately after the stop solution was added.

Statistical Analysis

All statistical analyses were completed with the support of the University of Michigan's Center for Statistical Consultation and Research (CSCAR). All data for aim 1 was analyzed using one-way ANOVA with Fisher's LSD post-hoc pairwise comparison ($P \leq 0.05$). A "lower case letter" will denote statistically significant results between compared groups. The analysis was completed using IBM SPSS 21.0 Software (IBM Corp, Armonk, New York, United States). At least three independent trials of each experiment were done in triplicate to verify reproducibility of results.

Specific Aim 2: To analyze the influence of biomechanical stimulation on the expression of periostin in different dental pulp cells

Analysis of periostin expression levels in DPSC, DPF and MDPC-23 were evaluated by qRT-PCR, Western blot and ELISA. Cells were cultured as previously described until a level of 75% confluence was reached. Total cell count was made to ensure (1×10^5 cells/well) were placed in 2ml of DMEM on flexible-bottomed BioFlex™ Culture Plates coated with Collagen I (Flexcell International Corp.) until they reached 75% confluence. Media was changed every 48 hours. At which point, cell cultures were subjected to the application of continuous or intermittent biomechanical stimulation. The experimental groups were run independently, each with a static control (no biomechanical stimulation). Cell collection occurred at 48 hours.

Application of continuous biomechanical stimulation: A cyclic mechanical force was applied to the dental pulp cells using the cell culture-loading station Flexcell® FX-5000™ System (Flexcell International Corp., Hillsborough, NC, USA). Fresh media was added every 48 hours. To apply the biomechanical stimulation to the cells, the flexible bottoms of the plates were deformed to 14% by a computer-operated vacuum system at six cycles/minute (i.e., 5 seconds on and 5 seconds off) for 48 hours. Non-stimulated cells were used as controls (24, 30, 40). Collection for RNA and total protein were at 48 hours after application of the biomechanical stimulation.

Application of intermittent biomechanical stimulation: A cyclic mechanical force was applied as previously described for continuous biomechanical stimulation. The application parameters were modified as follows: stimulation was applied for 8 hours at six cycles/minute (i.e., 5 seconds on and 5 seconds off) and then allowed to rest (no stimulation) for 16 hours. Two total cycles were completed. Non-stimulated cells were used as controls. RNA and total protein were collected at 48 hours after application of the biomechanical stimulation.

RNA Extraction

Cells were collected and RNA was purified as previously described.

Reverse Transcription

Messenger RNA was reverse transcribed to cDNA as previously described using the same Taqman mix/probes for GAPDH, Periostin, and Collagen I as previously described.

Quantitative Real-Time Polymerase Chain Reaction

qRT-PCR was performed as previously described.

Western Blot

Protein was collected, lysed, and quantified as previously described. Western blot was performed as previously described for continuous biomechanical stimulation only. Antibodies used were the same as previously described. Western blot for intermittent biomechanical stimulation was not performed since this experimental group was added after western blot and ELISA was completed for continuous biomechanical stimulation. Since, periostin protein expression was found for the continuous biomechanical stimulation group, it was determined to utilize an ELISA technique only for the intermittent group.

ELISA Protein Analysis

ELISA was performed as previously described and the ELISA kits were used according to manufacturer's protocol.

Statistical Analysis for Specific Aim 2

All statistical analyses were completed with the support of CSCAR. Messenger RNA data was analyzed using a Student's t-test for pairwise comparison between each control and type of biomechanical stimulation (continuous or intermittent) ($P \leq 0.05$). ELISA data was analyzed using ANOVA with Fisher's LSD post-hoc test ($P \leq 0.05$). A "lower case letter" will denote statistically significant results between compared groups. The analysis was completed using IBM SPSS 21.0 Software (IBM Corp, Armonk, New York, United States). At least three independent trials of each experiment were done in triplicate to

verify reproducibility of results.

Specific Aim 3: To analyze the effects of periostin on collagen expression by different dental pulp cells

Total Collagen Assay was used to evaluate collagen expression levels in DPSC, DPF and MDPC-23 odontoblast-like cells. Cells were cultured as previously described until a level of 75% confluence was reached. Total cell count was made to ensure (1×10^5 cells/well) were place in 2ml of DMEM in each well of a 6-well plate and sub-cultured with media changes every 48 hours to reach 75% confluence. At which point, cell cultures were subjected to supplementation with periostin. Group 1: control, cells in DMEM only (no supplementation); Group 2: treated with 50ng/ml periostin at time point 0 and 50ng/ml periostin at 24 hours; Group 3: treated with 100ng/ml periostin at time point 0 and 100ng/ml periostin at 24 hours.

Total Collagen Assay

Cells were washed twice with 2ml of PBS, and collected in 1ml of PBS by scraping vigorously for 2 minutes into 1.5ml microcentrifuge tubes. Cells/PBS mixture was centrifuged at 3,000rpm for 10 minutes at 4ºC to form a pellet. The PBS was aspirated by vacuum pipette and the cells were re-suspended and diluted 1:1 (100ul/100ul) with 12M HCl (final concentration 6M HCl). The QuickZyme Total Collagen Kit (QuickZyme Biosciences) was completed according to the manufacturer's protocol. Briefly, tubes were incubated for 20 hours at 95^{o}C in a thermoblock. After incubation, tubes were centrifuged for 10 minutes at 13,000g and then 35ul of hydrolyzed samples were pipetted into appropriate wells of a 96-well micro-plate. Next, 75ul of assay buffer was added to each well, the plate was covered and incubated for 20 minutes at room temperature while shaking. A volume of 75ul detection agent was then added to each well, the plate was sealed and incubated for 60 minutes in a 60^{o}C oven. The micro-plate was then cooled on ice to room temperature and the plate was read at 570nm.

Statistical Analysis for Specific Aim 3

All statistical analyses were completed with the support of CSCAR. All data was

analyzed using one-way ANOVA with a Fisher's LSD post-hoc pairwise comparison ($P \leq 0.05$). A "lower case letter" will denote statistically significant results between compared groups. The analysis was completed using IBM SPSS 21.0 Software (IBM Corp, Armonk, New York, United States). Two independent trials of each experiment were done in triplicate to verify reproducibility of results.

Chapter 3 - Results

Specific Aim 1: To determine if periostin is expressed by Dental Pulp Cells *in vitro* and the effect of TGF-β1 on its expression.

The results for aim 1 are presented for each cell line.

DPSC

Periostin mRNA expression was evaluated by qRT-PCR in DPSC for 3 experimental groups which included the control, 10ng/ml TGF-β1, and 20ng/ml TGF-β1. All results were normalized to the housekeeping gene GAPDH. Periostin mRNA was evident in each of the 3 experimental groups as seen in Figure 4. There was a statistically significant increase in periostin mRNA expression after exposure to 10ng/ml TGF-β1 group ($p<0.018$) for 48 hours. Where as there was a trend for increased periostin mRNA from the control group to the 20ng/ml TGF-β1 group ($p=0.218$), but at lower expression than in the 10ng/ml TGF-β1 group ($p=0.116$).

To demonstrate if DPSC were capable of expressing periostin protein, western blot was performed. Immunopositive bands for periostin were observed at approximately 90KDa. All samples were normalized to the housekeeping protein GAPDH, which showed immunopositive bands at approximately 37KDa. In DPSC, immunopositive bands were evident in the 10ng/ml TGF-β1 and 20ng/ml TGF-β1 groups with dose dependent increased expression as compares to the control group as seen in Figure 5.

To further evaluate and quantify periostin protein expression, an ELISA was utilized. Figure 6 shows periostin protein levels in DPSC at control, 10ng/ml TGF-β1 and 20ng/ml TGF-β1 groups. Periostin protein expression in DPSC was evident in all 3 groups. There was an overall trend for increased periostin protein expression with increasing concentrations of TGF-β1. There was a statistically significant increase in periostin protein from the control to 20ng/ml TGF-β1 ($p<0.032$). There were no statistically significant differences, when comparing the control to 10ng/ml TGF-β1 ($p=0.097$), or

comparing the 10ng/ml TGF-β1 group to the 20ng/ml TGF-β1 group (p=0.247).

In summary, DPSC express periostin mRNA and protein and TGF-β1 can be used to induce periostin mRNA and protein expression in DPSC.

DPF

The qRT-PCR for DPF showed periostin mRNA present in each of the 3 experimental groups. There were no statistically significant difference between any of the experimental groups, but there was a trend for increased periostin mRNA expression as the concentration of TGF-β1 increased as seen in Figure 7. Comparing periostin mRNA expression from the control group to the 10ng/ml TGF-β1 (p=0.556) and 20ng/ml TGF-β1 (p=0.255) showed no statistically significant differences. Comparing 10ng/ml TGF-β1 to 20ng/ml TGF-β1 (p=0.549) was not statistically significant.

To demonstrate if DPF were capable of expressing periostin protein western blotting was performed. Immunopositive bands for periostin were evident in the control, 10ng/ml TGF-β1 and 20ng/ml TGF-β1 groups. The band intensity was relatively consistent, as seen in Figure 8.

To evaluate and quantify periostin protein expression, an ELISA was utilized. Figure 9 shows periostin protein levels in DPF at control, 10ng/ml TGF-β1 and 20ng/ml TGF-β1 groups. Periostin protein expression in DPF was evident in all 3 groups. There was an overall trend for decreased periostin protein expression with increasing concentrations of TGF-β1. There was a statistically significant decrease in periostin protein from the control group to the 10ng/ml TGF-β1 group ($p<0.028$), as well as from the control to 20ng/ml TGF-β1 ($p<0.008$) group. There was not a statistically significant difference, when comparing 10ng/ml TGF-β1 to the 20ng/ml TGF-β1 (p=0.095).

In summary, DPF express periostin mRNA and protein at control levels. TGF-β1 can be used to induce DPF to express increased levels of periostin mRNA but decreased levels of periostin protein.

MDPC-23

The qRT-PCR for MDPC-23 shows periostin mRNA present in each of the 3 experimental groups as seen in Figure 10. There were statistically significant increases of periostin mRNA from the control group to 10ng/ml TGF-β1 ($p<0.003$) and to 20ng/ml TGF-β1 ($p<0.01$). However, there was not a statistically significant difference when comparing 10ng/ml TGF-β1 to 20ng/ml TGF-β1 ($p=0.158$).

To demonstrate if MDPC-23 were capable of expressing periostin protein western blot was performed. Immunopositive bands for periostin were evident in the control, 10ng/ml TGF-β1 and 20ng/ml TGF-β1 groups. The band intensity was relatively consistent, as seen in Figure 11.

To evaluate and quantify periostin protein expression, an ELISA protocol was utilized. Figure 12 shows periostin protein levels in MDPC-23 at control, 10ng/ml TGF-β1 and 20ng/ml TGF-β1 groups. Periostin protein expression in MDPC-23 was evident in all 3 groups, but at much lower levels than the other 2 cell lines. There was an overall trend for increased periostin protein expression with increasing concentrations of TGF-β1. There were no statistically significant differences between the following groups: control to the 10ng/ml TGF-β1 ($p<0.452$) and 20ng/ml TGF-β1 ($p<0.067$), as well as comparing 10ng/ml TGF-β1 to 20ng/ml TGF-β1 ($p=0.145$).

In summary, MDPC-23 express periostin mRNA and protein at control levels. TGF-β1 can be used to induce MDPC-23 to express increased levels of periostin mRNA and protein.

Figure 13 shows the relative levels of periostin mRNA between the cell lines, but since the experiments were completed independently the statistical analysis was not completed across cell lines. Figure 14 shows the quantification of periostin protein expression from the ELISA protocol, relative to each cell line. Box 1 shows the ELISA mean values for each experimental group.

Specific Aim 2: To analyze the influence of biomechanical stimulation on the expression of periostin in different dental pulp cells

The results for aim 2 are presented for each cell line. Messenger RNA data was analyzed using a Student's t-test for pairwise comparison between each control and type of biomechanical stimulation (continuous or intermittent) ($P \leq 0.05$). ELISA data was analyzed using ANOVA with Fisher's LSD post-hoc test ($P \leq 0.05$).

DPSC

Periostin mRNA expression was evaluated by qRT-PCR in DPSC for continuous and intermittent biomechanical stimulation, which each had their own static control. All results were normalized to the housekeeping gene GAPDH. Periostin mRNA was evident in each of the experimental groups. There were no statistically significant differences for either experimental group against their own static control. However, there was a trend for increased periostin mRNA expression from the static control to the continuous biomechanical stimulation group ($p<0.309$) as seen in Figure 15. There was also a trend for increased periostin mRNA expression from the static control to the intermittent biomechanical stimulation group ($p<0.064$) as seen in Figure 17.

To demonstrate if DPSC express periostin at the protein level, western blotting was performed for the static control and continuous biomechanical stimulation groups only. Immunopositive bands for periostin were observed at approximately 90KDa. All samples were normalized to the housekeeping protein GAPDH, which showed immunopositive bands at approximately 37KDa. Immunopositive bands were evident for the continuous biomechanical stimulated group, but were not clearly visible in the static control group as seen in Figure 29.

To evaluate and quantify periostin protein expression, ELISA was also utilized. Figure 16 shows periostin protein levels in DPSC at static control and in the continuous biomechanical stimulation group ($p<0.380$). Figure 18 shows periostin protein levels at

static control and in the intermittent biomechanical stimulation group ($p<0.250$). There was an overall trend for increased periostin protein expression in both of the biomechanical stimulation groups, but there were no statistically significant differences in any of the results.

In summary, DPSC can be induced by biomechanical stimulation to show a trend for increased periostin mRNA and protein expression.

DPF

Periostin mRNA expression was evaluated by qRT-PCR in DPF for continuous and intermittent biomechanical stimulation, which each had their own static control. All results were normalized to the housekeeping gene GAPDH. Periostin mRNA was evident in each of the experimental groups. There were no statistically significant differences for either experimental group against their own static control. However, there was a trend for increased periostin mRNA expression from the static control to the continuous biomechanical stimulation group ($p<0.223$) as seen in Figure 19. There was no change periostin mRNA expression from the static control to the intermittent biomechanical stimulation group ($p<0.776$) as seen in Figure 21.

To demonstrate if DPF express periostin at the protein level, western blotting was performed. Immunopositive bands were evident in the static control and continuous biomechanical stimulated groups as seen in Figure 29.

To evaluate and quantify periostin protein expression, ELISA was utilized. Figure 20 shows periostin protein levels at the static control and show a trend for decreased periostin in the continuous biomechanical stimulation group ($p<0.078$). Figure 22 shows periostin protein levels at static control and a trend to increase periostin in the intermittent biomechanical stimulation group ($p<0.055$). There were no statistically significant differences in any of the results.

In summary, DPF can be induced by continuous biomechanical stimulation and show a

trend for increased periostin mRNA, but decreased periostin protein expression. Whereas DPF shows a trend for increased periostin mRNA and protein expression when intermittently stimulated.

MDPC-23

Periostin mRNA expression was evaluated by qRT-PCR in MDPC-23 for continuous and intermittent biomechanical stimulation, which each had their own static control. All results were normalized to the housekeeping gene GAPDH. Periostin mRNA was evident in each of the experimental groups. There was a statistically significant decrease in periostin mRNA from the static control group to the continuous biomechanical stimulation group ($p<0.026$) as seen in Figure 23. However, there was only a trend for decreased periostin expression from the static control to the intermittent biomechanical stimulation group (0.216) as seen in Figure 25.

To demonstrate if MDPC-23 express periostin at the protein level, western blotting was performed. Immunopositive bands were evident in the static control and continuous biomechanically stimulated groups as seen in Figure 29.

To evaluate and quantify periostin protein expression, ELISA was utilized. There was an overall trend for decreased periostin protein expression in both experimental groups against the static control. Figure 24 shows periostin protein levels at static control and in the continuous biomechanical stimulation group ($p<0.227$), whereas in Figure 26 the periostin protein levels are present in the static control and in the intermittent biomechanical stimulation group ($p<0.078$).

In summary, MDPC-23 can be induced by biomechanical stimulation to decrease periostin mRNA, and likewise show a trend for decreased periostin protein expression in both experimental groups.

Figure 27 shows the relative levels of periostin mRNA between each of the cell lines, but since the experiments were completed independently the statistical analysis was not

performed. Figure 28 quantifies periostin protein expression from the ELISA technique, showing the relative levels of protein. Box 2 shows the ELISA mean values for each experimental group. Comparing protein levels across experimental groups the control levels of periostin expression are highest in DPF (Figure 30). When the cells are intermittently biomechanically stimulated there is a change in the behavior of DPF cells. The biomechanical stimulation increases the levels of periostin protein instead of decreasing ($p<0.005$).

Specific Aim 3: To analyze the effects of periostin on collagen expression by different dental pulp cell populations

The results for aim 3 are presented for each cell line. All statistical analysis was completed using ANOVA with Fisher's LSD for comparisons ($P\leq0.05$).

