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Validation of the FlexCell®FX-5000 Tension System to Evaluate the Expression of Cementum Protein-1 in Human Periodontal Ligament Cells While Under Cyclical Orthodontic Tensile Force

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Abstract

The pressure-tension theory of orthodontic tooth movement (OTM) states that there are differences in microenvironment between the tension and compressions side within the periodontal ligament, leading to differential gene expression within those zones. Cementum protein 1 (CEMP-1) is a human-specific protein localized to the root of a tooth and may play a role in OTM. The first objective was to develop a protocol to apply tensile force to human PDL cells similar to that within the tension zone during OTM. For this, the FlexCell®FX-5000 Tension system was used and fluorescent microscopy was used to confirm validity of the protocol. The second objective was to evaluate expression of CEMP-1 in human PDL cells under tensile force using RTqPCR. Expression of CEMP-1 appeared to decrease over time regardless of amount of force application or time point. More research is required to further investigate CEMP-1 expression in human PDL cells.

Keywords

Bone remodeling, periodontal ligament, orthodontic tooth movement (OTM), pressure-tension theory, periostin, collagen, tension, compression, gene expression, FlexCell®FX-5000 Tension system, TissueTrain®, reverse transcriptase-quantitative polymerase chain reaction (RTqPCR), periostin (PSTN), cementum protein 1 (CEMP-1), alkaline phosphatase (ALP)

Summary for Lay Audience

Orthodontics is the dental specialty that addresses the diagnosis, prevention and correction of malpositioned teeth and jaws. Tooth movement is just one of the tools used to address dental malocclusion. A specialized group of fibers, called the periodontal ligament (PDL), connects the tooth to the surrounding bone and is essential for tooth movement. When a tooth is moved through the bones of the jaw, one side of the PDL is compressed while the other side of the PDL is experiencing tension. Because the forces are different on either side of the tooth's PDL, this can lead to differences in the microenvironments between the zones of compression and tension.

Previous studies have shown that certain proteins – such as Periostin – are differentially expressed in the different zones and this suggests that other proteins might be differentially regulated as well. This study specifically wanted to look at how cementum protein 1 (CEMP-1) was expressed in the zone of tension. CEMP-1 is only found in humans and as such there is not much known about this protein. It is thought that it may help generate or regulate components that help repair teeth during orthodontic tooth movement.

In order to evaluate CEMP-1 expression in an environment as similar as possible to that found in humans, a protocol was developed using a specialized machine (FlexCell®FX-5000 Tension system) that could apply tension to human PDL cells isolated from extracted teeth in a 3-dimensional collagen gel. Using this protocol, it was determined that CEMP-1 expression decreased over time with or without tension applied. Additional research is required to more fully understand the role of CEMP-1 in orthodontic tooth movement.

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List of Abbreviations

ALP	alkaline phosphatase
BGLAP	bone gamma-carboxyglutamic acid-containing protein
BMP	bone morphogenic proteins
BSA	bovine serum albumin
BSP	bone sialoprotein
cAMP	cyclic adenosine monophosphate
CEMP-1	cementum protein-1
Cfba1	core-binding factor subunit alpha-1
DMEM	Dulbecco's modified Eagle's medium
EARR	external apical root resorption
ECM	extracellular matrix
Erk	extracellular signal-related protein kinase
GAG	glycosaminoglycan
GCF	gingival crevicular fluid
HERS	Hertwig epithelial root sheath
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPDF	human PDL-derived fibroblasts
hPDL	human periodontal ligament
IGF	insulin-like growth factor
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
NCC	neural crest cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OCIF	osteoclastogenesis inhibitory factor
OCN	osteocalcin
OPG	osteoprotegerin
OTM	orthodontic tooth movement
PBS	Phosphate-buffered saline

PDL	periodontal ligament
PG	proteoglycan
PGE_2	Prostaglandin E_2
PSTN	periostin
PTH	parathyroid hormone
RANK	receptor activator of NF-kB
RANKL	receptor activator of NF-kB ligand
RT-qPCR	reverse transcriptase-quantitative polymerase chain reaction
RUNX2	runt-related transcription factor 2
TGF-β	transforming growth factor-beta
TIMP-1	tissue inhibitor metalloproteinases
TNF	tumor necrosis factor
TNFRSF11B	tumor necrosis factor receptor superfamily member 11B
TRAF	TNF receptor-associated factor
TRAP	tartrate resistant acid phosphatase

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Chapter 1 – Introduction

1.1 Orthodontic tooth movement:

Orthodontics is the dental specialty that addresses the diagnosis, prevention, and correction of malpositioned teeth and jaws[1]. According to the Canadian Association of Orthodontists, the purpose of orthodontic treatment includes the "application and control of corrective appliances to bring teeth, lips and jaws into proper alignment and achieve facial balance"[2]. A stable bite can not only improve the patient's self-esteem but also improve multiple functional aspects of their life including improved sleep, mastication, swallowing and breakthing[1]. Figure 1 depicts an example of the results possible from comprehensive orthodontic treatment.

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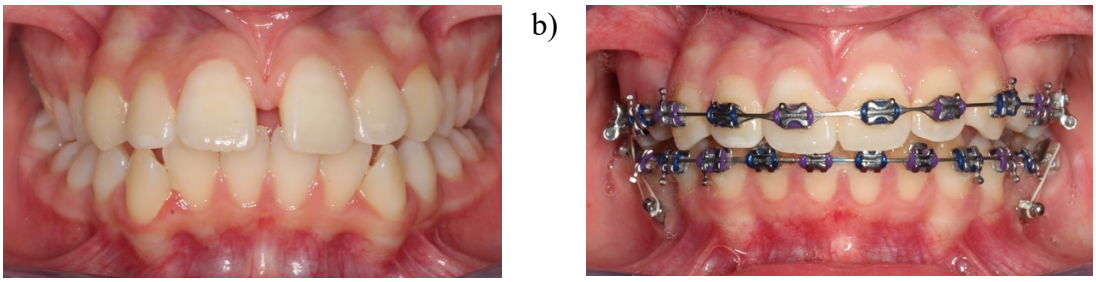

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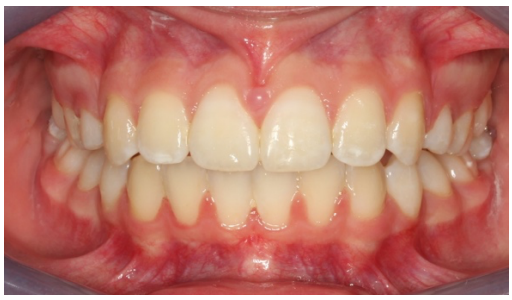


Figure 1: Example of results possible from comprehensive orthodontic treatment.

This patient had comprehensive orthodontic treatment at the Western University Graduate Orthodontics Clinic and their initial (a), progress (b) and final (c) photographs are shown. Their treatment plan included upper and lower orthodontic appliances, dental unit extractions and temporary anchorage devices.

Tooth movement is just one of the tools used to address dental malocclusion. When a prolonged force or mechanical stimuli is applied to a tooth, this initiates a series of complex cellular signalling processes within the periodontal ligament (PDL) that lead to tissue and bone remodeling around the tooth and eventual orthodontic tooth movement (OTM)[3].

In general, OTM occurs in three phases[1]. The first phase involves initial tooth movement 1-2 days after orthodontic force application accompanied by alterations in blood flow associated with tension sites (where blood vessels are stretched) and compression sites (where blood vessels are crushed) within the PDL. The second phase occurs 20 to 30 days after orthodontic force application, where there is a buildup of hyalinization and tissue necrosis in the compressed areas of the PDL that causes OTM to cease. Once necrotic tissue has been removed, the final phase begins and OTM resumes.

Three different models have been suggested to describe OTM. First, the "Pressure-Tension Theory" of orthodontic tooth movement is the most widely accepted model for OTM and a diagrammatic representation is seen in Figure 2. This theory states that tooth movement is the sum of inducing a tensile force in the PDL on one side of the tooth leading to bone deposition via osteoblasts and a compressive force on the other side of the tooth leading to bone resorption via osteoclasts[4]. However, the Finite Element Model developed by Cattaneo *et al.* showed that OTM could not be a result of symmetric compressive/tensile stress distribution within the PDL[5,6].

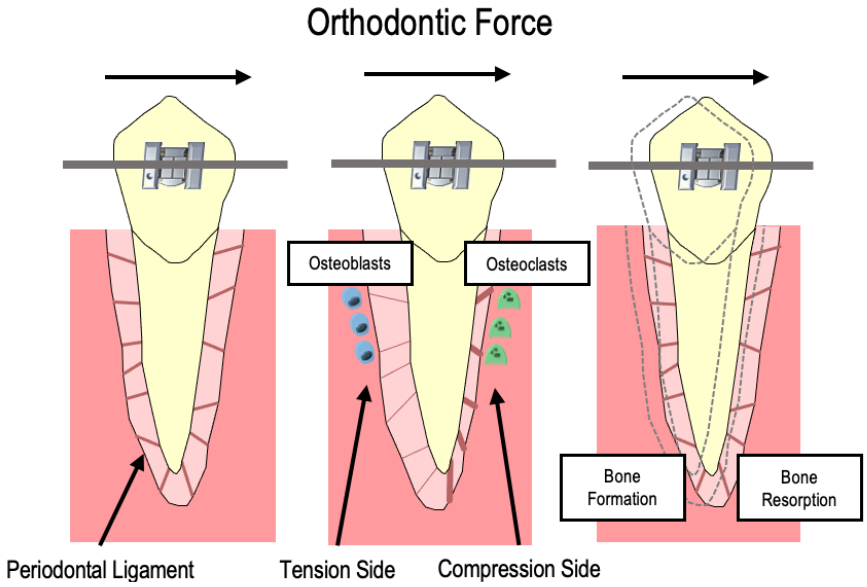


Figure 2: Diagrammatic representation of the Pressure-Tension theory.

The Pressure-tension theory is the most commonly accepted theory for OTM. Tooth movement is the sum of inducing a tensile force in the PDL on one side of the tooth leading to bone deposition (osteoblasts) and a compressive force on the other side of the tooth leading to bone resorption (osteoclasts). Given the differences in the microenvironment between the tension and compression sides, this can lead to differential gene expression within the PDL during OTM.

Secondly, the "Alveolar Bending Hypothesis" agrees that the PDL has a role in OTM but describes the importance of deformation within the alveolar bone as well[7]. By performing tooth movements in isotope-labeled mice, it was determined that the alveolar bone tooth socket acts like a cantilever for the tooth leading to a free end (crown of the tooth) and a fixed end (root of the tooth). Rather than the symmetric movement as described in the first theory, when an orthodontic force is applied, the free crown is displaced more than the fixed root leading to a bending of the alveolar bone lining the tooth socket[6,7].

Finally, the "Stretched Fibre Hypothesis" attempts to relate orthodontic tooth movement with orthopedic bone remodelling[8]. This hypothesis is based on the orthopedic mechanostat

theory that states that a low force applied to bone leads to bone resorption while a high force applied to bone leads to bone formation[9]. Tooth movement is thus due to the elastic fibers in the PDL that are stretched when under tensile forces (leading to a high force and bone formation) and squeezed when under compressive forces (leading to a low force and bone resorption).

While each of these hypotheses is based on a slightly different principle, there is one feature that is shared – the importance of the PDL. Ankylosed teeth – meaning teeth that lack a PDL – are not moved by orthodontic forces, which enforces the role and importance of the PDL and PDL-derived cells in OTM[4,10].

1.2 The Periodontal Ligament

Located between the root of a tooth and the inner wall of the alveolar bone within the tooth socket, the PDL is a vascular, highly fibrous, collagen-rich connective tissue that acts to protect a tooth against stresses during mastication and occlusion[11]. Not only does the PDL provide mechanical support against stress, it also contains several sensory and vascular cells that can modulate cell activity in response to various stimuli[12]. For example, when orthodontic appliances are activated at the level of the crown of the tooth, the forces are transmitted through the PDL to the alveolar bone, triggering a cascade of various chemical signalling molecules such as prostaglandins and cytokines, ultimately leading to activation of cells that mediate tissue and bone remodelling[1]. As a result, an in depth understanding of the components of the PDL is essential to evaluate orthodontic tooth movement.

1.2.1 PDL Development

The PDL is of mesenchymal origin, specifically the transient neural crest cells (NCCs)[13]. NCCs are first seen during embryonic neural tube closure and migrate to the craniofacial region[14]. Once in the cranial region, NCCs begin to proliferate and are termed neural crest derived-mesenchymal cells or ectodermal mesenchymal cells, where they are involved in the development of cranial facial bones, neural tissue and dental organs such as the dental

follicle, pulp, dentin and periodontium[14]. Specifically, odontogenic epithelium invaginates odontogenic mesenchymal tissue, which is the beginning of the start of the classic three-step tooth development pathway[13]:

(1) Bud stage involves dental cells condensing to form a bud shape, known as the enamel organ,

(2) Cap stage follows as further proliferation and agglutination of cell mass,

(3) Bell stage involves histodifferentiation and morphodifferentiation where there is specific development of dental tissues such as enamel and dentin[15].

Periodontal tissue begins to form during the cap stage and enamel matrix proteins such as amelogenin (secreted from the enamel epithelium) trigger the beginning of the bell stage[13]. During root development, collagen fibers (laid down by fibroblasts) are initially laid down fairly loosely in a direction parallel to the root and as the tooth erupts and makes final occlusal contact, these PDL fibers re-orient such that they become horizontally arranged within the coronal third of the root, obliquely arranged within the middle third of the root and arranged apically from the cementum surface to the alveolar bone in the apical third[17]. The terminal point of insertion of these collage fibers (whether on the tooth cementum side or on the alveolar bone side) occurs as Sharpey's fibers[17].

1.2.2 PDL Cells

1.2.2.1 Stem cells

The healthy PDL contains many different types of cell populations including fibroblasts, endothelial cells, epithelial cell rests of Malassez, cementoblasts, sensory cells and bone-associated cells (each described in turn below)[12]. Additionally, the PDL contains populations of undifferentiated mesenchymal stem cells (MSCs), also known as dental follicle stem cells.

MSCs have the ability to give rise to mature cell types that have characteristic morphologies and specialized functions[18]. In the case of the PDL, these cells include osteoblasts, cementoblasts and fibroblasts[18]. MSCs are of mesoderm origin and have the ability for self-renewal[19]. Within the PDL, various growth factors and signalling molecules will trigger an MSC to differentiate into a specific progenitor cell, namely pre-osteoblast, pre-cementoblast or a pre-fibroblast, prior to becoming committed to differentiating along their particular cellular developmental pathway to become an osteoblast, cementoblast or fibroblast[19]. Given the potential for self-renewal, there is much ongoing research evaluating the use of MSCs for regeneration of the PDL in the face of periodontal disease and re-establishment of the attachment apparatus[20].

1.2.2.2 Fibroblasts

The most abundant cell found within the PDL are fibroblasts, and they constitute ~50-60% of the total PDL cell composition[21]. These highly active cells are responsible for maintenance of the PDL and continuously remodel the collagen fibers[18]. In rodent molars, it has been shown that fibroblasts are in direct contact and communicate with neighboring fibroblasts, which facilitates electrical coupling and chemical signalling[12,22]. Within the PDL, fibroblasts are oriented parallel to the collagen fibers and apicocoronal to oxytalan fibers[23]. This fibroblast-oxytalan fiber complex adds to the elastic properties of the PDL and may also help keep blood vessels open under loading[12].

Interestingly, fibroblasts have also been shown to have the ability to produce the enzyme alkaline phosphatase (ALP)[12,24]. This membrane-bound enzyme plays a key role in phosphate metabolism, specifically in the mineralization and calcification of hard tissues (such as bone) and is a biomarker of osteoblast activity[12,25]. Therefore, it may be speculated that fibroblasts within the PDL may also have an “osteoblastic” function, on top of their role in collagen maintenance[26].

1.2.2.3 Endothelial cells

The PDL contains a well-defined vascular system originating from alveolar bone and gingiva and consist of two layers[27]:

(1) The inner layer containing capillaries that supply the PDL cells with oxygen and nutrients;

(2) The outer layer containing larger vessels that play a role in absorbing forces[27,28].

PDL endothelial cells can promote the proliferation of periodontal cells and thus function in the regeneration of the PDL[27,29].

1.2.2.4 Epithelial cell rests of Malassez

During the development of the periodontium, there is a collection of epithelial cells – Hertwig epithelial root sheath (HERS) or Epithelial Root Sheath – located on the cervical loop of the enamel organ[13]. By initiating the differentiation of odontoblasts from the dental papilla, the HERS cells are responsible for dentin formation within the root of a developing tooth[13]. Eventually, the HERS will disintegrate as the PDL forms, but some pieces do not completely resolve and remain within the PDL as epithelial cell rests of Malassez[30].