DPSC

Collagen Type I mRNA expression was evaluated by qRT-PCR in DPSC for the same 3 experimental groups as in aim 1 and the same 2 experimental groups as in aim 2. All results were normalized to the housekeeping gene GAPDH. Collagen type I mRNA was evident in all 5 experimental groups. There were no statistically significant differences, but all results show that collagen type I mRNA expression increased from the control group to the experimental group. Figure 32 shows the affect of TGF-β1 on collagen type I mRNA. There was a trend for increase collagen type I mRNA expression from the control group to 10ng/ml TGF-β1 ($p=0.394$) and 20ng/ml TGF-β1 ($p=0.183$). Comparing 10ng/ml TGF-β1 to 20ng/ml TGF-β1 ($p=0.577$) shows an upward trend for collagen type I mRNA expression as well. The experimental groups from aim 2 show a trend for increased collagen type I mRNA expression from static control to continuous biomechanical stimulation ($p=0.380$) as seen in Figure 33, as well as from static control to intermittent biomechanical stimulation ($p=0.250$) as seen in Figure 34.

To further evaluate collagen expression, a total collagen assay was utilized. The results from the total collagen assay may not be reliable since many of the experimental data

values were lower than the most diluted standard value. However, the total collagen assay was completed 2 times in triplicate for each sample and the same results occurred. The total collagen mean values are shown in Box 3. Figure 31 shows the mean total collagen levels in the control (1.66ug/ml), in the 50ng/ml periostin (1.42ug/ml) and in the 100ng/ml periostin (1.36ug/ml) delivery groups. Overall, there was a downward trend in total collagen expression from exogenous delivery of periostin.

DPF

Collagen Type I mRNA expression was evaluated by qRT-PCR in DPF for the same 3 experimental groups as in aim 1 and the same 2 experimental groups as in aim 2. All results were normalized to the housekeeping gene GAPDH. Collagen type I mRNA was evident in all 5 experimental groups. There was a statistically significant increase in collagen type 1 from control to 20ng/ml TGF-β1 ($p=0.009$) and from 10ng/ml TGF-β1 to 20ng/ml TGF-β1 ($p=0.027$) as seen in Figure 36. There was a trend for increased collagen type I mRNA expression from control to 10ng/ml TGF-β1 ($p<0.383$). There were no statistically significant differences in collagen type I expression for aim 2, but collagen type I mRNA expression showed a trend for decreased expression in both biomechanically stimulated groups. Comparing the experimental groups from aim 2, there was a trend for decreased collagen mRNA expression from static control to continuous biomechanical stimulation ($p=0.660$) as seen in Figure 37, as well as from static control to intermittent biomechanical stimulation ($p=0.391$) as seen in Figure 38.

To further evaluate collagen expression, a total collagen assay was utilized. The results from the total collagen assay may not be reliable since many of the experimental data values were lower than the most diluted standard value. However, the total collagen assay was completed 2 times in triplicate for each sample and the same results occurred. Figure 35 shows the mean total collagen levels in the control (1.74ug/ml), in the 50ng/ml periostin (2.27ug/ml) and in the 100ng/ml periostin (1.41ug/ml) groups. There was no change in total collagen expression with increasing concentrations of exogenous periostin delivery.

MDPC-23

Collagen Type I mRNA expression was evaluated by qRT-PCR in MDPC-23 for the same 3 experimental groups as in aim 1 and the same 2 experimental groups as in aim 2. All results were normalized to the housekeeping gene GAPDH. Collagen type I mRNA was evident in all 5 experimental groups. There were no statistically significant differences in any of the experimental groups. In aim 1, control levels to 10ng/ml TGF-β1 ($p=0.154$) and 20ng/ml TGF-β1 ($p=0.177$) showed trends for increased collagen type I mRNA expression as seen in Figure 40. Comparing 10ng/ml TGF-β1 to 20ng/ml TGF-β1 ($p=0.922$) shows almost no change in collagen type I mRNA expression. Comparing the experimental groups in aim 2 show a trend for decreased collagen type I mRNA expression from static control to the continuous biomechanical stimulation ($p=0.103$) as seen in Figure 41, and show a trend for increased collagen type I mRNA expression from static control to intermittent biomechanical stimulation ($p=0.178$) as seen in Figure 42.

To further evaluate collagen expression, a total collagen assay was utilized. The results from the total collagen assay may not be reliable since many of the experimental data values were lower than the most diluted standard value. However, the total collagen assay was completed 2 times in triplicate for each sample and the same results occurred. Figure 39 shows the mean total collagen levels in the control (1.14ug/ml), in the 50ng/ml periostin (1.72ug/ml) and in the 100ng/ml periostin (2.71ug/ml) groups. There was an overall trend for increased total collagen expression with increasing concentrations of exogenous periostin delivery.

Figure 43 represents the effect of TGF-β1 on collagen type I mRNA, with relative levels across cell lines. Figure 44, shows the effect from continuous biomechanical stimulation on collagen type I mRNA and Figure 45 shows the effect from intermittent biomechanical stimulation on collagen type I mRNA across all each cell line.

Chapter 4 – Discussion

The potential exists to create a clinical protocol in order to achieve a state of dental pulp regeneration. The practicality of this treatment approach would allow for the continued development of the entire tooth through soft and hard tissue formation, but could also lead to possible solutions for artificial tooth implantation. The difficulty lies in correctly identifying the sequence of events that must occur, as well as the appropriate constituents that are needed. There have been different mature and progenitor cell lines studied, as well as unique ECM bioactive molecules (3), 3D scaffolds (4, 5), and biomaterials (6). However, for the first time, the bioactive molecule, periostin, was explored as to how it relates to the dentin-pulp complex. Historically, there has been ambiguous localization of periostin within dental pulp tissue, and only accurately identified in the periodontal ligament (22, 23, 27, 31), as well as other load bearing tissues throughout the body (21). Even though periostin has been acknowledged in the pulp proper, previous studies have lacked to identify which specific cell types are responsible for its expression.

In this study, we set out to identify the dental pulp cell populations responsible for the expression of periostin and suggest a potential role for its expression. Our experimental design allowed for the *in vitro* investigation of 3 cell types, which are located in the dental pulp. DPSC, DPF, and MDPC-23 odontoblast-like cells were used since these cells compromise the majority of cells present in the heterogenic population of the dental pulp. We first examined the capacity of each cell line's ability to express periostin mRNA and protein at control levels and compared those results to periostin mRNA and protein expression after the cells were treated with different concentrations of TGF-β1. We observed that DPSC, DPF, and MDPC-23 cells each express periostin mRNA at control levels and observed significant differences in periostin mRNA in DPSC and MDPC-23 after TGF-β1 treatment. Periostin protein expression in DPSC also showed significant increases after TGF-β1 treatment, and the western blot supports this by showing an increase in immunopositive band intensity from the control to the TGF-β1 treatment groups. In DPF there were no significant differences in periostin mRNA expression, but the ELISA data showed a significant decrease in periostin protein expression in the TGF-

β1 treatment groups. The MDPC-23 cells also showed periostin protein expression at control levels and significant differences when treated with TGF-β1, but overall, at much lower levels compared to the DPSC and DPF.

Periostin mRNA and protein were identified in the control groups of DPF and MDPC-23 cells, but the DPSC only expressed periostin after TGF-β1 treatment. The DPF and MDPC-23 cell lines are both well characterized and are terminally differentiated cells, whereas DPSC are adult stem cells with the ability to differentiate into cells of odontogenic, osteogenic, adipogenic or chondrogenic pathways (34). It was previously thought that periostin was only expressed during tooth development (23, 27, 31) and was not expressed in the mature pulp. Our results indicate that DPF and MDPC-23 cells have the capacity to express periostin without any stimulation, whereas DPSC shows induction potential by stimulation with TGF-β1 in order to express periostin. Our results also show that the mean values for periostin protein quantification in MDPC-23 were extremely low, compared to DPSC or DPF. Recently, periostin was identified in the pulp proper after mechanical drilling with peak expression at 24 hours, and visibility up to 7 days (32). These results suggested that dental pulp cells express periostin, but did not specify the exact cell type. This leads us to speculate that either DPF cells were stimulated to express periostin, or DPSC were activated, allowed to differentiate and then expressed periostin. Expression was not evident in the odontoblast zone (32). The mechanical drilling model supports our findings, since TGF-β1 is expressed as a result from stress to the dental pulp. TGF-β1 may also be released during carious demineralization of the dentin matrix and be come available in the mediation of dental repair processes (38, 51). Its main function is to regulate cell growth and differentiation (38, 41). TGF-β1 usually has stimulatory effects for cells of mesenchymal origin and inhibitory effects for cells of epithelial or neuroectoderm origin (52). DPSC, DPF and MDPC-23 are all of mesenchymal origin and therefore should have stimulatory effects if the treatment concentrations are appropriate.

In aim 2 of this experiment, each cell line was biomechanically stimulated in either a continuous or intermittent pattern. The continuous group underwent 48 straight hours of

14% strain in 6 cycles per minute using the Flexcell® FX-5000™ System. Whereas, the intermittent group was stimulated under the same conditions for 8 hours, allowed to rest for 16 hours, then stimulated for an additional 8 hours and allowed to rest for 16 more hours before collection at 48 hours. The experimental parameters were designed to incorporate ideal conditions for periostin expression in hPDL cells (53), as well as being a suitable stimulus to DPC (40, 44, 45) Biomechanical stimulation has been shown to regulate the expression of periostin and ECM incorporation via TGF-β1 modulation (24, 25, 27), as well as playing an important role in regulating the function of mesenchymal stem cells (54). The type of mechanical stress to dental pulp may vary, but includes fluid shear stress, compression, hydrostatic pressure, and uniaxial vertical and horizontal stretching (55). There are conditions where the dental pulp is stretched vertically, such as during tooth eruption and in orthodontic forces. These forces can also transfer horizontal stretch to DPSCs and PDL tissues (56). Previous studies have also suggested that the mechanical stress of drilling acts in the same way as mechanical stress of mastication to induce periostin expression (32). Continuous biomechanical stimulation was an attempt to mimic clinically relevant situations such as chronic dental trauma, uncontrolled orthodontic movements, iatrogenic trauma and chronic para-functional forces. The intermittent biomechanical stimulation was designed to mimic the nocturnal para-functional forces of chronic bruxism. Since, biomechanical stimulation is modulated via TGF-β1 and we wanted to relate the effects of biomechanical stimulation to that of exogenous TGF-β1 delivery. The results from aim 2 show similar trends in periostin mRNA and protein expression, for each cell line, as in aim 1. There were no significant differences in the DPSC, but both periostin mRNA and protein showed trends for increased expression. The DPF cells showed similar results as when treated with TGF-β1, but none of the differences were statistically significant. There were trends for increased periostin mRNA in both biomechanical groups and increased periostin protein in the intermittent group. However, there was a trend for decreased periostin protein in the continuous group. The periostin mRNA and protein levels in MDPC-23 cells show an overall trend for decreased periostin expression in both types of biomechanical stimulation. The only statistically significant result was a decrease in periostin mRNA from the static control to continuous biomechanical stimulation group.

Biomechanical stimulation activates several intracellular signaling pathways through mechanoreceptors (56, 57), thus leading to activation of cells. Studies have shown that mechanical stress can regulate the differentiation of DPSC into odontoblasts (40), while other studies claim mechanical stress has no effect on differentiation of DPSC (58). Our results suggest that biomechanical stimulation may be used to induce expression of periostin mRNA and protein. Further evaluation is needed, and potential changes to the experimental parameters may be warranted. The parameters for biomechanical stimulation were 14% strain in 6 cycles per minute (24). Other investigators have used DPCs subjected to 6%, 12%, or 15% strain in 6 cycles per minute for 3-48 hours and showed that mechanical stress could stimulate DPCs (45). However, cyclic strain can also cause cellular damage and studies have shown that when 15% cyclic strain is applied it may lead to cell death (45). Others have shown that a maximum of 15% cyclic strain in 6 cycles per minute mimics physiological occlusal loading or the effects of moderate orthodontic force inducing stress across a PDL with 40kPa. They found that mechanical stress up-regulates the mRNA levels of encoding key markers for differentiation of DPC to odontoblasts (BSP, OPN, DSPP, DMP-1) (40).

The final aim of this experiment was to identify periostin's affect on collagen. The experimental model included treating each cell line with different concentrations of periostin for 48 hours. Collagen fibrillogenesis is a complex, highly regulated, multistep process involving many proteins. Collagen is the main structural component of the ECM and stabilizes the dental pulp, as well as being the precursor to dentinogenesis. Periostin is co-localized and directly binds to Collagen Type I (29). Therefore if periostin is expressed in the dental pulp, identifying which cells express it will give us clues as to how it affects collagen synthesis and ultimately repair and regeneration. In general, we observed no statistically significant differences in total collagen expression. There were trends for decreased total collagen expression in DPSC, no change in DPF, and increased collagen expression in MDPC-23. TGF-β1 has similar mRNA expression patterns as Collagen Type I, and when odontoblasts express TGF-β1 it is sequestered within the pulpal ECM where it can participate in repair and homeostasis after pulp injury (37, 41).

TGF-β1 has been shown to be an active component of the dental ECM associated with regulation of cell growth, differentiation and matrix biosynthesis (59). Therefore we also evaluated collagen type I after each of the experimental parameters in aim 1 and 2. Collagen Type I mRNA showed trends for increased expression from static control, after treatment with different concentrations of TGF-β1, or when biomechanically stimulated in DPSC. The DPF cells showed a statistically significant increase in collagen type I mRNA after treatment with different concentrations of TGF-β1, but showed trends for decreased collagen type I mRNA expression when biomechanically stimulated. The MDPC-23 cells showed a trend for increased collagen type I mRNA after treatment with different concentrations of TGF-β1 and intermittent biomechanical stimulation, but a trend for decreased collagen type I after continuous biomechanical stimulation. There were differences in the result from qRT-PCR probing for Collagen Type I and the total collagen assay. It is possible that the total collagen assay is not sensitive enough to identify specific collagen, therefore not showing a difference in active collagen fibrillogenesis. The total collage assay identifies all collagen, including degraded collagen and immature collagen. Additionally, exogenous periostin delivery to the cells, actually have no effect on influencing or activating its expression potential of collagen.

Overall, DPSC are very responsive to TGF-β1 and have the ability to induce periostin expression. Interestingly, the western blot data does not show immunopositive bands for the control group in aim 1. In this group, there is no stimulus, so cells may still be in an undifferentiated state and lack the ability to express periostin protein. Whereas, when DPSC are treated with TGF-β1, the cell may be activated and differentiate into a mature cell type that is capable of expressing periostin. DPSC are readily induced to express periostin by TGF-β1. The question remains what cell type has the DPSC differentiated into, since they have the ability to differentiate into cells of odontogenic, osteogenic, adipogenic or chondrogenic pathways (34), Unfortunately, we were not able to identify this cell type and there is no way to verify this since no additional evaluations were completed. In an active state of differentiation the cells would have to under go 1 full cell cycle, which is about 12-16 hours to complete. This would allow enough time for maturation of the cellular processes, stimulation to have an affect, and periostin to be

expressed at the 48-hour collection. However, if the confluence levels during cell culture have already caused differentiation of the DPSC, we may observe that periostin expression would be completely dependent on TGF-β1 concentration and activation of the cells. TGF-β1 mRNA has been expressed by preodontoblasts and odontoblasts, and is also implicated in odontoblast differentiation and dentin formation (41). Therefore, these cells could be influenced to take an odontogenic differentiation pathway. (41). Other studies have suggested DPSCs physiologically receive mechanical stress by mastication and swallowing, thus our results suggest that mechanical stretch may have an essential role in the maintenance of DPSCs through increasing the proliferation, while suppressing osteogenic differentiation (44). The total collagen assay showed little change in collagen expression of DPSC after exogenous periostin delivery. This is an interesting finding, since DPSC have been a very responsive cell. It is possible that periostin does not regulate or activate DPSC to differentiate, and therefore collagen expression is unchanged or even decreased. These cells are not being stimulated or induced, and therefore in a stable state, so there is no need for ECM stabilization or collagen formation. It would be interesting to see how the cells would respond to biomechanical stimulation and then exogenous periostin delivery.

In general, DPF show similar results comparing aim 1 to aim 2. There is a general trend for increased periostin mRNA, and decreased periostin protein, except when observing the intermittent biomechanical stimulation group for periostin protein which shows increased expression. There is much speculation as to why there would be increases in mRNA and decreases in protein for both aim 1 and 2. There are several possible explanations for this observation. One possibility is that there is a problem post-translational, not allowing periostin protein to be transcribed. Another possibility is that 48 hours may be too long of an evaluation period to assess periostin protein expression. If the pulp proper expresses periostin that peaks at 24 hours following stimulation (32), we can speculate that these cells may have expressed more periostin at 24 hours in order to achieve homeostasis, and then periostin expression decreased as time went on. There is no doubt that DPF express periostin, as is evident in the control, but the cells may be extremely sensitive to TGF-β1 concentrations or biomechanical stimulation. The TGF-β1

concentrations and the parameters for 14% strain for a total of 48 hours may be too long to stimulate these cells and may be causing damage to the cells. These stimulations to DPF may initially be stimulatory as the cells react, but may change to inhibitory after some time. By overloading DPF with TGF-β1 it may inhibit and down regulate any periostin expression and the homeostatic effects of periostin. These effects may only affect periostin post-translational, inhibiting its transcription into protein, and inhibiting its secretion extracellular. As TGF-β1 stimulated DPSC to migrate, differentiate and proliferate, it may also act to inhibit DPF, allowing the progenitor cells to be activated. Interestingly, when the DPF are intermittently biomechanically stimulated, there are no changes in periostin mRNA, but a trend for increased periostin protein. The intermittent stimulation, may allow the DPF cells to rest and rebound, in order for intracellular processes to take place and lead to the expression of periostin protein. The affects on total collagen following exogenous periostin delivery to DPF show no change in total collagen. The qRT-PCR data from aim 1 shows that collagen type I is significantly up-regulated following increasing concentrations with TGF-β1, however, collagen type I is down-regulated following biomechanical stimulation in both groups.