In addition to helping maintain PDL width, epithelial cell rests of Malassez may also play a role in cementum regeneration and repair, and they have been implicated in development of cysts such as the gingival or periodontal cyst[30,31].

1.2.2.5 Sensory Cells

The main sensory cells within the PDL originate from the trigeminal ganglia and these neurons are concentrated mainly in the cervical and middle root areas[32]. The PDL continuously receives a variety of sensory and mechanical input throughout the day such

as occlusal forces, masticatory forces and nociceptive input which is then sent from the tooth to the central nervous system[32].

Mechanoreceptors at the neuronal endings in the PDL convert this mechanical stimulus into a biological response, and input from the periodontal mechanoreceptors can influence a variety of reflex systems within the orofacial region such as unconscious sensory feedback during mastication and proprioceptive information regarding bite force[32]. These mechanoreceptors become very important in the face of orthodontic forces as orthodontic tooth movement alters their signalling state and leads to an inflammatory response with the release of inflammatory neuropeptides such as substance P and calcitonin Gene-related peptides[32–34].

These signalling molecules then interact with PDL endothelial cells which in turn leads to the activation, migration and adherence of leukocytes[32]. Cytokines, growth factors and colony-stimulating factors that are then secreted by the leukocytes trigger the cell-signalling cascade required to remodel bone and other dental tissue[32,35].

1.2.2.6 Cementoblasts

Cementoblasts are the ectomesenchymal cells responsible for forming the cellular cementum around the apical two thirds of the root of a tooth[36]. Progenitor cementoblast cells can be found within the PDL and are triggered to become cementoblasts in the face of periodontal tissue reconstruction and repair[37].

Note that cementoclasts (cells responsible for the breakdown of cementum) have been shown to mediate the apical external root resorption commonly seen in orthodontically moved teeth[38]. Consequently, during cementum repair, local cytokines and growth factors such as insulin-like growth factor-1, fibroblast growth factor, epidermal growth factor, bone morphogenic proteins (BMP) and transforming growth factor-beta (TGF-β) trigger cementoblast precursors in the PDL to become mature cementoblasts which promote cementogenesis[39,40].

1.2.2.7 Bone cells

While there are multiple hypotheses describing orthodontic tooth movement, the most widely accepted hypothesis is the "Pressure-Tension Theory"[1]. On the side of the tooth experiencing tension, cell signalling pathways within the PDL are induced that lead to activation of osteoblasts (cells that build up bone) while on the side of the tooth experiencing compression, different cell signalling pathways are induced that lead to activation of osteoclasts (cells that break down bone)[1]. Each of these bone-related cells are discussed in detail below.

1.2.2.7.1 Osteoblasts

Osteoblasts are specialized mononucleated cells of mesenchymal origin that are responsible for bone apposition[41]. Osteoblasts are similar in function to fibroblasts in that they both are capable of synthesizing type I collagen; however, osteoblasts distinguish themselves from fibroblasts in that they express a core-binding factor subunit alpha-1 (Cbfa1) and Osteocalcin[41,42]. Cbfa1 – also known as Runt-related Transcription Factor 2 (RUNX2) is a transcription factor which acts as a key regulator of osteoblast differentiation and function[42,43]. A study by Komori *et al.* showed that there is complete lack of bone formation in Cbfa1-deficient mice which demonstrates the importance of Cbfa1for osteoblast driven ossification[42,44]. Specifically, one of the roles of Cbfa1 is to regulate the expression of Osteocalcin (OCN), which is a gene only expressed in terminally differentiated osteoblasts[42].

In addition to Cbfa1 and OCN, osteoblasts rely on various other factors and proteins for activation and maturation. For example, BMPs have been shown to commit mesenchymal progenitor cells to become osteoblasts as well as stimulating the maturation of committed osteoblasts[45,46]. Furthermore, PTH controls the expression of BMPs by activating the extracellular signal-related protein kinase (Erk) 1/2 and nuclear factor (NF)-κB signaling pathway[47,48].

TGF-β is a superfamily of cytokines that regulates bone and cartilage proliferation, maturation and homeostasis. It is deposited in bone by osteoblasts during bone formation and is released from bone during osteoclastic activity[49,50]. By inhibiting TGF-β in transgenic mice, Filvaroff *et al.,* showed that osteocyte (mature osteoblasts) density was reduced and there was an imbalance between bone formation and resorption during remodelling[51]. Furthermore, cultured osteoblast-like cells have a significant reduction in mobility after treatment with TGF-β_1, which is a characteristic of mature bone cells[52]. Other critical factors for osteoblast development include the Wnt signaling pathways, insulin-like growth factors (IGFs), osterix and fibroblast growth factor[53].

As mentioned previously, mature osteoblasts are responsible for bone apposition and do so by expressing and secreting type I collagen, ALP and OCN[29]. Recall that ALP is a glycoprotein and enzyme found within the gingival crevicular fluid (GCF) of periodontal tissue that releases phosphate ions from organic phosphate leading to the precipitation of calcium phosphate salt[54]. During OTM, there is an increase in GCF ALP which peaks at about 1 to 3 weeks of force application and as such, measurement of ALP has been considered as a marker for bone turnover in OTM[55,56]. Interestingly, Parinetti *et al.* (2002) demonstrated that ALP activity was temporally and spatially regulated in tension and compression zones during OTM[57].

OCN – or bone gamma-carboxyglutamic acid-containing protein (BGCAP) – is a small, non-collagenous, protein exclusively found in osteoblasts that is necessary for the formation and mineralization of osteoid[42,43,58]. Studies using an osteocalcin knock-out in mice initially revealed a potential role for osteocalcin as an inhibitor of bone maturation as these null mice had an increased mineral to matrix ratio[59]. However, more recent studies have shown that OCN binds to hydroxyapatite and forms a complex with collagen through osteopontin (a matrix protein)[60]. While the exact mechanism of action of osteopontin has not yet been elucidated, it is known that osteocalcin can directly bind calcium and concentrates in bone and as such has been used as a marker for bone formation[61].

1.2.2.7.2 Osteocytes

The type I collagen produced by osteoblasts eventually forms an organized organic matrix with proteoglycans and glycoproteins now termed osteoid[62]. Bone formation and maturation continues with deposition of calcium phosphate, which mineralizes the osteoid[63]. During this process, osteoblasts become terminally differentiated into osteocytes, which are then incorporated into the mineralized osteoid[64].

Osteocytes reside within lacunae and have long cytoplasmic extensions that transverse canaliculi for which exchange of nutrients and waste can occur through gap junctions (transmembrane channels)[62,65]. Of note, these gap junctions may be involved in bone response to mechanical strain – such as OTM – as mechanical forces have been shown to cause fluid flow through these channels[66].

1.2.2.7.3 Osteoclasts

Contrary to osteoblasts, which are of mesenchymal origin, osteoclasts are multinucleated cells from the monocyte/macrophage lineage[67]. It has been shown that osteoclast differentiation is supported by osteoblasts, reinforcing that there is balance between bone resorption and bone apposition[68,69].

The signalling pathways that trigger osteoclasts to commence differentiating from precursor cells are complex and involve various factors such as 1a,25-dihydroxyvitamin D3 and PTH, which leads to the production of receptor activator of NF-kB ligand (RANKL)[70,71]. RANKL binds to receptor activator of NF-kB (RANK) on osteoclast precursor cells which triggers a cell-signalling cascade[71,72].

This cascade begins with the recruitment of TNF receptor-associated factor (TRAF) family proteins that then activate NF-kB and mitogen-activated protein kinase (MAPKs) which subsequently activates transcription factors required for osteoclast differentiation (c-fos, PU.1, NFATc1)[72]. The RANK-RANKL system is essential for osteoclast regulation as

disruption of either RANK or RANLK results in osteopetrosis (abnormally dense bone) due to impaired osteoclast differentiation[73,74].

Once mature, various interleukins (IL-1β, IL-6) and Prostaglandin E_2 (PGE_2) signal osteoclasts to start resorbing bone[75]. Activated osteoclasts are found within Howship's lacunae, which are pits in the surface of the bone they are resorbing[67]. Here, the osteoclasts create an acidic microenvironment and produce vacuoles filled with various enzymes required to break down the collagen within bone (demineralization) such as tartrate resistant acid phosphatase (TRAP), matrix metalloproteinases (MMPs) and Cathepskin K (which breaks down collagen within bone)[67,76]. At the site of bone resorption, osteoclasts form a specialized ruffled border to increase surface area for maximum secretion of these bone-resorbing proteases[67,76].

Another connection between osteoclast and osteoblast function is osteoprotegerin (OPG). OPG – also known as osteoclastogenesis inhibitory factor (OCIF) or tumor necrosis factor receptor super family member 11B (TNFRSF11B) – is largely expressed by osteoblasts and acts as a decoy for RANKL[77,78]. OPG binds to RANKL preventing RANKL from binding to its receptor, RANK, eventually leading to reduction of osteoclast function and subsequent apoptosis[79]. Of note, there has been some research in using recombinant OPG or other inhibitors of RANKL as a means to block osteoclast function in the face of OTM to provide additional orthodontic anchorage[80].

1.3 PDL Extracellular Matrix (ECM)

The main structural component of the PDL is collagen that is organized into cross-banded fibrils[12]. Specialized Type I and III collagen fibers (Sharpey's fibers) firmly attach the periosteum of the alveolar bone and the cementum on the root of the tooth[12,81]. Note that Type V, VI and XII collagen can also be found associated with, buried within or found in the spaces between main Sharpey's fibers fibril bundles[81–84]. In addition to providing structural support for the PDL, collagen can mediate cell-signalling events and provide substrates for cell adhesion [85,86].

Another fiber type present in the PDL are oxytalan fibers. As mentioned previously, these fibers help protect blood vessels by keeping them patent during loading and typically run from the cementum to outside of the blood vessels[12].

In addition to various fibers, the PDL ECM is also comprised of various proteoglycans and glycoproteins.

Proteoglycans (PG) are comprised of a core protein with one or more highly anionic glycosaminoglycans (GAG). They are present in virtually all connective tissue extracellular matrices and as such, it is no surprise that PGs are found within the PDL[87]. Given the highly anionic GAGs, PGs help provide hydration to the ECM by attracting cations (such as sodium, potassium, calcium) and water, which in turn helps cushion and protects against compressive forces, provides lubrication and nutrient delivery[88].

Glycoproteins, such as undulin, tenascin and fibronectin, are non-collagenous proteins that contain a covalently bonded glycan (oligosaccharide chain) and can also be found within the PDL ECM[89]. These molecules play a role in ECM organization and signal transduction[89]. Undulin is localized between densely packed mature collagen fibrils and likely plays a role in the supramolecular and functional organization of collagen fibrils into flexible bundles thus allowing for limited gliding between individual collagen fibrils[90,91]. Contrarily, tenascin is localized between loosely packed collagen fibres and is expressed during development, disease or injury[91]. Fibronectin is more broadly distributed than both undulin and tenascin, and it shows a close association with type I and III collagen and can bind fibroblasts[92]. Additionally, it has been shown that tenascin can interact with fibronectin thus reducing its ability to bind fibroblasts[93].

More recently, a novel glycoprotein classified as a matricellular protein family member – Periostin (PSTN) – was found to be expressed by fibroblasts in the periodontal ligament and osteoblasts in the alveolar bone and is responsible for the maturation, stability and cell signalling within the PDL[94–96]. Additionally, periostin has been implicated in a variety of

disease processes such as tumorigenesis and metastasis, cardiac repair, diabetes and obesity, bronchial asthma as well as wound healing[97–101].

Relating to the field of orthodontics, this 90-kDa extracellular matrix protein has been specifically implicated in promoting tooth movement during orthodontic treatment[94]. Using cultured human periodontal ligament (hPDL) cells, Xu *et al.* determined that periostin expression increases when under orthodontic forces[94]. This same research group also determined that periostin expression levels increase in murine periodontal ligaments during OTM[94].

Additional research has shown that PSTN is upregulated specifically in the compression zone of the PDL compared to that within the tension zones of the PDL and that mice lacking the periostin gene have reduced rates of OTM[95,102]. Note that expression of periostin did increase in both the compression and tension zones, however PSTN increased to a greater degree in the zone of compression relative to the zone of tension[95,102]. If periostin is differentially expressed in compression versus tension zones, it is important to discuss other proteins/products that are differentially and spatially regulated during OTM.

Table 1 summarizes the components of the PDL ECM.

Table 1: Summary of PDL ECM components.

ECM Component	Function	
Fibroblasts	Most abundant cell in the PDL. Continuously remodels collagen[21].	
Collagen Type I & III	Sharpey's fibers. Core fibril of the PDL ECM. Connect periosteum to bone[12].	
Collagen Type V	Minor collagen associated with the Sharpey's fibers. Is found buried within the core or found in between the spaces of the core fibrils[12].	
Collagen Type VI & XII	Minor collagen. Help contribute to the construction of the 3-dimensional fibril arrangement[12].	
Oxytalan Fibers	Protection of blood vessel integrity while under loading[23].	
Proteoglycans	Helps provide hydration to the ECM by attracting cations[88].	
Glycoproteins:	**Undulin**	Localized between densely packed mature collagen fibrils. Likely plays a role in the functional organization of collagen fibrils[89].
	Tenascin	Localized between loosely packed collagen fibrils. Likely involved in wound healing[89].
	Fibronectin	Broadly distributed. Closely associated with type I and III collagen[92].
	Periostin	Implicated in a variety of disease processes such as tumorigenesis and metastasis, cardiac repair, diabetes and obesity, bronchial asthma as well as wound healing[94].

1.4 Gene expression during OTM:

As mentioned previously, "Pressure-Tension Theory" of OTM states that tooth movement is the sum of inducing a tensile force in the PDL on one side of the tooth leading to bone deposition, and a compressive force on the other side of the tooth leading to bone resorption[4]. Within minutes of orthodontic force application, blood vessels within the PDL are dilated on the tension side and partially compressed on the pressure side[1].

This changes the blood flow to those areas, leading to changes in oxygen tension and release of prostaglandins and cytokines and if force is sustained, secondary cellular messengers such as cyclic adenosine monophosphate (cAMP) are upregulated by 4 hours[1]. cAMP then goes onto regulating differentiation of osteoclasts and osteoblasts as well as

leading to multiple downstream effects such as production of mediators related to inflammation[4,103]. As can be imagined, given the differences in the microenvironment between the tension and compression side, this can lead to differential gene expression within the PDL.

By applying compressive force to human PDL-derived fibroblasts for 6 days, Rankovic *et al.,* was able to evaluate the temporal expression of various genes related to mechanosensing (cFOS, HB-GAM), inflammation (COX2, IL-6, TNF-α), bone remodelling (RUNX2, P2RX7)[103]. For this experiment, human PDL cells were isolated from the roots of extracted teeth, cultured *in vitro* and compressed using a glass cylinder for varying amounts of time[103,104]. Next, total mRNA was isolated and quantitative RT-PCR was performed in order to evaluate relative amounts of expressed of the genes listed above[103].

In summary the above article showed that when under compressive forces cFOS was up-regulated; HB-GAM expression was only up-regulated on day 5 of compression; IL-6 and COX2 reached maximum expression after 3 days of compression; TNF-α was overall up-regulated except on day 2 of compression; P2RX7 was down-regulated on days 2, 4, 5, 6 of compression; and RUNX2 was down-regulated on days 2 and 5 of compression[103].

Complementing the data concerning gene expression in PDL cells under compressive force, another research group evaluated gene expression with PDL cells under tensile force[105]. For this, human subjects were treated with a palatal expander after which premolars were extracted immediately after expander removal. By performing RT-qPCR on PDL cells isolated from the palatal side of the extracted tooth, this gave researchers a sampling of the mRNA in cells exposed to tensile force. It was determined that there is increased expression of IL-10, tissue inhibitor metalloproteinases (TIMP-1), COL-I, osteoprotegerin, and osteocalcin which all exhibit anti-inflammatory properties[105].

Interestingly, this group also investigated gene expression while under compressive force as well by evaluating PDL cells on the labial side of the extracted tooth and revealed higher

expression of pro-inflammatory cytokines such as NFκB, RANKL and MMPs which corroborates the data by Rankovic *et al.,* that also showed up-regulation of pro-inflammatory cytokines.

A limitation to both these studies is that a set of pre-selected genes to evaluate during compressive or tensile forces were used; however, it can be imagined that there are many other genes also expressed during orthodontic tooth movement that were not evaluated. More specifically, much of the research presented involves looking at gene expression specifically within the PDL during OTM, but perhaps there are other factors at play, such as tooth root-specific factors such as those involving the cementum.