The MDPC-23 shows periostin protein expression is at much lower levels than the DPSC and DPF at control, after treatment from TGF-β1 and biomechanical stimulation. These results suggest that MDPC-23 has the ability to express periostin at controls levels, and those levels can be influenced when stimulated. However, the levels of periostin expression may be at insignificant levels and may not have a clinical application. The MDPC-23 cells are odontoblast-like cells and are a terminally differentiated cell line. Odontoblasts typically have a more specialized function than the other 2 cell lines in this study. The primary function of odontoblasts, regulated by TGF-β1, is dentinogenesis. TGF-β1 has been shown to induce secretion of dentin extracellular matrix components associated with primary dentinogenesis and to play a role in tertiary or reparative dentinogenesis (41). This process includes the synthesis of procollagen fibrils in the odontoblasts' cell bodies, where it aggregates and increases its fibril diameter. The procollagen then migrates through the predentin where is will undergo mineralization at the mineralization front. This procollagen matures during this migration and will be

mineralized there after and incorporated into dentin as tertiary dentin. The majority of collagen biosynthesis takes about 2 hours to reach the predentin (33). Simulations to MDPC-23 cells react by initiating dentinogenesis and not by regulating ECM stability or soft tissue homeostasis. Recent experiments show that during cavity preparation, periostin expression is identified in the pulp proper, but not in the odontoblast layer (32). This supports our results, and suggests that even though odontoblasts possess the ability to express periostin, the in vivo application may be inappropriate since odontoblast extracellular secretions are into pre-dentin that ultimately become mineralized. The role of periostin to control ECM stability and soft tissue homeostasis during stress may be regulated through the expression of a different cell type. Biomechanical stimulation to the MDPC-23 cells resulted in down-regulation of periostin expression. It is possible that under the influence of mechanical stress, odontoblasts do not express periostin, since they function to in hard tissue stabilization. Another explanation is that the biomechanical stimulation was too strong and caused the MDPC-23 cells to die. The exogenous periostin delivery model shows a trend for increased total collagen expression. This result is in line, since periostin is closely related to collagen production and MDPC-23 cells primary function is to make collagen as a pre-cursor to mineralization. The influence of TGF-β1 to increase collagen type I mRNA is supported since; TGF-β1 is a known regulator of dentinogenesis as well.

Proposed Hypothesis

We hypothesize that biomechanical stimulation of the dental pulp causes localized inflammation leading to the release of local mediators, such as TGF-β1. This growth factor regulates intracellular and extracellular pathways of dental pulp cells. Periostin is expressed by dental pulp fibroblasts throughout the pulp to stabilize the ECM and maintain pulpal homeostasis. TGF-β1 also aids in migration, differentiation and proliferation of progenitor cells to the area of repair, which also express periostin for ECM stabilization prior to collagen formation. Biomechanical stimulation may also activate existing odontoblast cells to express periostin at low levels prior to collagen formation. Periostin will bind to the collagen fibers in the ECM to increase tissue strength and structural stability. It is suggested that periostin expression regulates soft tissue

stabilization, and if periostin is down regulated it leads to increased mineralization of the pulp space (32). Therefore, this may further confirm that periostin expression by DPSC and DPF stabilizes soft tissue formation during the healing phase and prevents soft tissue mineralization from occurring. Therefore, periostin's role is extremely important in maintaining the pulp's homeostasis and structural stability through supporting a competent collagen fibrillar system and avoiding complete pulp mineralization due to biomechanical forces.

It is speculated that mineralization of dental pulp tissue is feasible, but resisted by a negative regulation system. The expression of periostin has been shown to be up-regulated by cavity preparation, suggesting that the mechanical stress of drilling acts in the same manner as other mechanical stressors such as mastication as to induce periostin expression (32). Periostin expression was distributed throughout the pulp tissue, and seemed to localize between cells rather than inside them. Suggesting that periostin is secreted from dental pulp cells and stored in the ECM. Periostin expression is highly up regulated during early events of repair in several tissues. Whereas, when faced with particularly intense stimuli, such as replantation, this mechanism fails to keep the structure of dental pulp tissue intact, leading to canal space obliteration (60). After mastication, the incisors of periostin-null mice show massive increase in dentin formation and reduction in pulp space compared to age-matched controls. It has also been shown that over expression of TGF-β1 has a significant reduction in tooth mineralization and defective dentin formation (61). Findings in developing mouse mandibles have suggested that periostin serves as adhesive equipment at the sites of cell-to matrix interaction and aids against potentially harmful mechanical forces that include occlusal forces and tooth eruption (31).

Limitations and Future Directions

The findings derived from this study contribute to the ongoing effort of describing the basic elements involved in pulpal pathogenesis. Current clinical reality reflects the lack of a comprehensive understanding of the molecular and cellular events that lead to pulp disease, often times limiting treatment only when symptoms appear and normal tissue

function is altered or lost. Insights into pulpal pathogenesis incorporate gene, protein, and metabolite data into dynamic biologic networks that include disease-initiating and progression mechanisms. New evidence is emerging that periostin may play a greater role in regulating pulpal homeostasis and preventing complete mineralization of the pulp space (32, 41). Ultimately, the data generated may have significant implications to aid in the understanding of pulpal biology relevant to cell-matrix dynamics and homeostasis. This data adds supporting scientific information that will help capture the dynamic nature of extracellular biochemical events involved in the transition between pulpal health, disease and the ability to repair and/or regenerate.

The realization is that there are several limitations to extrapolate any findings using an *in vitro* culture methodology to the clinical treatment in humans. These methods are challenging, with multiple variables such as cellular behavior, culturing techniques and operator error influencing the ability to generate consistent and reproducible results. However, *in vitro* testing is key for understanding and predicting what might happen *in vivo*. It is also an irreplaceable process in order to test different conditions that might influence the final *in vivo* outcomes. Furthermore, future transitions from *in vitro* to *in vivo* experiments will be looking at effects that are the result of complex systemic, local, and environmental interactions. Therefore, understanding the *in vitro* and *in vivo* experiences is key in order to propose specific ways or concentrations for a possible exogenous delivery of periostin. Furthermore, *in vivo* animal studies could be proposed in the future based on the results.

Conclusion

To conclude, Dental Pulp Cells (DPCs) such as: Stem cells, Fibroblasts and Odontoblast-like cells are capable of expressing periostin. We have successfully demonstrated that periostin mRNA and protein are expressed by all these dental cell populations *in vitro*. We have also confirmed that the expression of periostin is regulated by TGF-β1. In addition, biomechanical stimulation may be used as a model to stimulate DPCs *in vitro* with either stimulatory or inhibitory effects. Lastly, exogenous delivery of periostin to DPCs yields inconclusive effects on total collagen expression. Overall, DPSCs show the

most responsiveness and predictable behavior to stimulus. We propose that activation of DPCs through TGF-β1 and biomechanical stimulation may regulate the potential therapeutic effects of these cells. Beyond the limits of this study, periostin may have a potential therapeutic effect; in order to stabilize the ECM during dentinogenesis, while pulpal repair and regeneration is occurring.

Tables and Figures

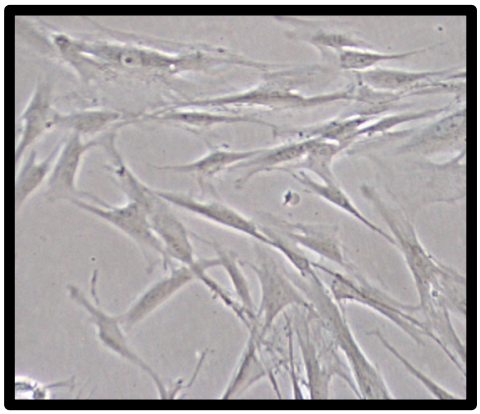

Figure 1: Phase contrast image of human dental pulp stem cells (DPSC) at 100x magnification. Cells were grown in Dulbecco's Modified Eagle's medium supplemented with heat inactivated 10% FBS, 1% penicillin/streptomycin and 1:1000 fungizone. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

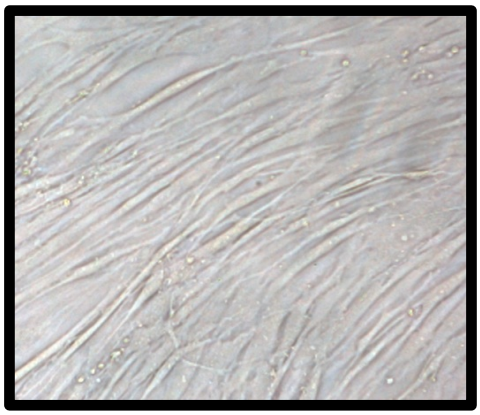

Figure 2: Phase contrast image of human dental pulp fibroblast cells (DPF) at 100x magnification. Cells were grown in Dulbecco's Modified Eagle's medium supplemented with heat inactivated 10% FBS, 1% penicillin/streptomycin and 1:1000 fungizone. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

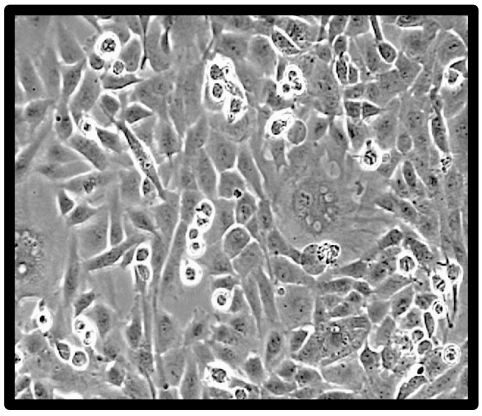

Figure 3: Phase contrast image of MDPC-23 odontoblast-like cells at 100x magnification. Cells were grown in Dulbecco's Modified Eagle's medium supplemented with heat inactivated 10% FBS, 1% penicillin/streptomycin and 1:1000 fungizone. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

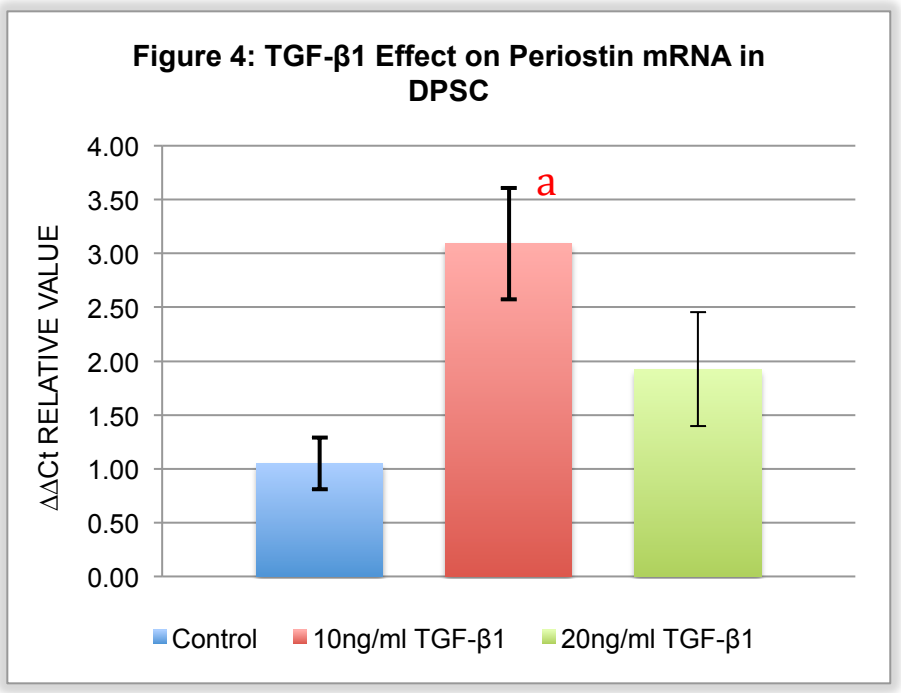

Figure 4: TGF-β1 Effect on Periostin mRNA in DPSC after 48 hours. Statistically significant difference from control to 10ng/ml TGF-β1 [a:(P=0.018) Fisher's LSD]

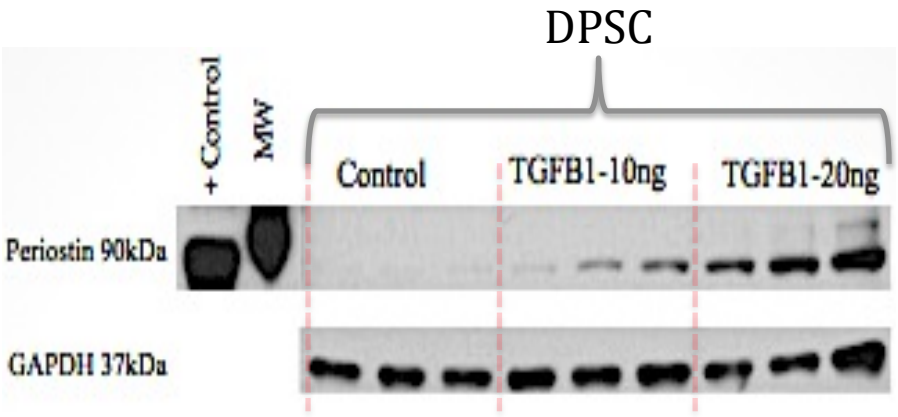

Figure 5: Western Blot for periostin protein in DPSC; GAPDH as housekeeping protein; comparing control to treatment with TGF-β1 after 48 hours. Molecular Weight (MW) and hPDL cells used for positive control.

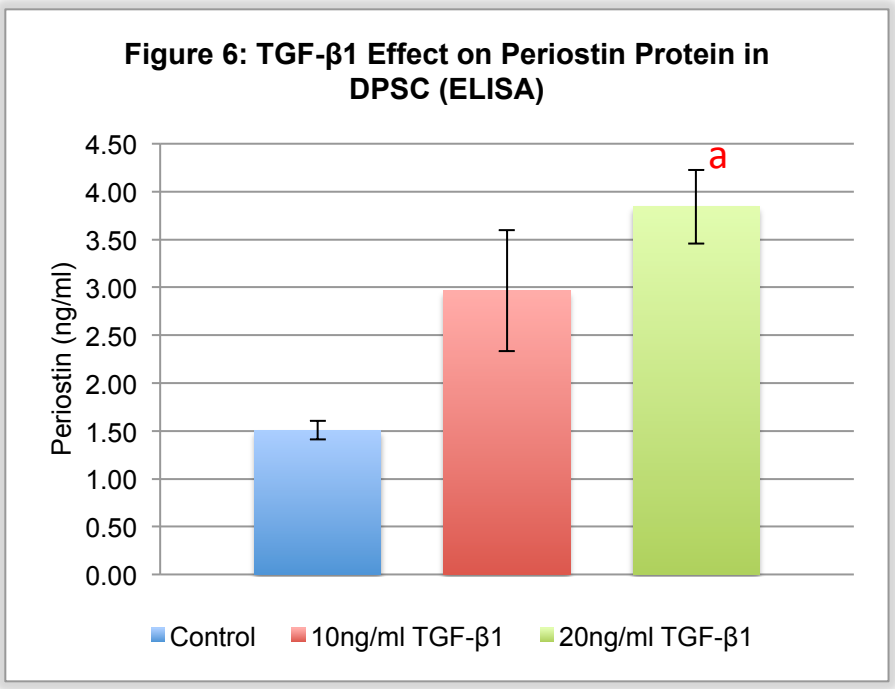

Figure 6: TGF-β1 Effect on Periostin Protein in DPSC after 48 hours. Statistically significant difference from control to 20ng/ml TGF-β1 [a:(P=0.032) Fisher's LSD]

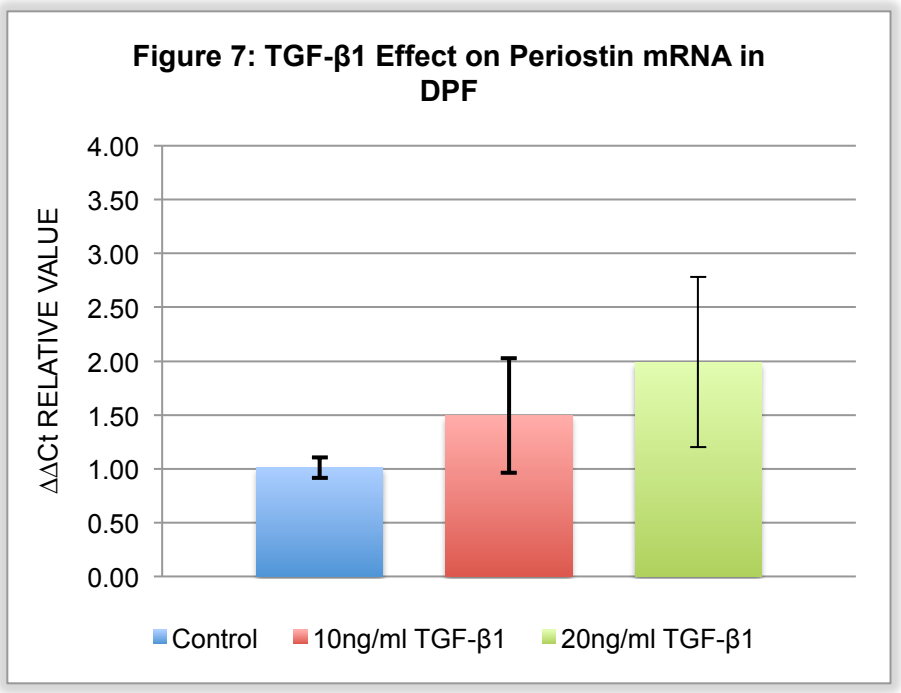

Figure 7: **TGF-β1 Effect on Periostin mRNA in DPF after 48 hours.** No statistically significant differences between groups [(P>0.05) Fisher's LSD]

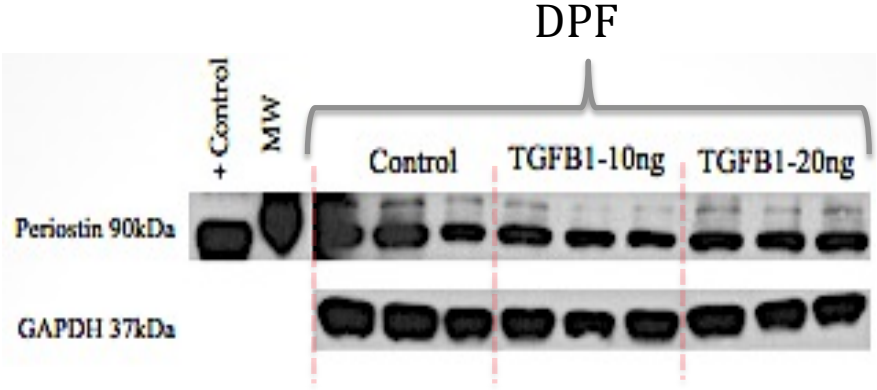

Figure 8: Western Blot for periostin protein in DPF; GAPDH as housekeeping protein; comparing control to treatment with TGF-β1 after 48 hours. Molecular Weight (MW) and hPDL cells used for positive control.