Table 2 summarizes gene expression during OTM.

Table 2: Summary of components involved in gene expression during OTM.

Gene	Gene Name	Role in OTM
cFOS	Fos proto-oncogene, AP-1 transcription factor subunit	Involved in PDL mechanosensing[103].
COX2	Cyclooxygenase-2	Enzyme involved in the inflammatory cascade[103].
HB-GAM	Heparin-binding growth-associated molecule, Pleiotrophin	Involved in PDL mechanosensing[103].
IL-6	Interleukin-6	Pro-inflammatory cytokine[103].
IL-10	Interleukin-10	Anti-inflammatory cytokine[105].
MMPs	Matrix metalloproteinases	Protease that degrades extracellular matrix components such as collagen. Involved in destructive periodontal diseases[106].
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells	Pro-inflammatory cytokine[67].
OCN	Osteocalcin	Only expressed in terminally differentiated osteoblast. Binds calcium directly. Biomarker of bone formation[58].
OPG	Osteoprotegerin	Expressed by osteoblasts. Acts as a decoy ligand for RANK, blocking RANK-L therefore reducing osteoclast differentiation and activation[105].
P2RX7	P2X purinoceptor 7	ATP-dependent ion channel. Plays a role in bone remodeling and inflammation. Mediates the release of pro-inflammatory mediators that are involved in the regulation of bone homeostasis[107].
RANK	Receptor activator of nuclear factor- κB	Receptor for the RANK-L. Part of the pathway that regulates osteoclast differentiation and activation[105].
RANK-L	Receptor activator of nuclear factor-κB ligand	Ligand for RANK. Part of the pathway that regulates osteoclast differentiation and activation[105].
RUNX2	Runt-related transcription factor 2	Key transcription factor associated with osteoblast differentiation[79].
TIMP-1	Tissue inhibitor of metalloproteinase-1	Non-specifically inhibits almost every member of the MMP family. Plays a physiologic role in the turnover of periodontal tissue[105].
TNF-α	Tumor necrosis factor-α	Pro-Inflammatory cytokine[103].

1.5 Cementum repair during OTM:

As described previously, OTM leads to an area of reduced oxygen tension within the compression side of the PDL, which then leads to a localized area of ischemia within the PDL itself, and the adjacent alveolar bone[108]. This leads to an area of necrosis/hyalinization which must eventually be removed by osteoclasts/cementoclasts, potentially leading to collateral damage of the superficial layer of acellular cementum in that same area[109]. Cementoclasts resorb cementum and have a similar structure as osteoclasts in that they are multi-nucleated, have ruffled borders and produce TRAP[110]. However, unlike osteoclasts, the molecular pathways that drive their differentiation and activity are not as well understood[40].

Acellular cementum resorbed by cementoclasts can be replaced with new cellular cementum from cementoblasts and depending if the balance in cementoclast/cementoblast activity is unfavourably skewed in favour of cementoclasts, external apical root resorption (EARR) can result[40]. While approximately 80% of patients experience EARR during OTM (particularly on maxillary incisors), the affected cementum is typically repaired to an acceptable degree with cementoblast-derived cellular cementum[40,111]. However, in approximately 5% of EARR cases, there is more than 5mm of apical root resorption and EARR is considered to be severe[111,112]. In these instances, the underlying dentin is affected and there is permanent loss of root length, potentially increasing the risk of tooth loss[1].

Determining which patients are at an increased risk for severe EARR is complicated given the number of intrinsic genetic factors, polymorphisms in gene expression, local anatomical factors and hormonal differences that all play a role in risk assessment[40]. For example, Sharab *et al.,* 2014 determined that a specific genotype of P2RX7 (SNP rs208294) was significantly associated with EARR demonstrating a genetic susceptibility severe resorption[112]. From this and other research, it is clear that reducing the risk of EARR is multifactorial and involves maintaining a proper balance of cementum resorption and deposition during OTM.

Recently, molecular cloning, bacterial expression and immunolocalization identified a novel human cementum-derived protein (CP-23) that is expressed by cementoblasts and subpopulations of PDL cells[113]. This glycosylated, phosphorylated and thermostable protein – also known as cementum protein 1 (CEMP-1) – is localized to the root of a tooth as well as paravascularly to the blood vessels within the PDL and is proposed to be a candidate for a PDL-cementum specific marker[113,114].

Research has shown that CEMP-1 promotes attachment of PDL fibroblasts, is implicated in regulating HAP crystal deposition and can increase the expression proteins implicated in cementogenesis such as ALP, osteopontin, BSP and OCN[115–118]. Additionally, another research group used immunocytochemical analysis to evaluate the effects of CEMP-1 over-expression in human periodontal cells retrieved from extracted teeth with clinically healthy periodontium and determined that CEMP-1 enhances cementoblast differentiation and attenuated osteoblastic phenotypes[119].

This is a key finding in the field of periodontal disease and OTM-related EARR as perhaps CEMP-1 may activate cementoblast progenitors to promote cementogenesis and could thus skew the balance of cementoblasts/cementoclasts in favour of cementogenesis.

Chapter 2 – Rationale, Hypothesis and Objectives of the Research

2.1 Rationale

CEMP-1 is a protein involved in cementogenesis that is expressed by cementoblasts and subpopulations of PDL cells; however, unlike other periodontal proteins/elements (such as periostin) that are found both in humans and in an animal experimental model, CEMP-1 is only found in humans[120,121].

Because CEMP-1 is only found in humans, this makes studying its role in OTM more challenging as PDL cells must be retrieved from a human source rather than using a well-established murine cell line (for example). Given the challenges of studying a protein only found in human cells combined with the relatively recent discovery of CEMP-1, much less is currently known about this protein, making it a prime target for further research.

2.2 Hypothesis

Given that OTM involves replacement of resorbed acellular cementum with new cellular cementum, perhaps CEMP-1 is also involved in the complex process of OTM. Specifically, it is proposed that CEMP-1 might be differentially regulated under tensile and compressive forces – such as those seen in OTM[95,102].

2.3 Objectives

2.3.1 To establish a technique to apply tension to human PDL cells in a 3-Dimensional collagen gel.

Knowing that genes are differentially expressed in zones of compression and zones of tension, a protocol was developed to place human PDL cells isolated from extracted teeth into a collagen gel and cyclically stretched using the FlexCell®FX-5000 Tension system

to apply a tensile force similar to what would be experienced in OTM. Note that previous experiments evaluating gene expression in PDL cells often use a 2-dimensional flat plate for experimentation; however, the PDL *in vivo* is 3-dimensional[94]. Accordingly, the development of this protocol involved plating hPDL cells in a 3-dimensional collagen gel to better approximate the condition seen *in vivo*.

Also, this portion of the experiment can be considered one-sided with regards to the overarching goal of differential gene expression during OTM as this experiment only focuses on the tension component. Another experimental protocol will need to be developed in the future focusing specifically on the compression component.

To confirm validity of the tension-applying protocol and ensure the hPDLs were still viable, fluorescent microscopy was completed. Given that Oortgiesen, *et al.,* demonstrated that PDL fibroblasts align perpendicular to the force of application, it is hypothesized that when hPDL cells have been exposed to tension, they will also appear aligned perpendicular to the direction of force application whereas cells that have not been exposed to tension will be more randomly dispersed[122].

2.3.2 To evaluate CEMP-1 gene expression during cyclical tensile force.

Using the FlexCell®FX-5000 Tension system and the protocol established in Objective 1, tension forces were applied to the hPDL cells for various amounts of time at various force levels. Once the mRNA was isolated, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) was used to look specifically for CEMP-1 expression.

It is hypothesized that CEMP-1 will be up-regulated when under tensile force, thus providing a cementum-protective effect or perhaps promoting cementogenesis (mirroring how osteoblasts promote bone apposition on the tension side). If CEMP-1 does have a cementum-protective effect, perhaps this could be a novel target to evaluate which patients

might be at higher risk for EARR during OTM by skewing the balance in favour of cementogenesis over cementum resorption.

Note that other genes with known and expected responses to tension were also evaluated at the same time as CEMP-1 as further confirmation of the validity of the FlexCell®FX-5000 Tension system protocol. As previously mentioned, hPDL-derived Periostin activity is known to increase in the zone of tension (recall that an increase is seen both in the zone of tension and zone of compression during OTM but it increases more in the zone of compression relative to the zone of tension)[95,102].