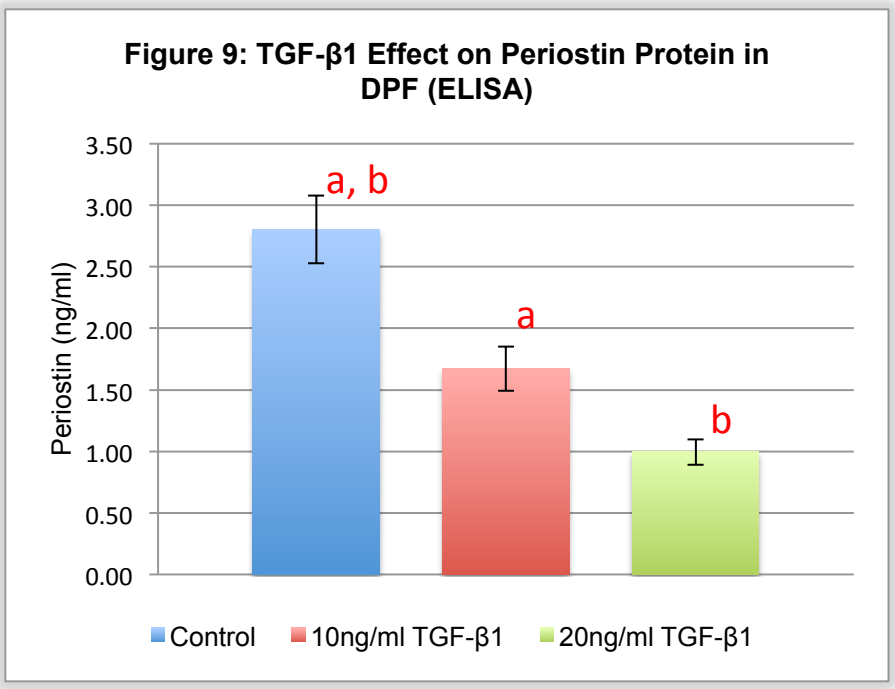

Figure 9: **TGF-β1 Effect on Periostin Protein in DPF after 48 hours.** Statistically significant difference from control to 10ng/ml TGF-β1 and 20ng/ml TGF-β1 [a:(P=0.028), b:(P=0.008) Fisher's LSD]

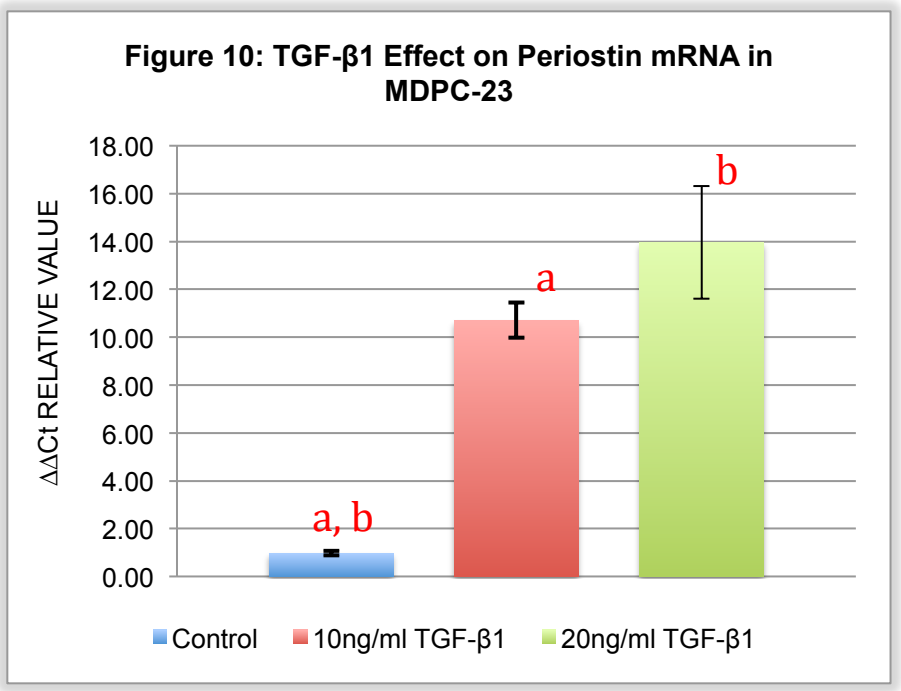

Figure 10: TGF-β1 Effect on Periostin mRNA in MDPC-23 after 48 hours. Statistically significant difference from control to 10ng/ml TGF-β1 and 20ng/ml TGF-β1 [a:(P=0.003), b:(P=0.001) Fisher's LSD]

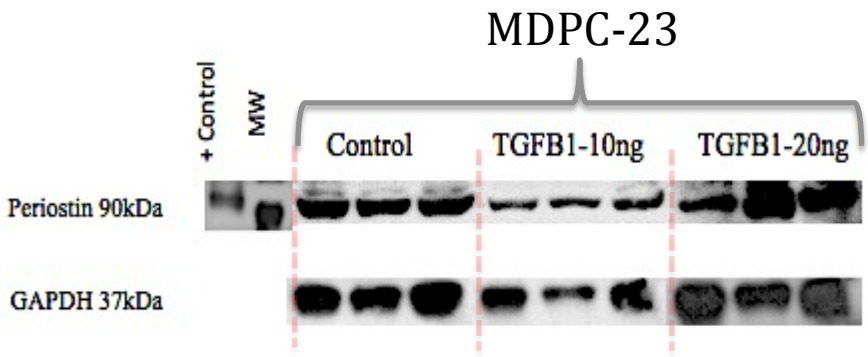

Figure 11: Western Blot for periostin protein in MDPC-23; GAPDH as housekeeping protein; comparing control to treatment with TGF-β1 after 48 hours. Molecular Weight (MW) and hPDL cells used for positive control.

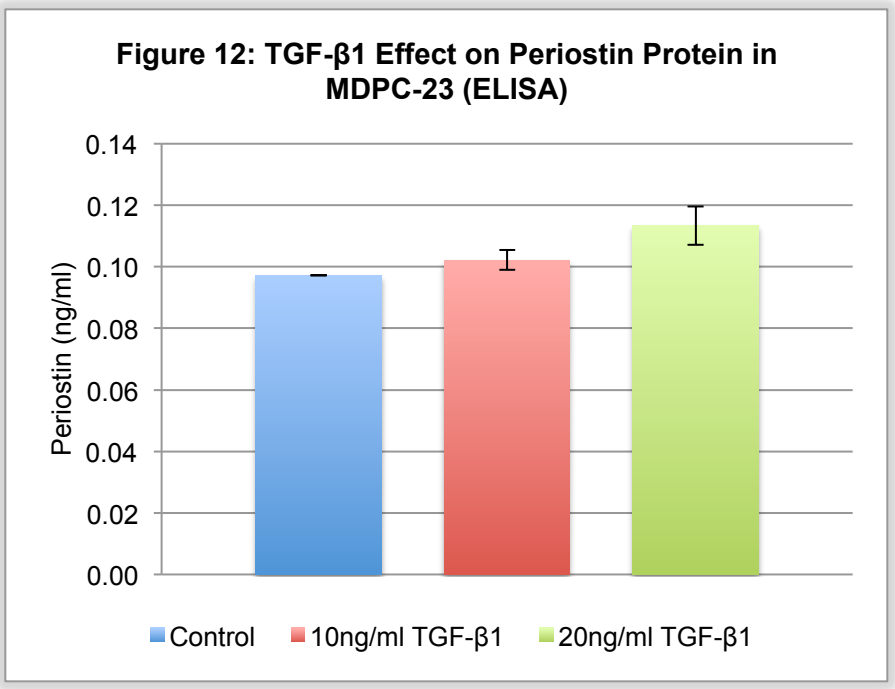

Figure 12: TGF-β1 Effect on Periostin Protein in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

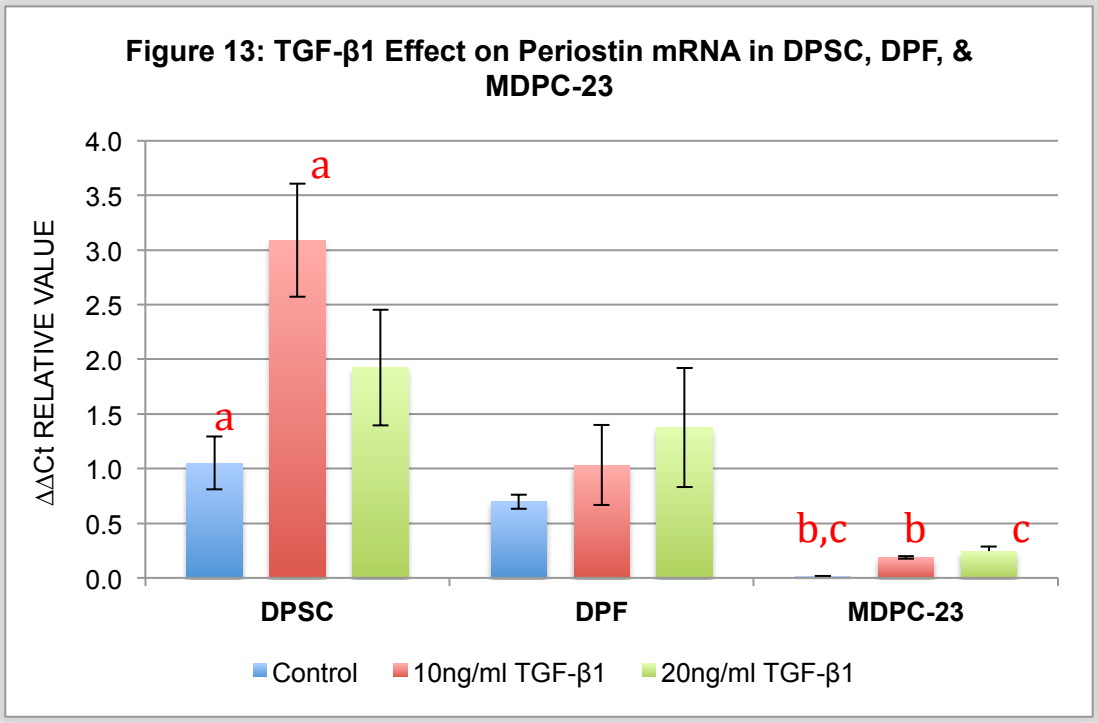

Figure 13: TGF-β1 Effect on Periostin mRNA in DPSC, DPF, & MDPC-23 after 48 hours. Statistically significant differences [a:(P=0.018); b:(P=0.003); c:(P=0.001) Fisher's LSD]

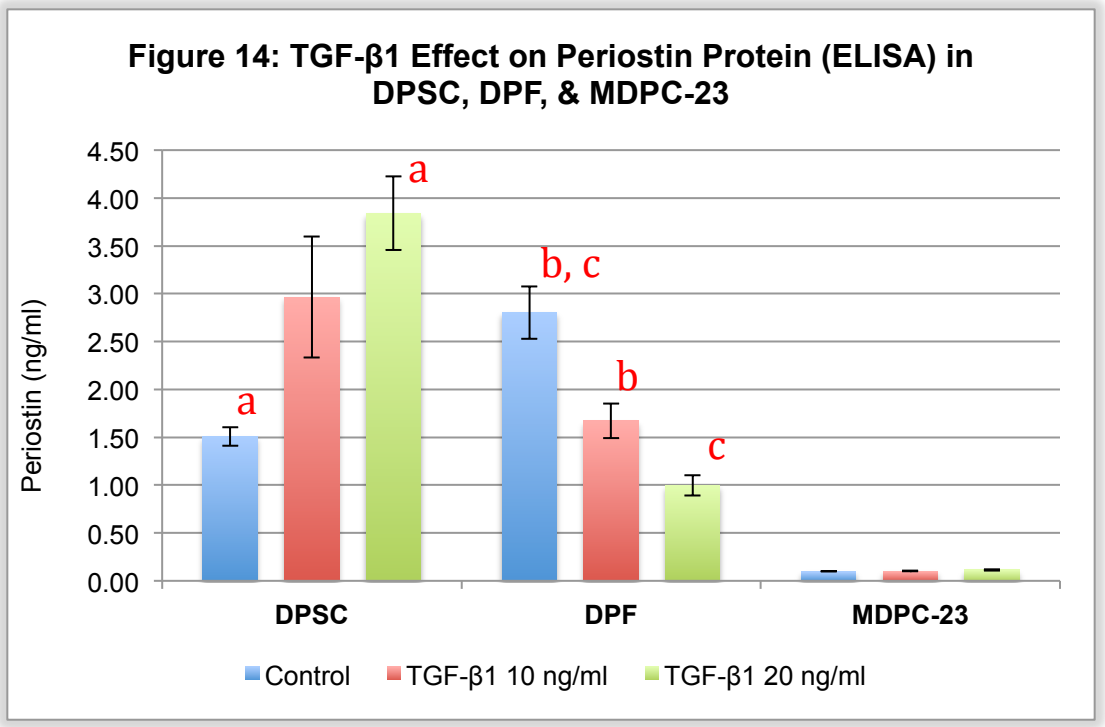

Figure 14: TGF-β1 Effect on Periostin Protein (ELISA) in DPSC, DPF, & MDPC-23 after 48 hours. Statistically significant differences [a:(P=0.032); b:(P=0.028); c:(P=0.008) Fisher's LSD]

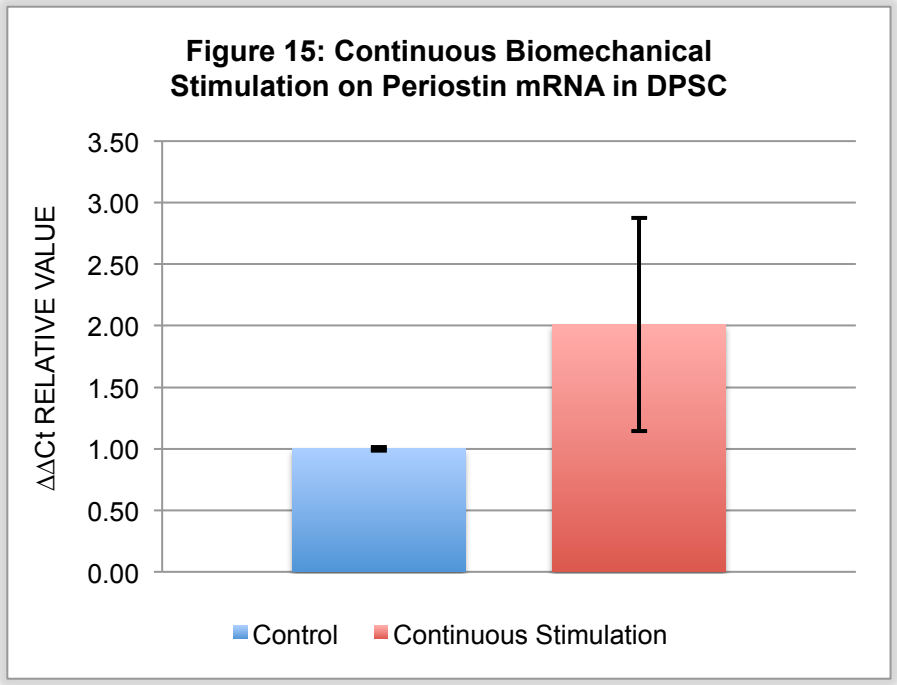

Figure 15: Continuous Biomechanical Stimulation on Periostin mRNA in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Student's t-test]

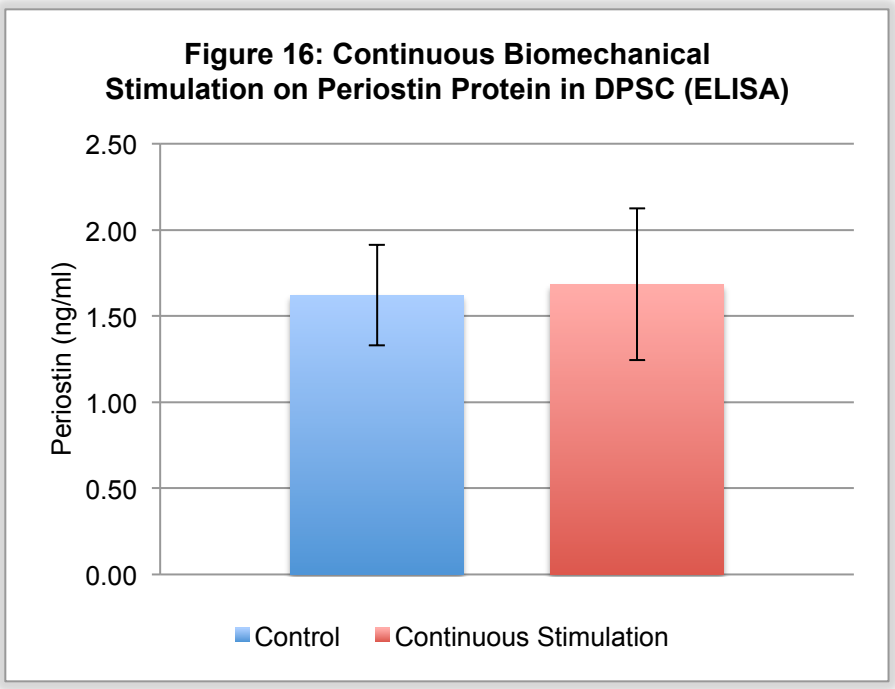

Figure 16: Continuous Biomechanical Stimulation on Periostin Protein in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

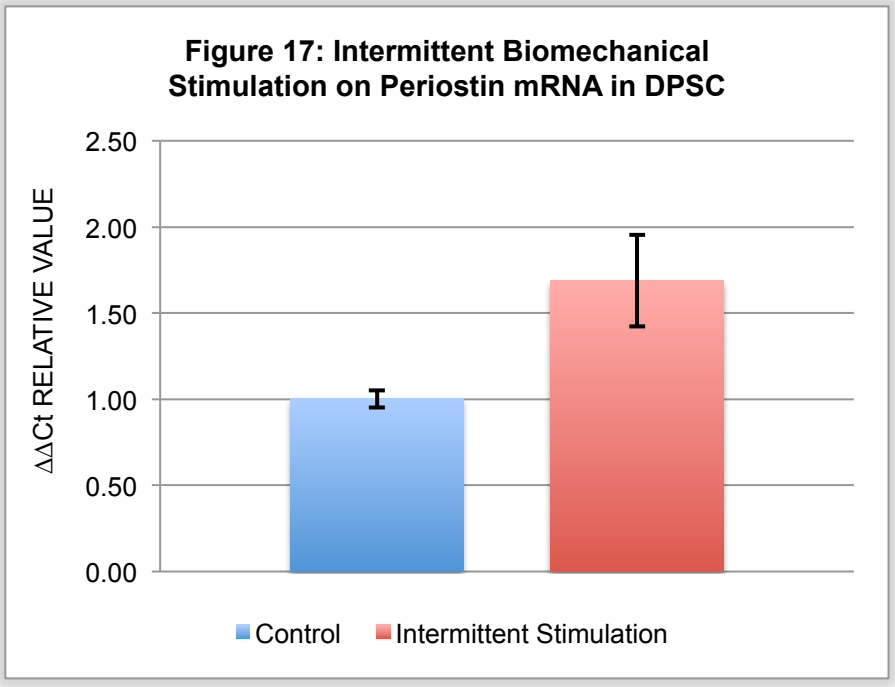

Figure 17: Intermittent Biomechanical Stimulation on Periostin mRNA in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Student's t-test]

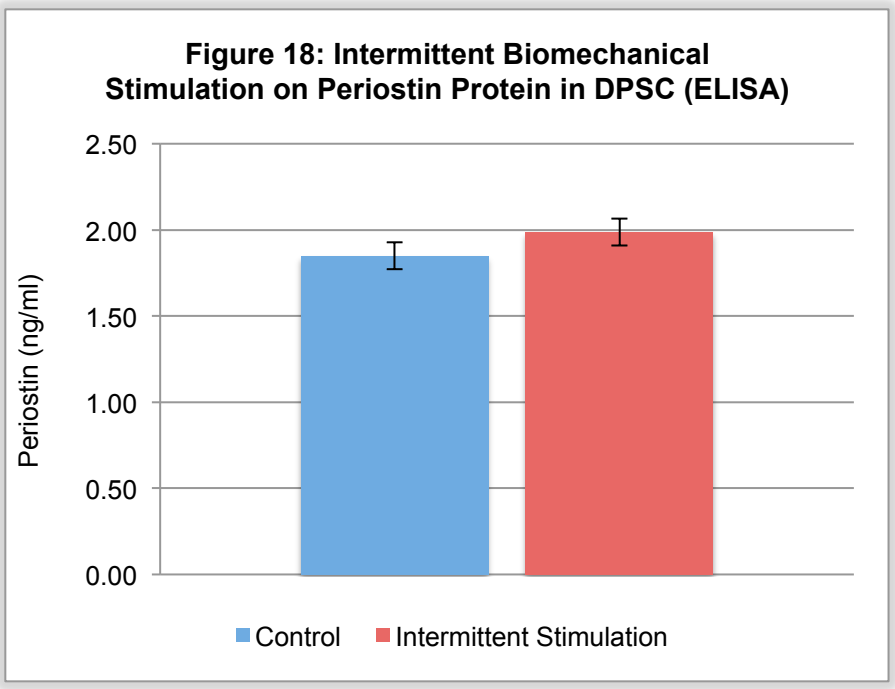

Figure 18: Intermittent Biomechanical Stimulation on Periostin Protein in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

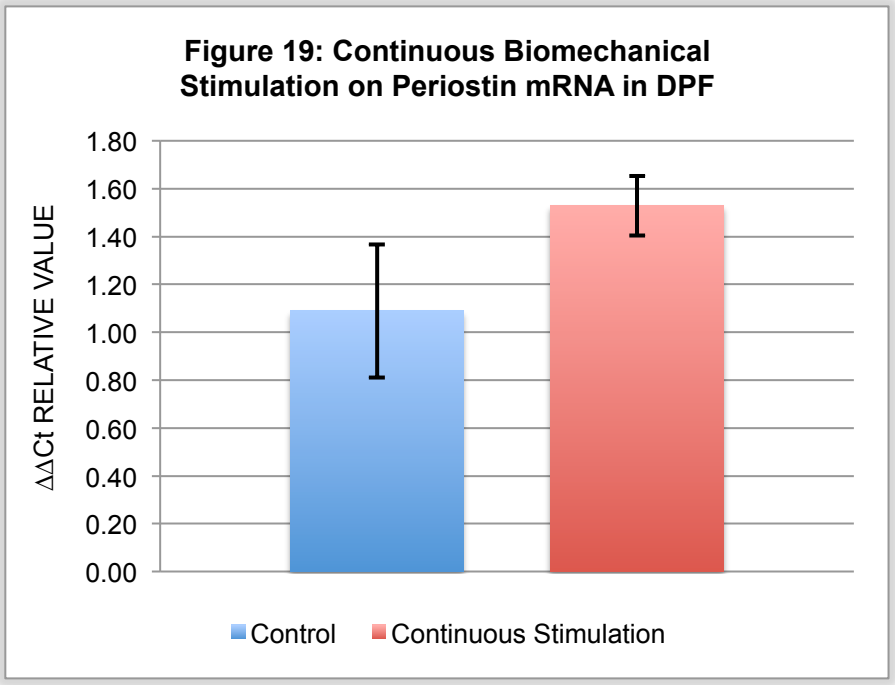

Figure 19: Continuous Biomechanical Stimulation on Periostin mRNA in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Student's t-test]

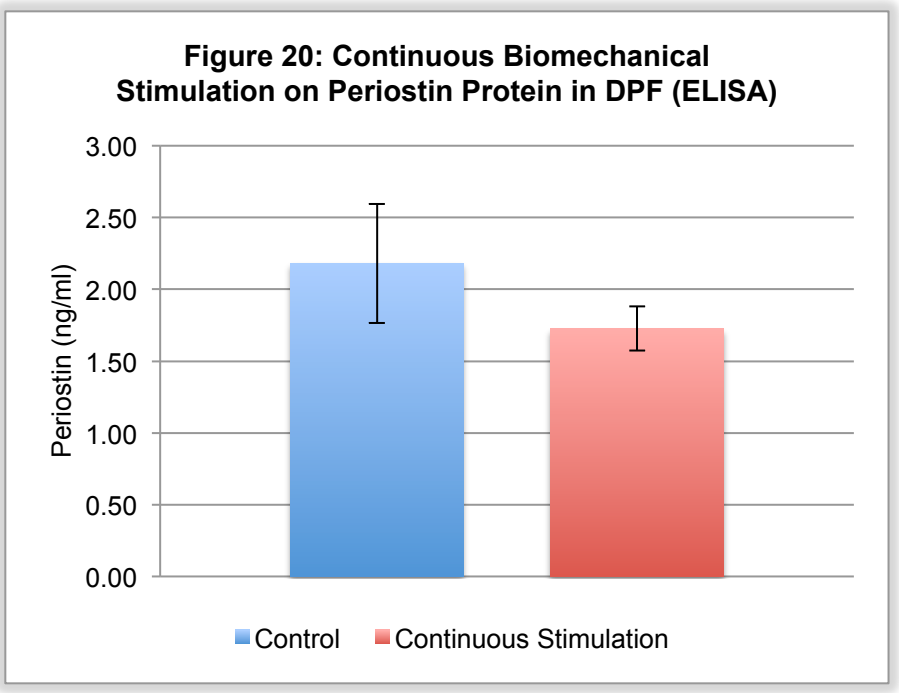

Figure 20: Continuous Biomechanical Stimulation on Periostin Protein in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

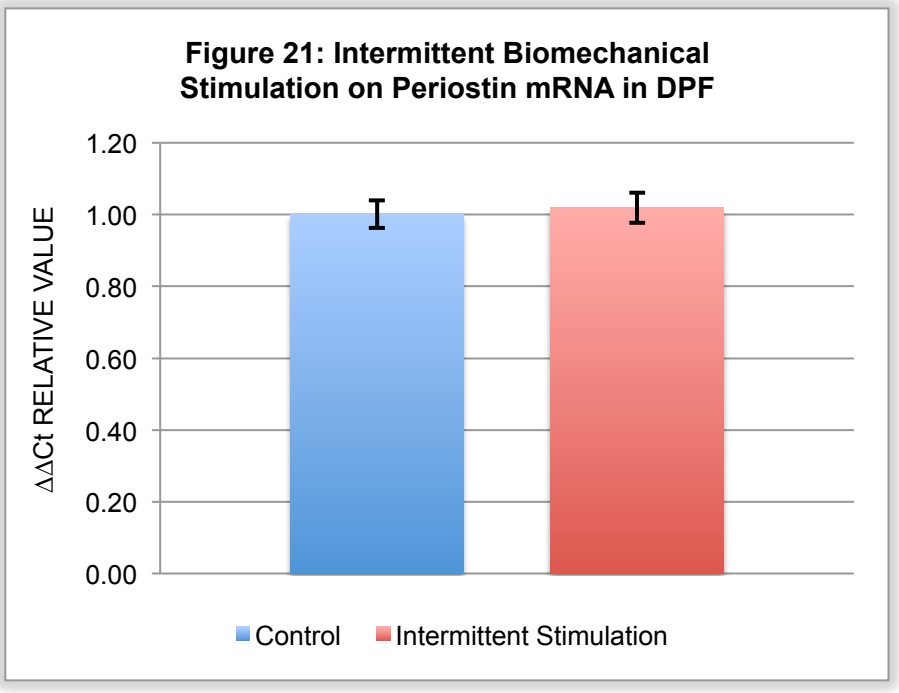

Figure 21: Intermittent Biomechanical Stimulation on Periostin mRNA in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Student's t-test]

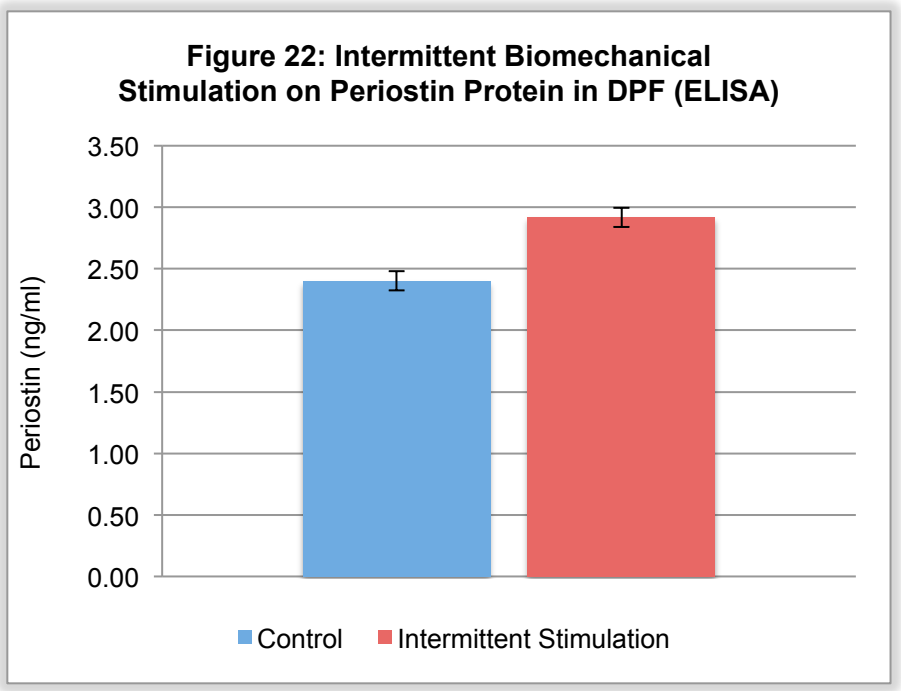

Figure 22: Intermittent Biomechanical Stimulation on Periostin Protein in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

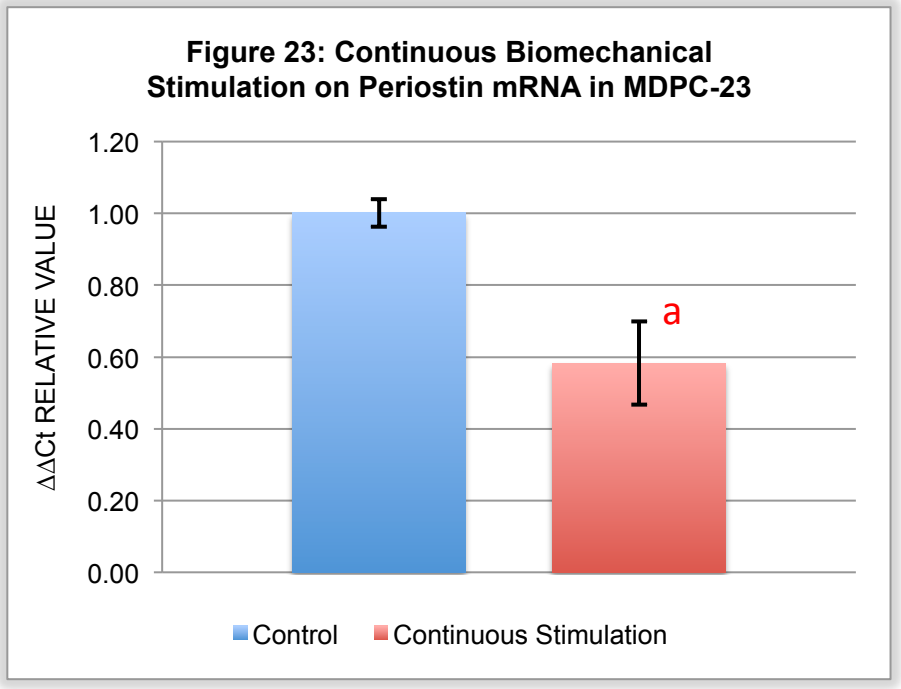

Figure 23: Continuous Biomechanical Stimulation on Periostin mRNA in MDPC-23 after 48 hours. Statistically significant differences between groups [a:(P=0.026) Student's t-test]