It is also known that similar to Periostin, hPDL-derived bone sialoprotein (BSP) (a component of mineralized tissue such as bone, dentin, cementum and calcified cartilage) also increases with the application of tensile force[123].

Contrary to Periostin, hPDL-derived alkaline phosphatase (ALP) (a protein enzyme involved in mineralization and calcification of hard tissue and is a biomarker of osteoblast activity) is known to be modulated by orthodontic forces and has been shown to decrease with the application of tension[54].

Chapter 3 – Materials and Methods

3.1 Materials and Solutions

Dulbecco's Modified Eagle Medium (DMEM), CO_2-Independent Medium, heat-inactivated fetal bovine serum (FBS), antibiotic solution (100U/ml penicillin and 100 µg/ml streptomycin), trypsin solution and phosphate buffered saline (PBS) were obtained from GIBCO (Life Technologies Inc., Burlington, ON, Canada). TRIzol reagent, UltraPure distilled water (DNase/RNase-free), Rhodamine phalloidin (lot 2053476) and Hoechst 33342, Trihydrochloride, Trihydrate, 10 mg/mL Solution (lot 1830258) were obtained from Invitrogen (Life Technologies Inc., Burlington, ON, Canada). Collagen (Nutragen® Type 1 Bovine Collagen Solution 6mg/mL) was obtained from Advanced Biomatrix (Cedarlane, Burlington, ON, Canada). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), paraformaldehyde, bovine serum albumin (BSA) and Triton™ X-100 were obtained from Sigma-Aldrich (Sigma-Aldrich Canada, Oakville, ON, Canada). Linear TissueTrain® sterile cell culture plates and the FlexCell®FX-5000 Tension system was obtained from FlexCell® International Corporation (Burlington, NC, United States). RNeasy Mini Kit was obtained from QIAGEN (Toronto, ON, Canada). iTaq Universal Probes One-step Kit and 2xiTaq™ Universal probes one-step reaction mix was obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). Probes for CEMP-1 (Hs04185363_s1, lot P210623-Q00), BSP (Hs00913377_m1, lot 188351), ALP (Hs0129144_m1, lot 1967391), PSTN (Hs01566750_m1, lot 1472190) and 18S rRNA ((DQ) Oligo mix, lot 1907042) were obtained from Applied Biosystems (Life Technologies Inc., Burlington, ON, Canada).

3.2 Cell Culture

Healthy periodontal ligament was obtained after routine extraction of third molars and premolars in the Oral Surgery Clinic at The University of Western Ontario. Each patient had no signs of inflammation in the periodontal tissues (no bleeding, probing depth < 3mm, no visible plaque). Explant cultures were initiated to derive the fibroblastic cells as

previously described by Silverio-Ruiz *et al.,* 2007[124]. Procedures involving human tissue were approved by the Western University Review Board for Health Sciences Research Involving Human Subjects and are in accordance with the 1964 Declaration of Helsinki. All tissues were collected with consent from patients and with approval from the Western University Health Science Research Ethics Board.

For fluorescent microscopy, hPDL cells isolated from a 43-year-old female (43F, originally preserved at P1 on 27.08.18) were used.

For mRNA isolation and RTqPCR, hPDL cells isolated from a 28-year-old male (28M, originally preserved at P3 on 14.08.18), a 38-year-old-female (38F, originally preserved at P1 on 24.08.18) and a 31-year-old male (31M, originally preserved at P3 on 01.07.20) were used.

The hPDL cells were maintained every 72 hours using DMEM supplemented with 10% FBS and 1% antibiotic solution at 37°C in a humidified atmosphere of 5% CO_2. The cells were washed using PBS, dissociated from the surface of the culture flask using 0.25% trypsin and split/resuspended for future experiments. Primary cells were passaged at least four times prior to using for any experimentation.

3.3 TissueTrain®

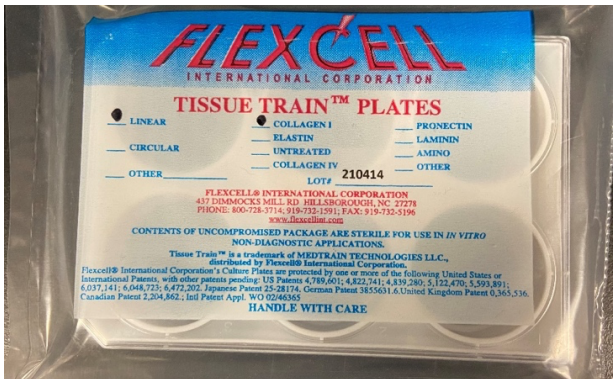
Once hPDL cells reached at least their fourth passage, hPDL cells were seeded into TissueTrain® 3D culture system plates (Figure 3a) at a concentration of 75,000 cells/TissueTrain® in a 3mg/mL purified bovine type 1 collagen (Nutragen®, 6mg/mL) gel using 1.58M HEPES (pH 8.0), both stored at 4°C or on ice prior to using. These reagents were added to each 150µL TissueTrain® as follows: 72.12µL of type 1 collagen, 5.7µL of HEPES and 72.12µL of hPDL cells in DMEM.

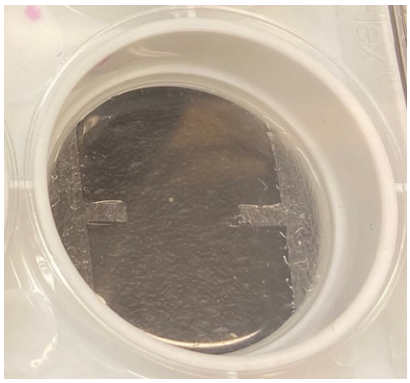
Note that cells were seeded into the TissueTrain® plates using a vacuum suction (Figure 3b) and were left to set for 1 hour prior to adding 3mL DMEM culture medium to each

well. At this point, the vacuum suction was removed and the collagen gels were left to set for 48 hours at 37°C in a humidified atmosphere of 5% CO_2 (Figure 3c).

Once the gels were completely set, the TissueTrain® plates were transferred to the FlexCell®FX-5000 Tension system after being washed using PBS and the media was replaced with CO_2-independent culture media.

a) b)





c) d)

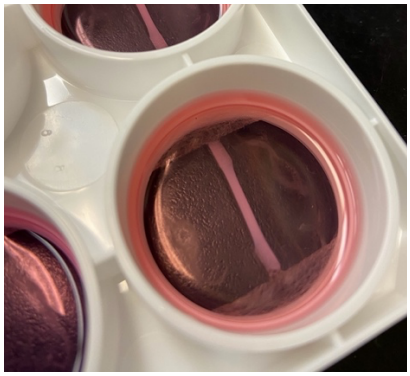


Figure 3: Photographs of the TissueTrain® culture system.

The FlexCell® TissueTrain® plates (a) have a flexible bottom with collagen tabs (b) that when combined with the vacuum suction apparatus (c) creates a mould for the 3D collagen gels. Once the collagen gels have set, media is added (d) and the gels are ready for tension application.

3.4 FlexCell®FX-5000 Tension system

Cells prepared in the TissueTrain® plates described above were placed under cyclical tensile stress loading using the FlexCell®FX-5000 Tension system. Tension protocol included a 5 second stretch followed by a 5 second release, repeated 6x/minute at 37°C in a humidified atmosphere without CO_2.

When using the TissueTrain® plates for fluorescent microscopy, the cells were loaded at a 10% extent for 72 hours. Cells cultured without tensile stress loading under the same conditions served as the control. Both the experimental and control conditions were completed in triplicate.

When using the TissueTrain® plates for mRNA isolation and RTqPCR, the cells were loaded at 10% or 20% for either 0, 24 or 72 hours. Cells cultured without tensile stress loading under the same conditions served as the control. Both the experimental and control conditions were completed in triplicate with three different sets of hPDL cells.

3.5 Fluorescent Microscopy

Fluorescent microscopy was used to visualize how hPDL cells were responding to the tension force applied by the FlexCell®FX-5000 Tension system and to ensure that the cells were still viable. For this experiment, 43F hPDL cells (P6) were plated in the TissueTrain® plates and loaded as described above.

After the plates were loaded for 72 hours, the cells (still within the collagen gels) were rinsed with PBS and fixed using a 4% paraformaldehyde (PFA) solution for 8 minutes. Following an additional three rinses with PBS, the cells were stained for fluorescent microscopy.

hPDL cell membranes were permeabilized with 0.1% Triton X-100 and blocked with 1.0% bovine serum albumin (BSA) for 30 minutes. 1:100 Rhodamine phalloidin and 1:1000

Hoechst was added and incubated in the absence of light for an additional 45 minutes. A final rinse with PBS was completed prior to imaging.

Images of hPDL cells within the collagen gels were taken on a Carl Zeiss Imager M2m microscope (Carl Zeiss; Jena) using ZenPro 2012 software at 10x magnification. The DsRed channel (558nm) was used to evaluate the Rhodamine phalloidin stain and then DAPI channel (405nm) was used to evaluate the Hoechst stain. An image representative of the whole collagen gel was taken as Z-stacks and presented as either the DsRed channel alone, as the DAPI channel alone or as a combination of both.

3.6 mRNA Isolation

mRNA isolation and RTqPCR was used to evaluate gene expression in hPDL cells after cyclical tensile loading from the FlexCell®FX-5000 Tension system. For this experiment, hPDL cells from three different individuals – 28M (P5), 38F (P4) and 31M (P5) – were plated in the TissueTrain® plates in triplicate and loaded as described above. Refer to Figure 4 for an overview of this portion of the protocol.

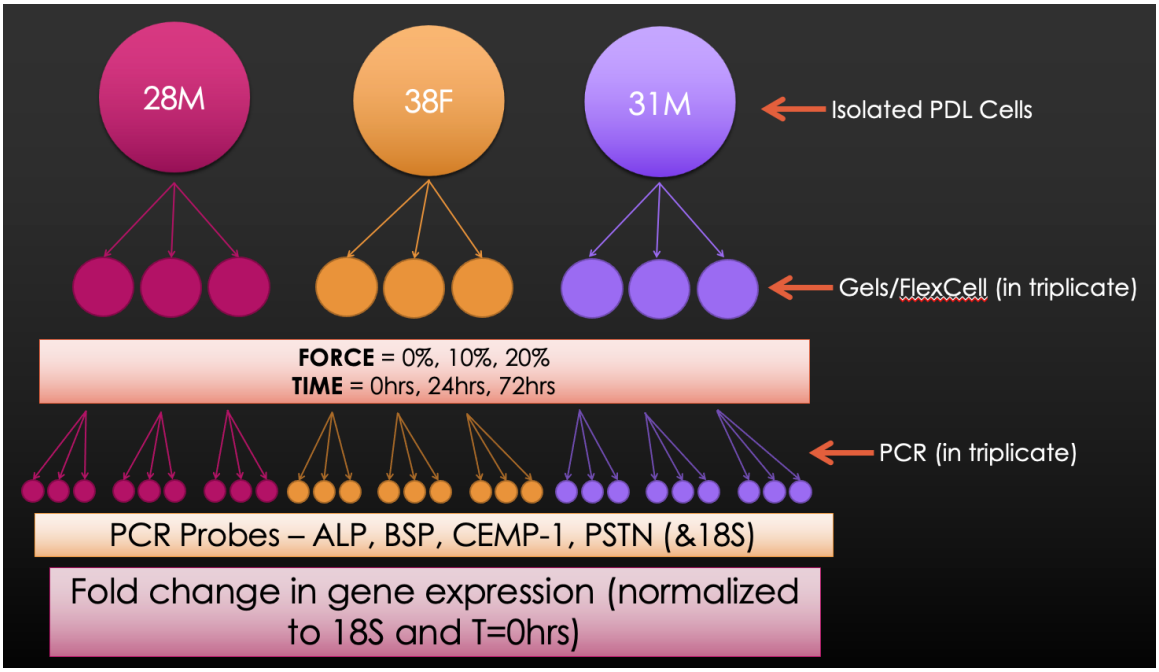


Figure 4: Overview of samples used for mRNA isolation and RTqPCR.

hPDLs from three samples (28M [P5], 38F [P4] and 31M [P5]) were propagated and plated into TissueTrain® gels. Each TissueTrain® condition (0%, 10% and 20% force at 0hrs, 24hrs and 72hrs) was repeated in triplicate for each hPDL sample for a total of 81 TissueTrain® gels. For the RTqPCR experiments, each sample was subsequently run in triplicate for each gene evaluated (ALP, BSP, CEMP-1, PSTN, 18S RNA). Data was normalized to 18S mRNA and presented as a fold change in expression relative to 0hrs.

To isolate total mRNA from the hPDL cells in the TissueTrain® gels, the TRIZOL® reagent (Invitrogen, Paisley, UK) and RNeasy kit was used to extract total RNA, using the manufacturer's instructions. The amount of RNA was determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific) by measuring absorbance at 260 nm. RNA quality was deemed adequate if the absorbance ratio of A260/A280 was within 1.9-2.1 and if the absorbance ratio of A260/A230 was greater than 2. RNA samples were normalized to 8ng/µL using RNase free water and then stored at -80°C until ready for amplification using RTqPCR.

Note that there was a building-wide power failure during the total RNA isolation from the hPDL cells isolated from the 31-year-old male, resulting in the mRNA being left on the RNeasy column for 1 hour prior to elution rather than the recommended 2 minutes. As such, the overall yield of total mRNA from this sample was less than the other two samples and further implications of this is discussed below.

3.7 Reverse Transcriptase-Quantitative Polymerase Chain Reaction

RT-qPCR was completed using the ViiA 7 Real-Time PCR System (ThermoFisher) and Quant Studio v1.2.4 software (Life Technologies) located at the Robarts Research Institute at the University of Western Ontario. Each reaction was performed in triplicate using a 15µL final volume containing 32ng of each RNA sample, 2xMasterMix, iScript Reverse transcriptase and one of the pre-mixed 10µM primers and probes (ether CEMP-1, PSTN, ALP, BSP) on a 384-well plate. An additional set of reactions was completed using 18S rRNA primers and probes as a normalization target. Reverse transcription was performed at 50°C for 10 min followed by 40 cycles of amplification at an annealing temperature of 60 °C. Fold change in gene expression was calculated by normalizing to the time zero control.

3.8 Statistical Analysis

Data is presented individually for each set of hPDL cells. While each PCR reaction and TissueTrain® gels were performed in triplicate, more data needs to be collected to increase the sample size (ie. More sets of hPDL cells) so that a proper statistical analysis can be completed.

Chapter 4 – Results

4.1 Validation of the FlexCell®FX-5000 Tension system Protocol using fluorescent microscopy

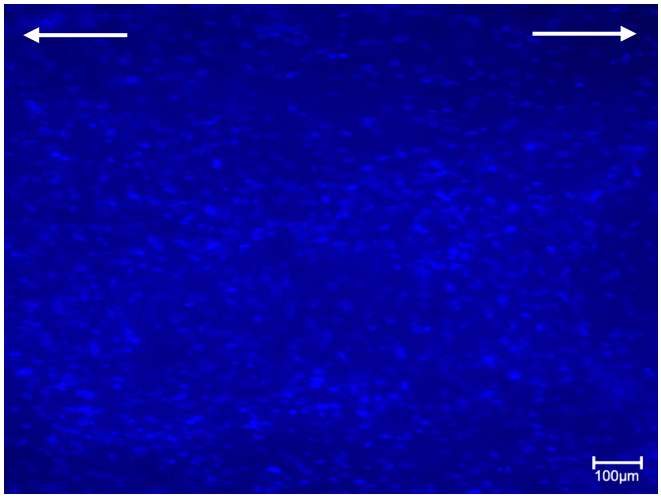
Fluorescent microscopy was used to visualize hPDL cells isolated from a 43-year-old female (P6) in a 3mg/mL collagen gel TissueTrain® incubated in a 37°C in a humidified atmosphere without CO_2 for 72 hours, either with a cyclical tension force application (5 second stretch at 10% tension force followed by a 5 second release, repeated 6x/minute using the FlexCell®FX-5000 Tension system) or without tension force (0% tension force).

Hoechst was used to evaluate the viability of the hPDL cells as it stains DNA and allows assessment of nuclear morphology[125]. Figure 5 demonstrates that the hPDL cells from both the experimental condition (Figure 5a, 10% cyclical tension force application) and the control condition (Figure 5b, 0% tension force application) are viable as the Hoechst staining reveals nuclei that are intact, ovoid and uniform.

Rhodamine phalloidin was used to visualize the shape and appearance of the hPDL cells through F-actin (an abundant component of the cell cytoskeleton)[126]. Figure 6 demonstrates that the hPDL in the experimental condition (Figure 6a, 10% cyclical tension force application) are more aligned in the direction of force application when compared to the control condition (Figure 6b, 0% tension force application).