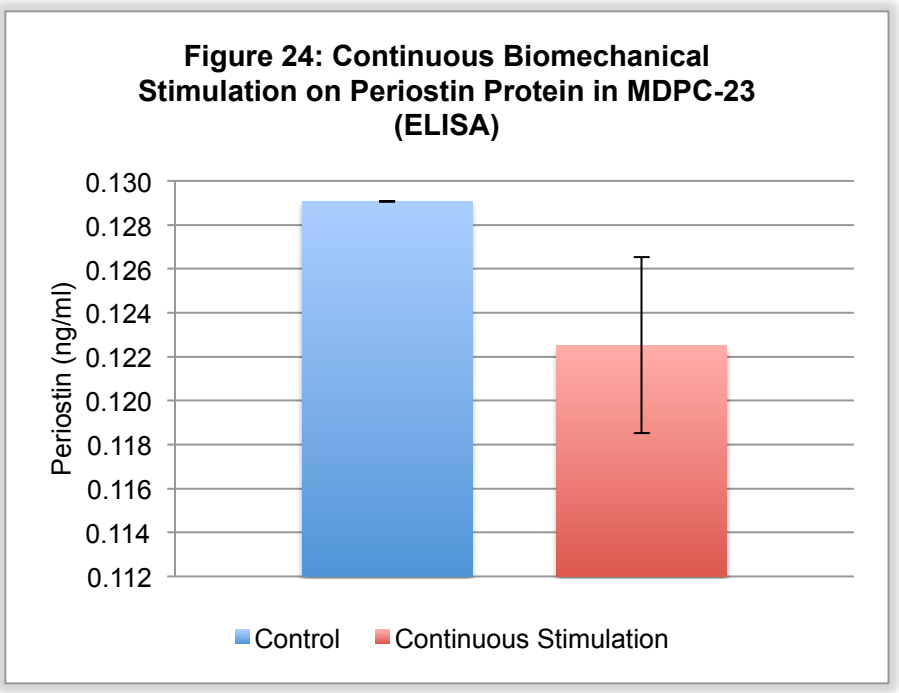

Figure 24: Continuous Biomechanical Stimulation on Periostin Protein in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

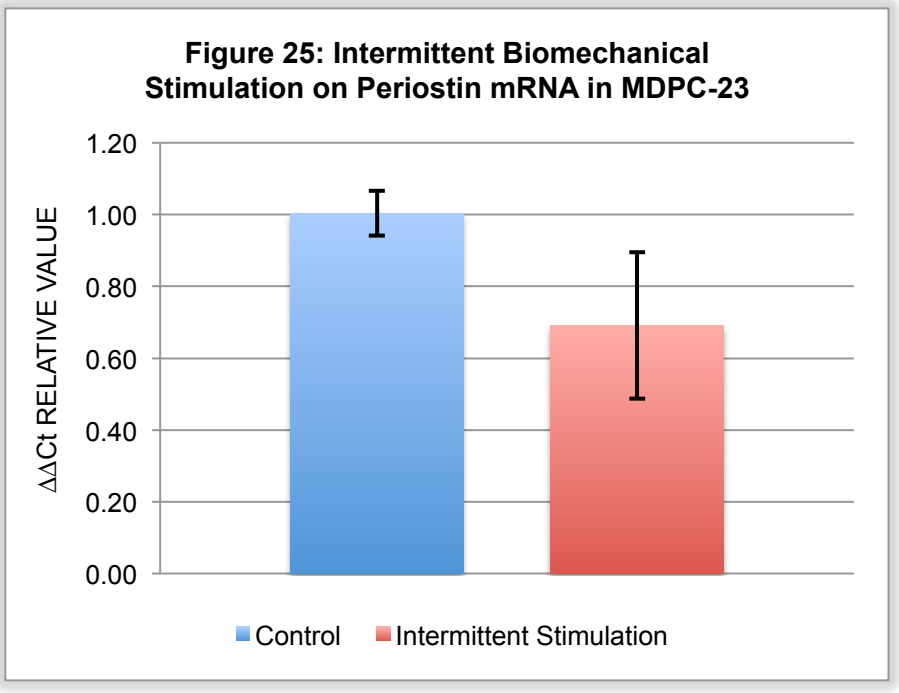

Figure 25: Intermittent Biomechanical Stimulation on Periostin mRNA in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Student's t-test]

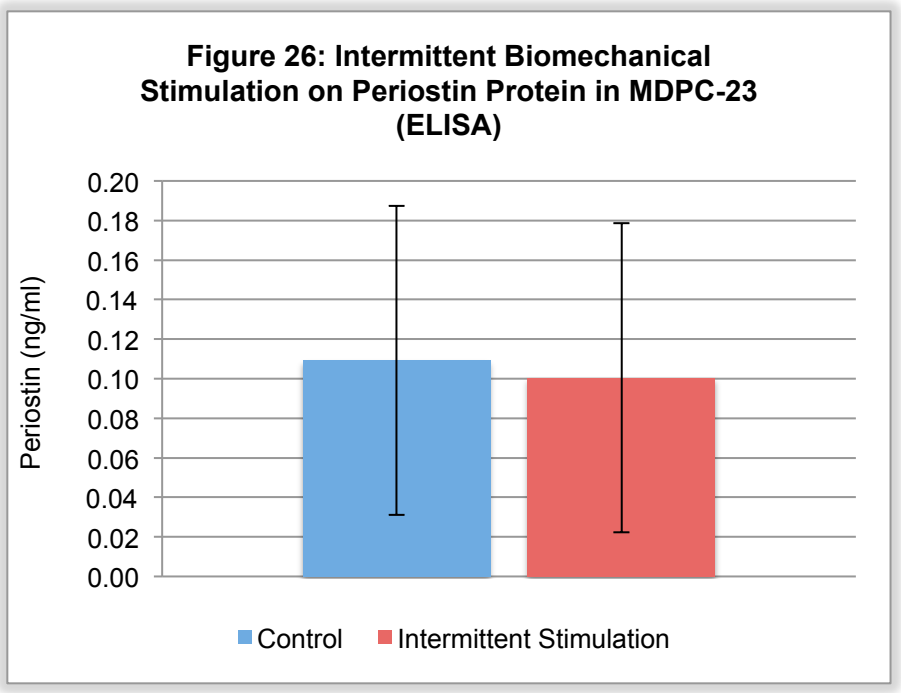

Figure 26: Intermittent Biomechanical Stimulation on Periostin Protein in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

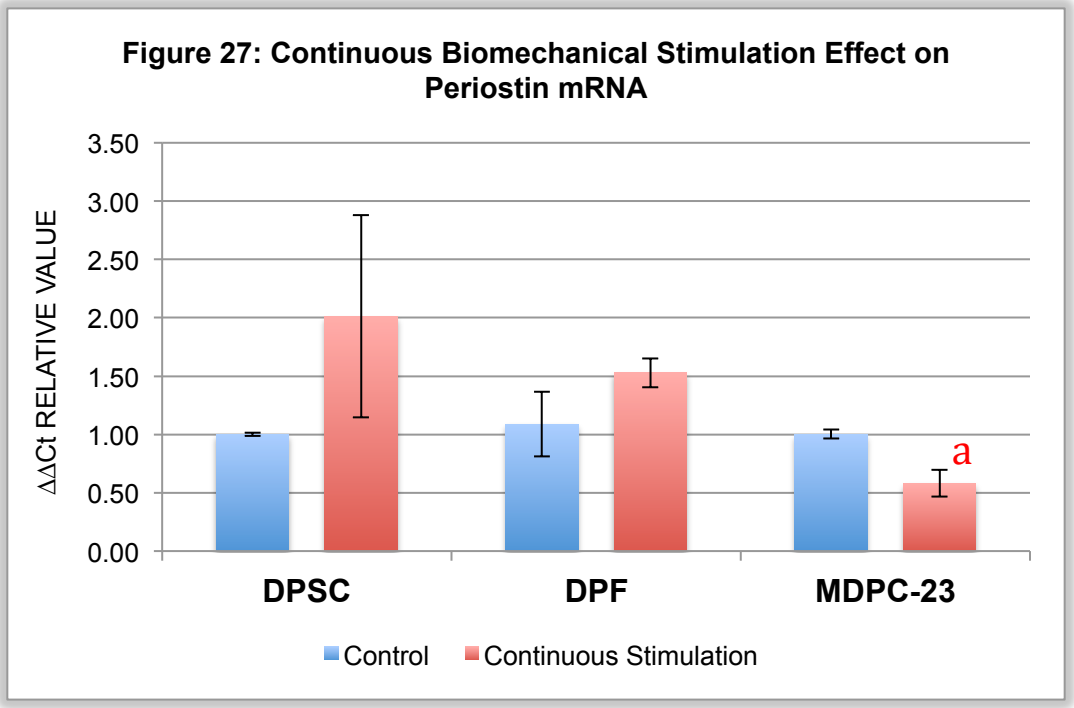

Figure 27: Continuous Biomechanical Stimulation Effect on Periostin mRNA in DPSC, DPF, & MDPC-23 after 48 hours. Statistically significant difference [a:(P=0.026) Student's t-test]

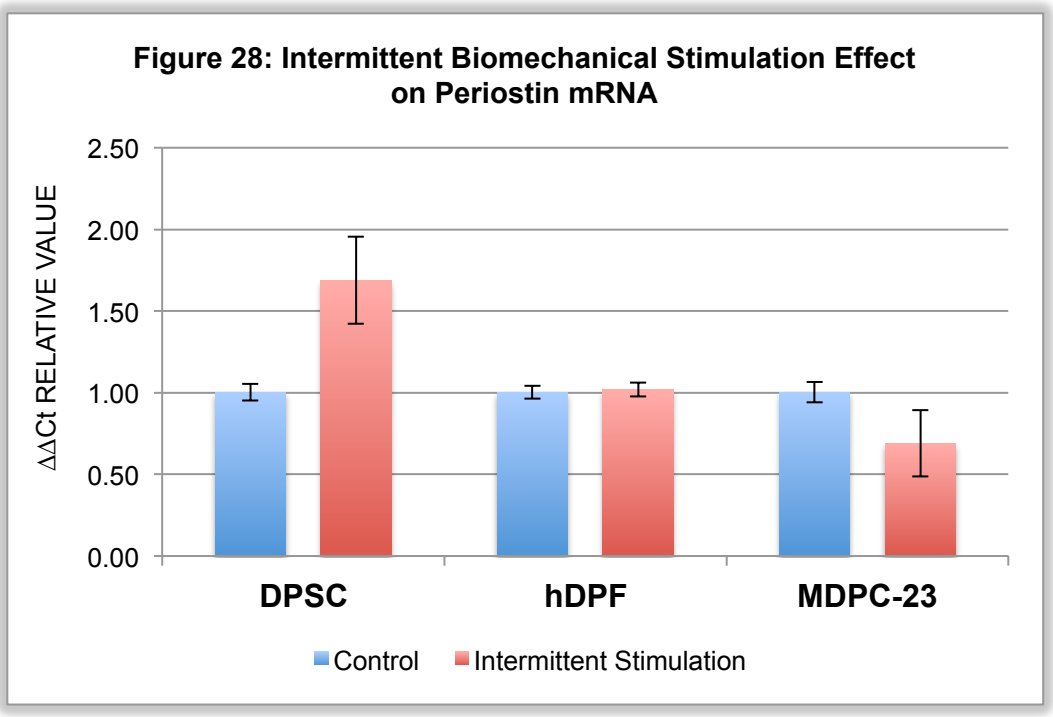

Figure 28: Intermittent Biomechanical Stimulation Effect on Periostin mRNA in DPSC, DPF, & MDPC-23 after 48 hours. No statistically significant findings between groups [(P>0.05) Student's t-test]

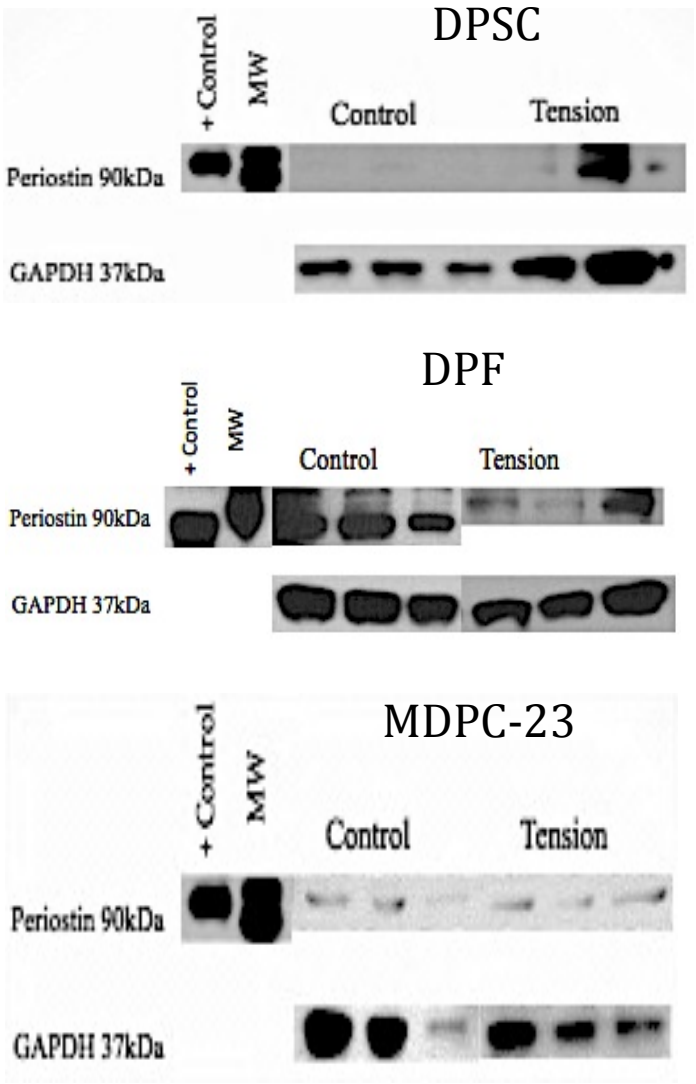

Figure 29: Western Blot for DPSC, DPF, & MDPC-23 probing for periostin, GAPDH as housekeeping protein; comparing at baseline levels to biomechanical stimulation after 48 hours. Molecular Weight (MW) and hPDL cells used for positive control.

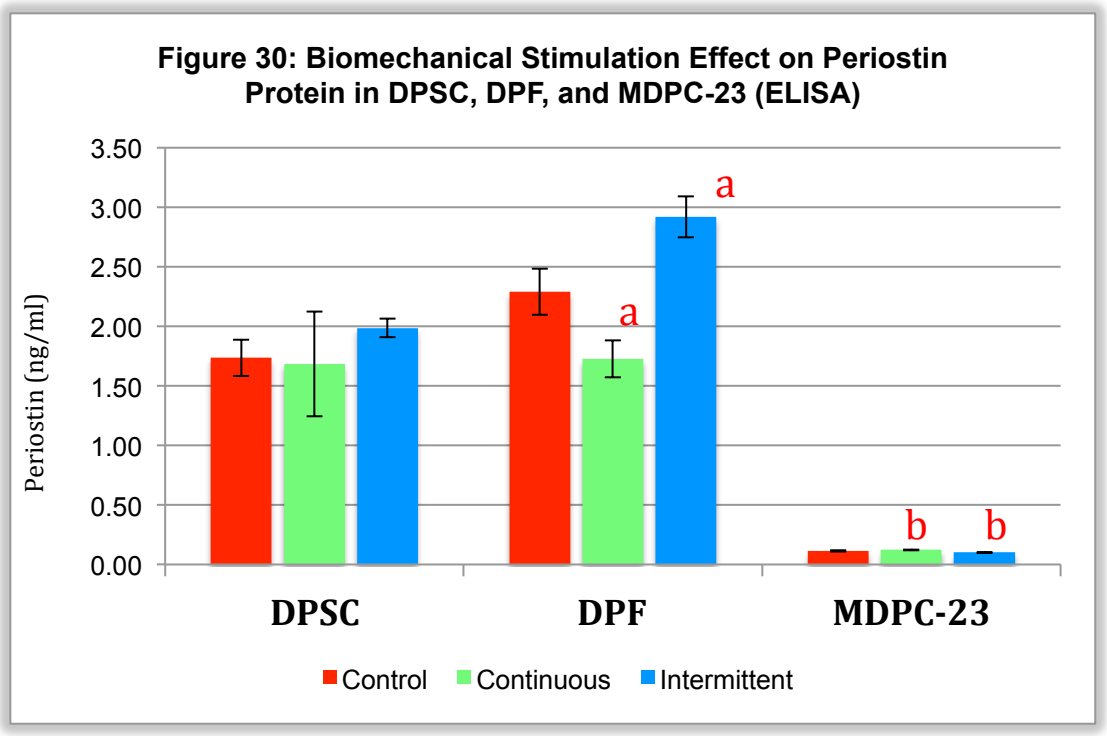

Figure 30: Biomechanical Stimulation Effect on Periostin Protein in DPSC, DPF, and MDPC-23 (ELISA) after 48 hours. Statistically significant differences [a:(P=0.005); b:(P=0.012) Fisher's LSD]

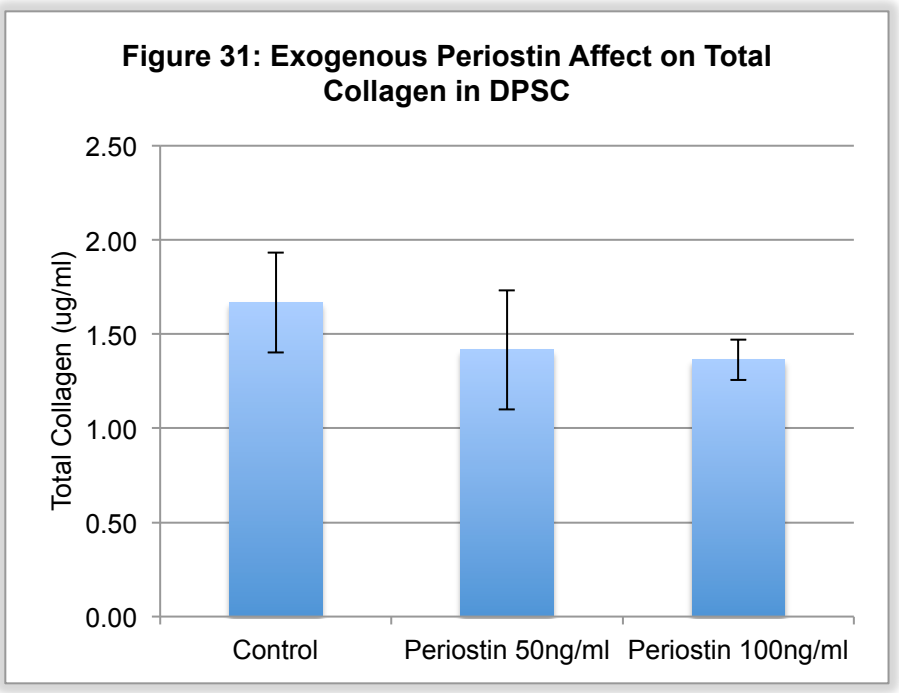

Figure 31: Exogenous Periostin Affect on Total Collagen in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

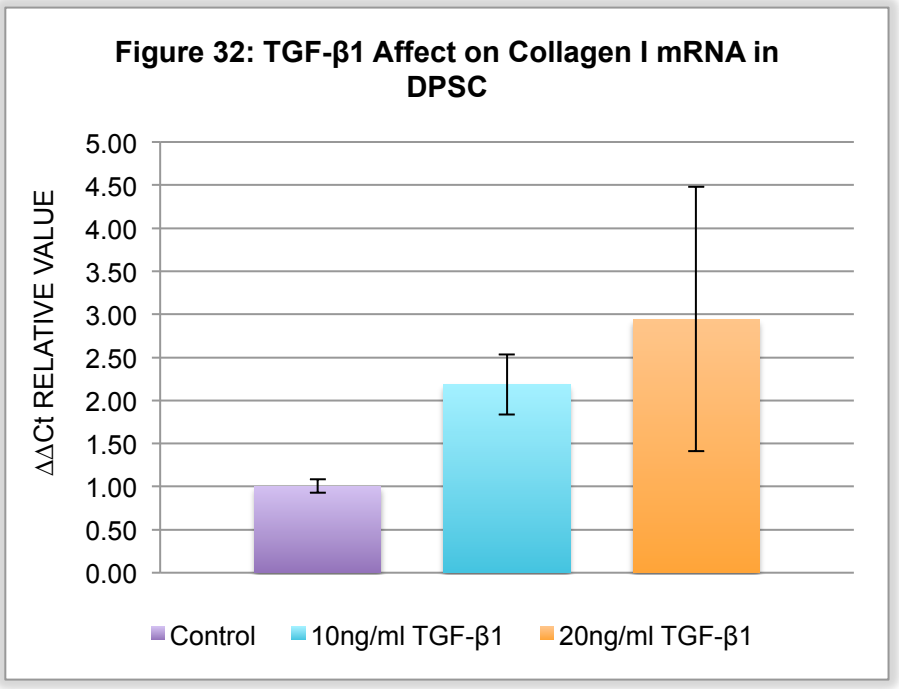

Figure 32: TGF-β1 Affect on Collagen I mRNA in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

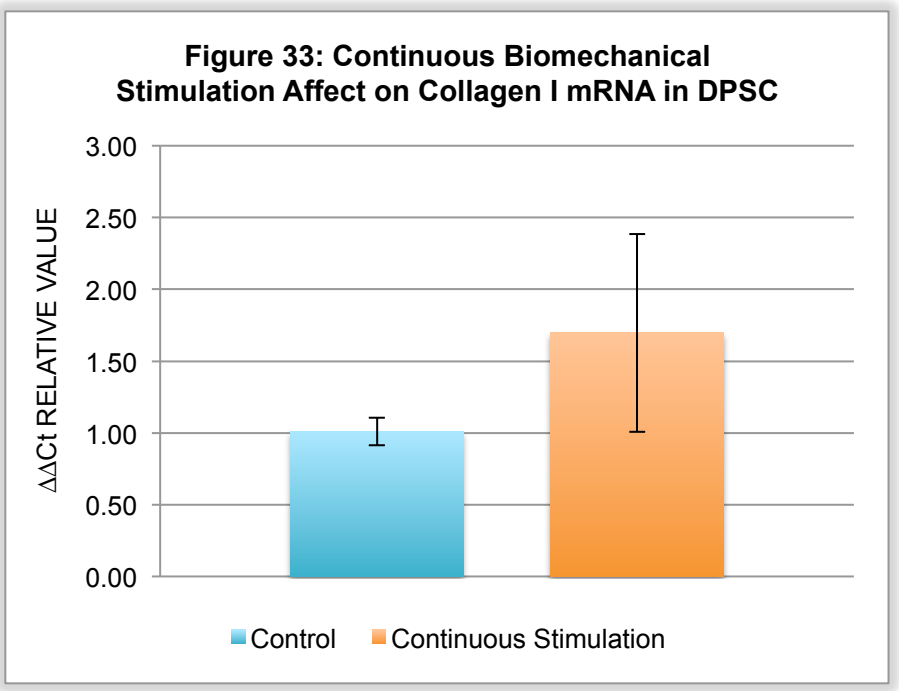

Figure 33: Continuous Biomechanical Stimulation Affect on Collagen I mRNA in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

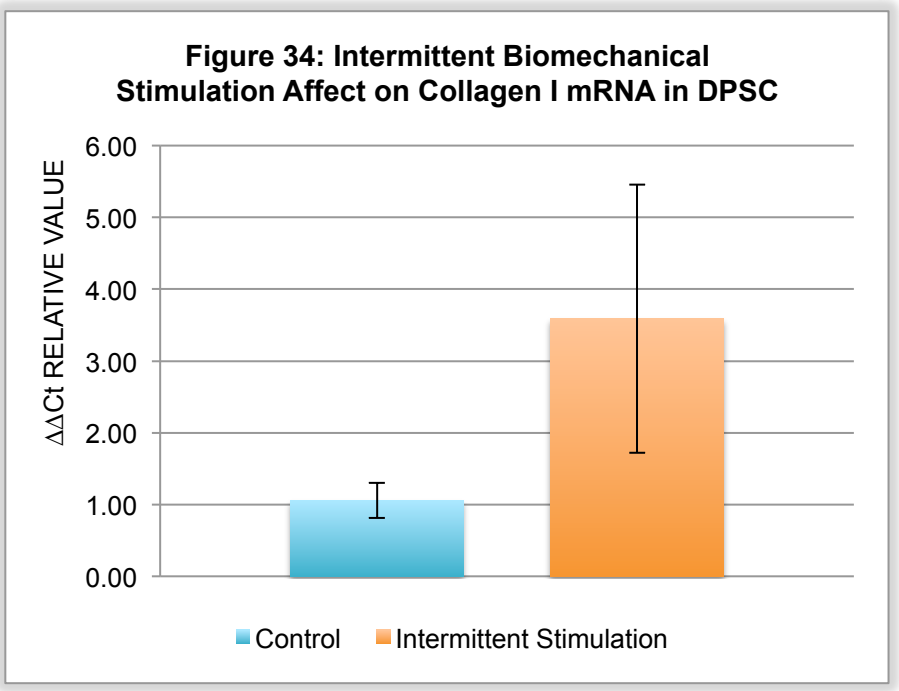

Figure 34: Intermittent Biomechanical Stimulation Affect on Collagen I mRNA in DPSC after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

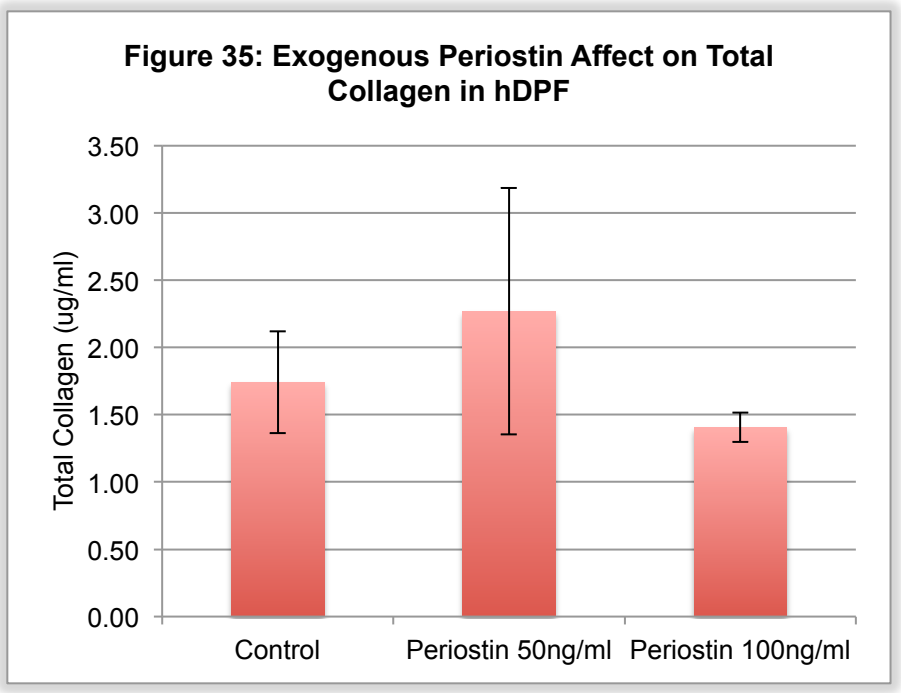

Figure 35: Exogenous Periostin Affect on Total Collagen in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

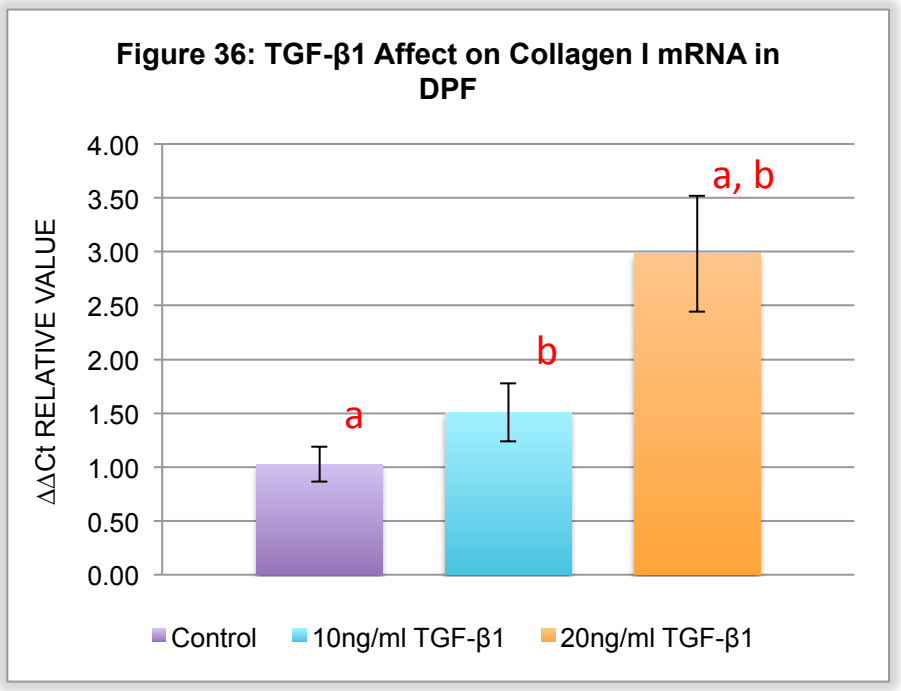

Figure 36: TGF-β1 Affect on Collagen I mRNA in DPF after 48 hours. Statistically significant differences [a:(P=0.009), b:(P=0.027); ANOVA LSD]

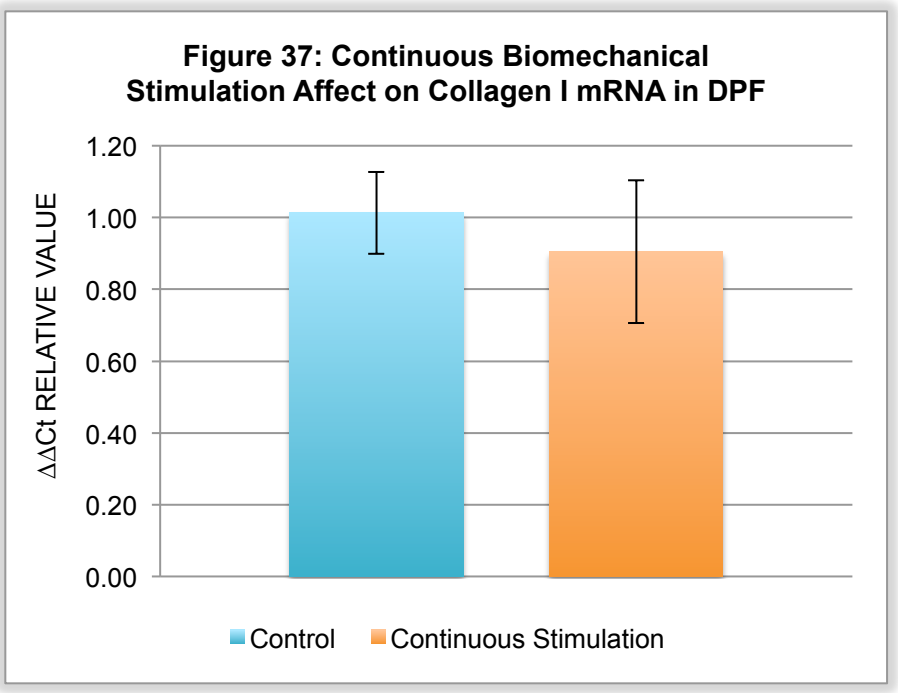

Figure 37: Continuous Biomechanical Stimulation Affect on Collagen I mRNA in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

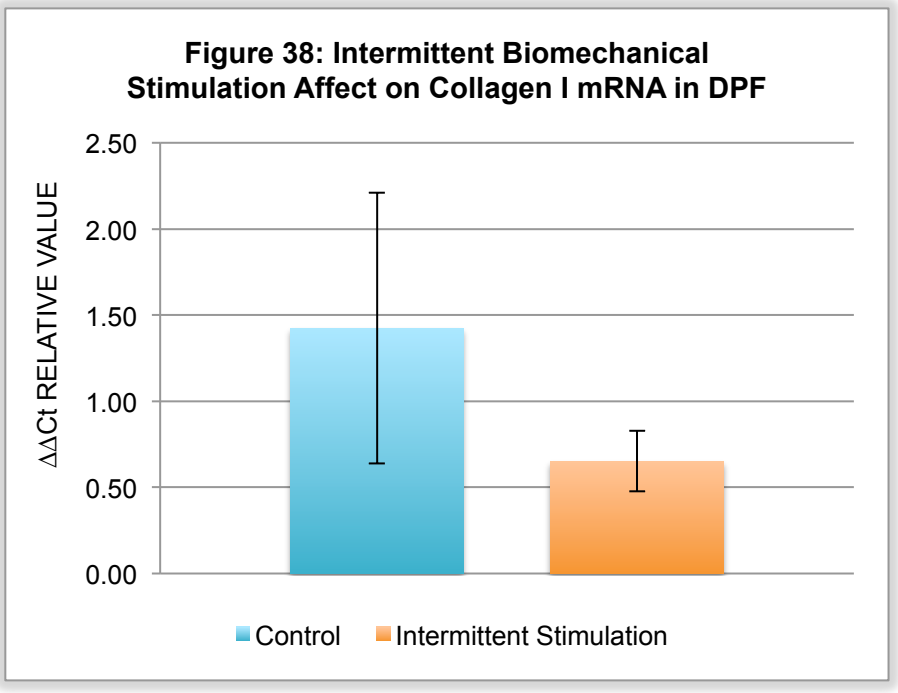

Figure 38: Intermittent Biomechanical Stimulation Affect on Collagen I mRNA in DPF after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

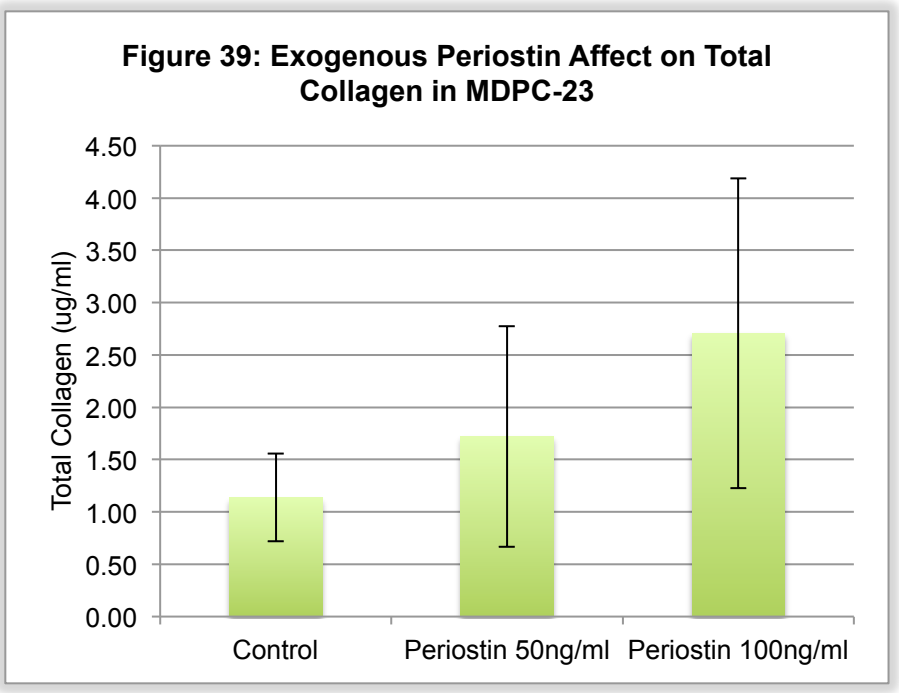

Figure 39: Exogenous Periostin Affect on Total Collagen in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

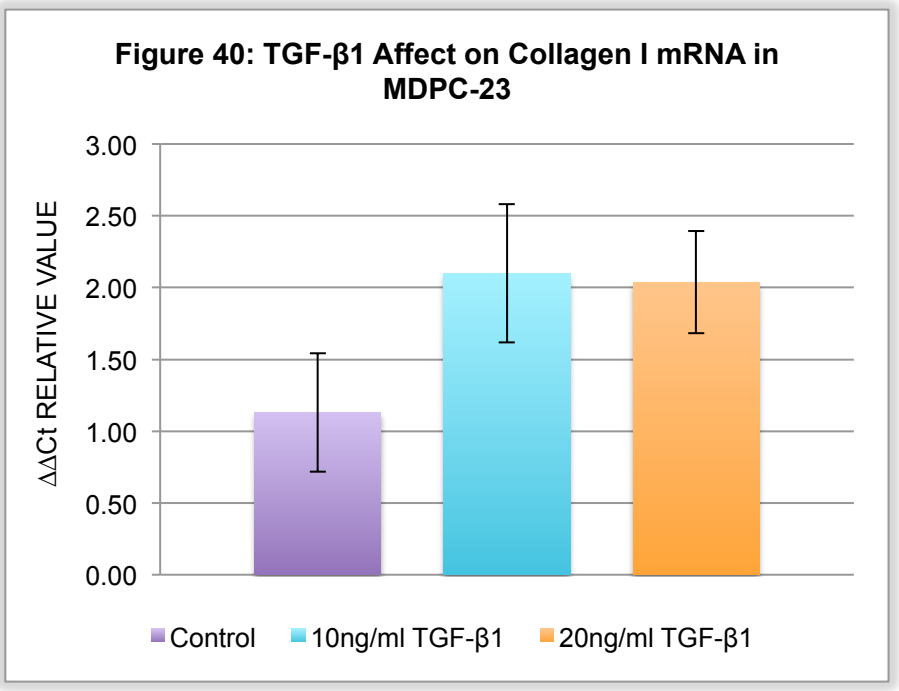

Figure 40: TGF-β1 Affect on Collagen I mRNA in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Fisher's LSD]

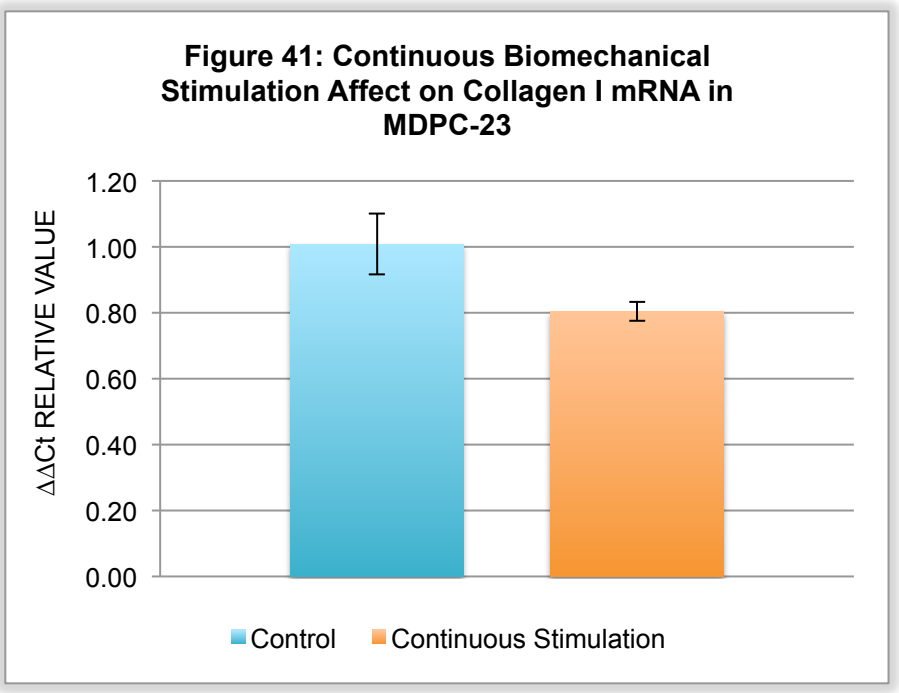

Figure 41: Continuous Biomechanical Stimulation Affect on Collagen I mRNA in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

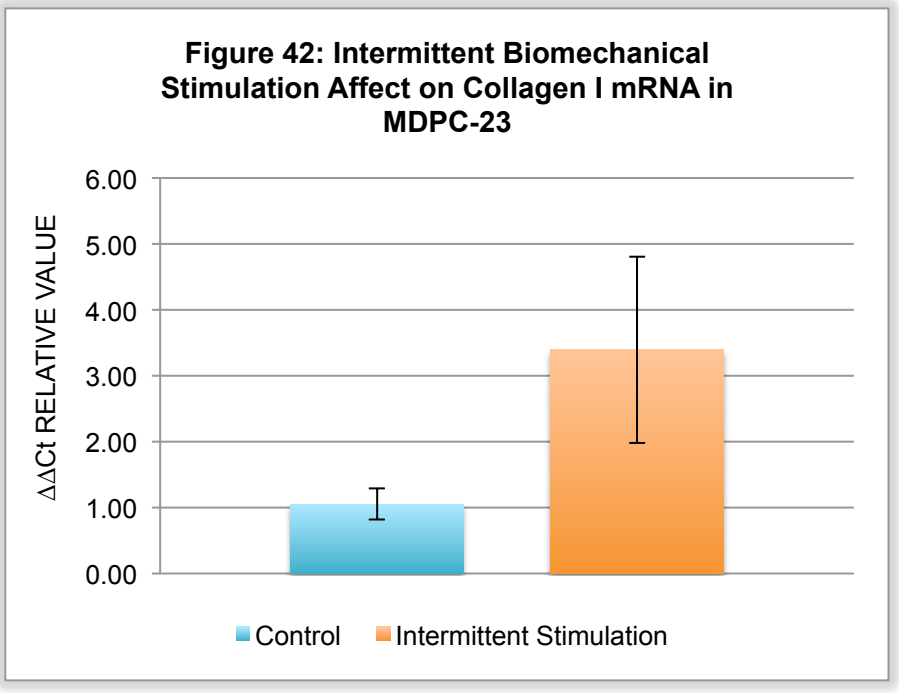

Figure 42: Intermittent Biomechanical Stimulation Affect on Collagen I mRNA in MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

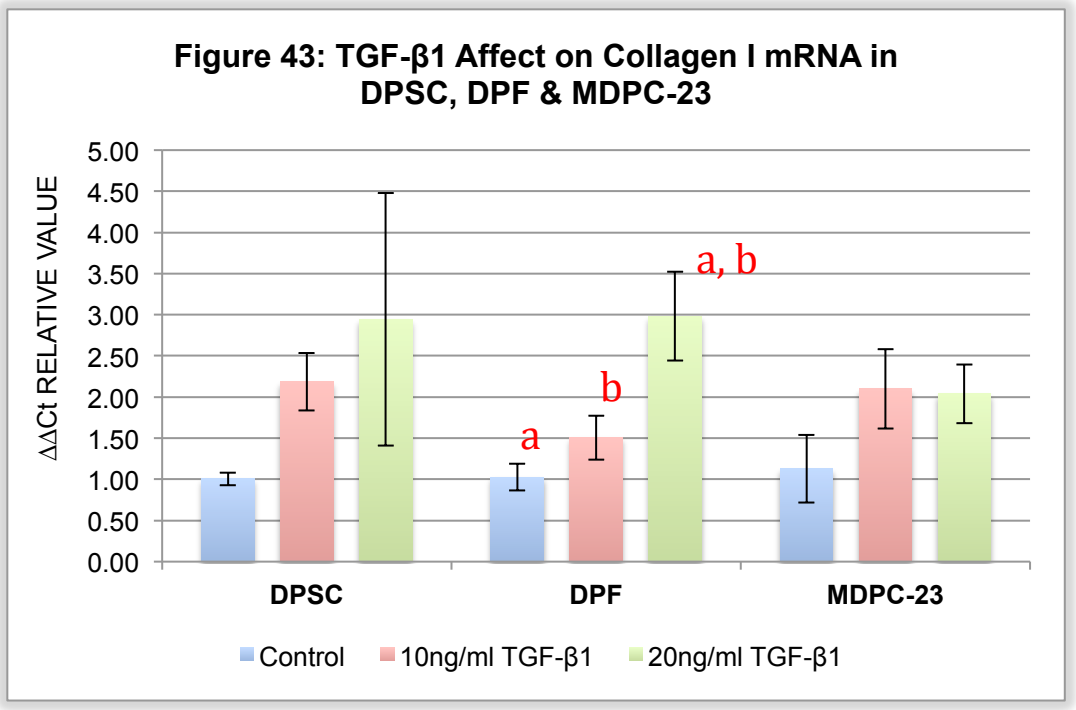

Figure 43: TGF-β1 Affect on Collagen I mRNA in DPSC, DPF & MDPC-23 after 48 hours. Statistically significant differences [a:(P=0.009), b:(P=0.027); ANOVA LSD]

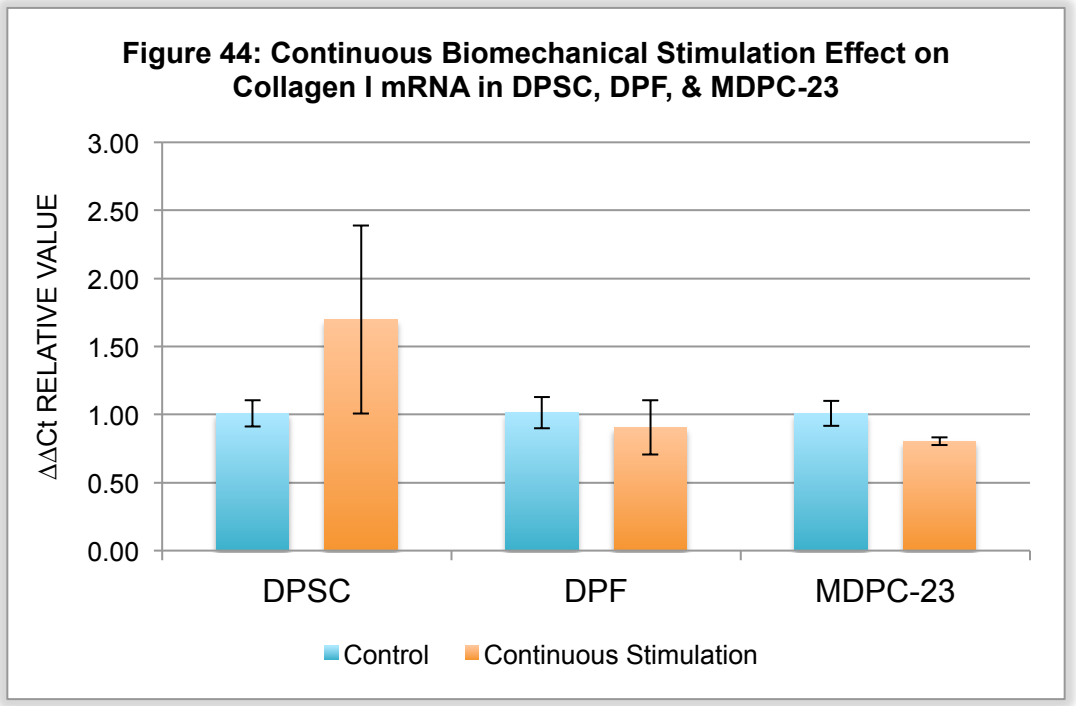

Figure 44: Continuous Biomechanical Stimulation Effect on Collagen I mRNA in DPSC, DPF, & MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

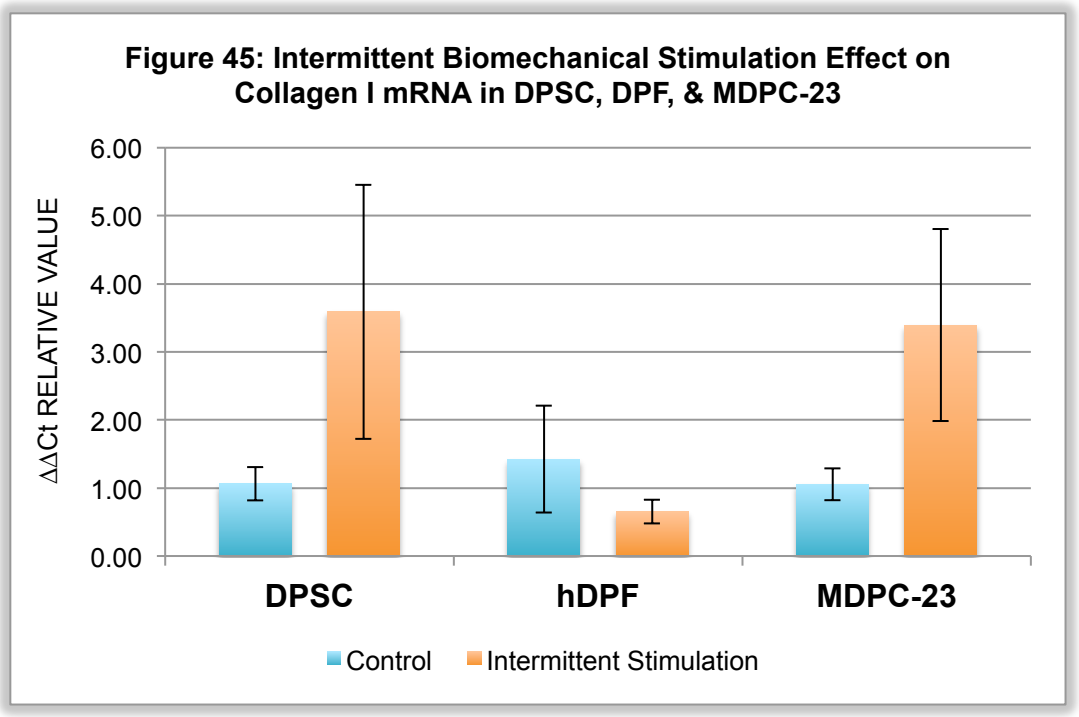

Figure 45: Intermittent Biomechanical Stimulation Effect on Collagen I mRNA in DPSC, DPF, & MDPC-23 after 48 hours. No statistically significant differences between groups [(P>0.05) Student T-test]

Box 1: ELISA Mean Values - TGF-β1 Effect on Periostin Protein (ng/ml)								
	DPSC			DPF			MDPC-23	
	MEAN	SEM		MEAN	SEM		MEAN	SEM
Control	1.5081	0.0965		2.8027	0.2739		0.0972	0.0000
10ng/ml TGF-β1	2.9647	0.6324		1.6725	0.1811		0.1022	0.0032
20ng/ml TGF-β1	3.8412	0.3871		0.9958	0.1041		0.1134	0.0063

Box 2: ELISA Mean Values - Biomechanical Stimulation Effect on Periostin Protein (ng/ml)								
	DPSC			DPF			MDPC-23	
	MEAN	SEM		MEAN	SEM		MEAN	SEM
Control	1.7352	0.1519		2.2912	0.1944		0.1143	0.0055
Continuous Stimulation	1.6848	0.4402		1.7261	0.1542		0.1225	0.0040
Intermittent Stimulation	1.9864	0.0782		2.9182	0.1729		0.1005	0.0037

Box 3: Total Collagen Assay Mean Values - Periostin Affect on Total Collagen (ug/ml)								
	DPSC			DPF			MDPC-23	
	MEAN	SEM		MEAN	SEM		MEAN	SEM
Control	1.6665	0.2649		1.7414	0.3782		1.1368	0.4190
50ng/ml Periostin	1.4163	0.3163		2.2708	0.9169		1.7216	1.0546
100ng/ml Periostin	1.3629	0.1065		1.4069	0.1085		2.7082	1.4826

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