Figure 7 overlays both Hoechst and Rhodamine phalloidin stained images to demonstrate that the intact nuclei are present within intact cells in both the experimental condition (Figure 7a, 10% cyclical tension force application) and the control condition (Figure 7b, 0% tension force application). In summary, the FlexCell®FX-5000 tension system protocol developed and described above does apply adequate force to align the cells in the direction of force application while maintaining cell viability. Therefore, this protocol was used for the next set of experiments described below.

a)



b)

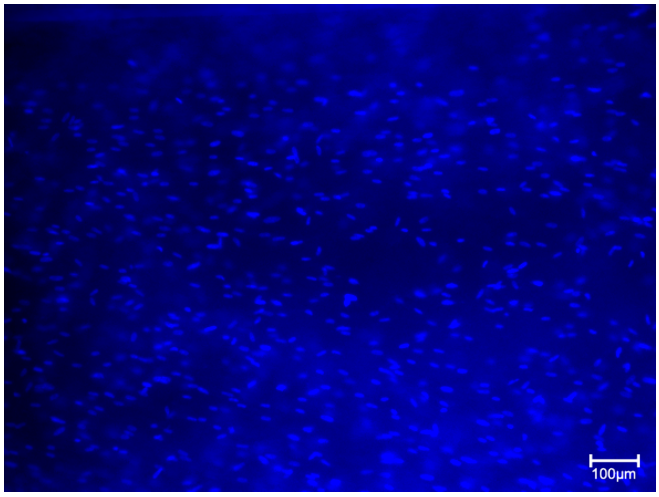
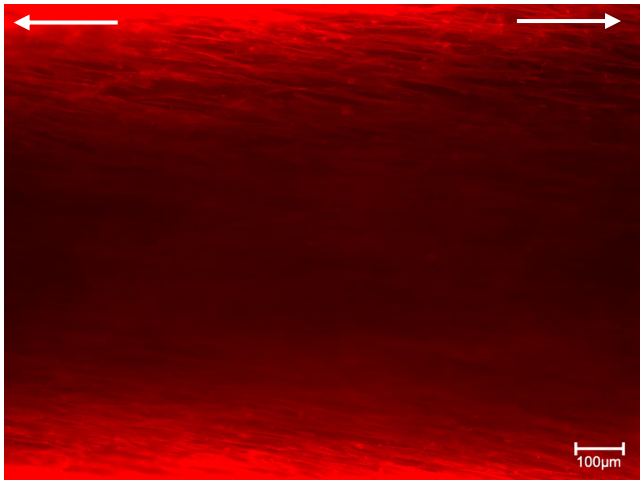


Figure 5: Hoechst staining of 43F (P6) hPDL cells in a 3mg/mL collagen gel after 72 hours with or without 10% cyclical tension force application.

hPDL cells were plated in a TissueTrain® in a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours in CO_2-independent media with either a 10% cyclical tension force application (a) or a 0% tension application (b). White arrows represent direction of force application. Cells were stained with Hoechst, visualized using the DAPI channel (405nm) at 10x magnification. Hoechst staining of the cell nuclei/DNA demonstrates that both conditions result in viable cells as seen by the intact nuclei. Note that this experiment was repeated in triplicate and representative data of one set of samples is presented here. Refer to Appendix A and B for data representative of all three sets of samples.

a)



b)

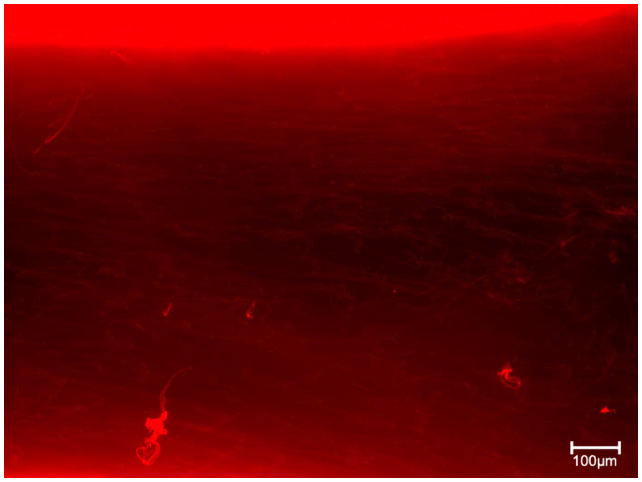
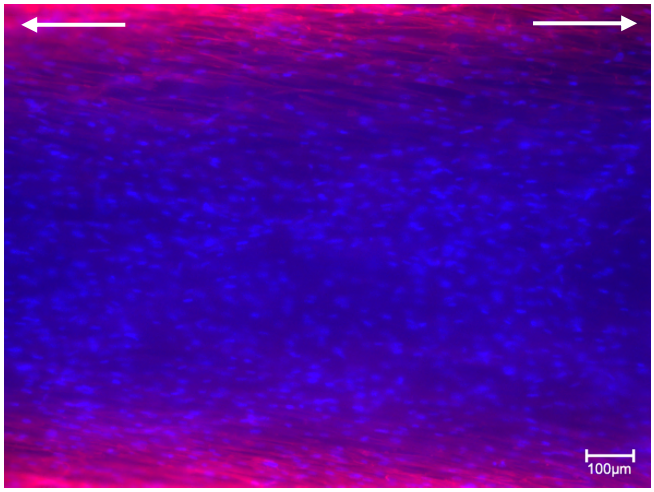


Figure 6: Rhodamine phalloidin staining of 43F (P6) hPDL cells in a 3mg/mL collagen gel after 72 hours with or without 10% cyclical tension force application.

hPDL cells were plated in a TissueTrain® in a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours in CO_2-independent media with either a 10% cyclical tension force application (a) or a 0% tension force application (b). White arrows represent direction of force application. Cells were stained with Rhodamine phalloidin, visualized using the DsRed channel (558nm) at 10x magnification. Rhodamine phalloidin staining of f-Actin within the cell cytoskeleton demonstrates that the hPDL cells are more aligned in the direction of force application than without force application. Note that this experiment was repeated in triplicate and representative data of one set of samples is presented here. Refer to Appendix A and B for data representative of all three sets of samples.

a)



b)

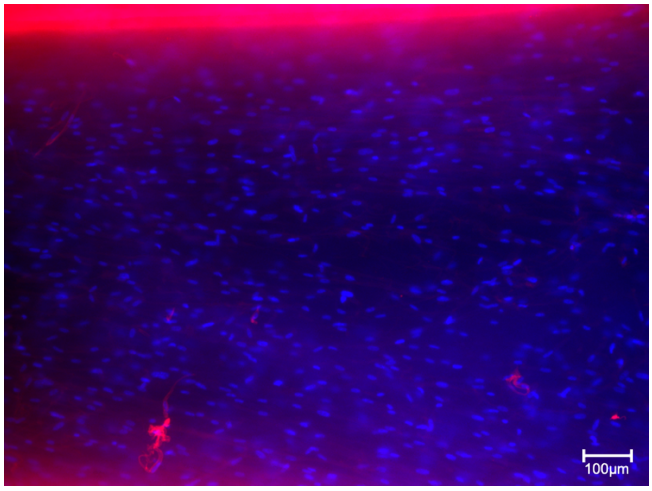


Figure 7: Overlay of Hoechst and Rhodamine phalloidin staining of 43F (P6) hPDL cells in a 3mg/mL collagen gel after 72 hours with or without 10% cyclical tension force application.

hPDL cells were plated in a TissueTrain® in a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours in CO_2-independent media with either a 10% cyclical tension force application (a) or a 0% tension application (b). White arrows represent direction of force application. Hoechst and Rhodamine phalloidin staining were visualized together to demonstrate that viable nuclei are present within intact cells. Refer to Appendix A and B for data representative of all three sets of samples.

4.1.1 Troubleshooting the FlexCell®FX-5000 Tension system protocol

While developing the FlexCell®FX-5000 Tension system protocol, it was determined that two key elements were essential for the success of the experiment: concentration of collagen and culture medium.

Firstly, a concentration of at least 3mg/mL collagen (Nutragen®) gel is required. Initially, the protocol completed was using a collagen concentration of 1mg/mL which was too dilute to even solidify in the TissueTrain®. A modification to the protocol included increasing the collagen gel concentration to 2mg/mL and while the gels did solidify adequately in the TissueTrain®, once 10% force application was added when in the FlexCell®FX-5000 unit, the collage gels ripped apart. Finally, increasing the collagen concentration to 3mg/mL allowed the gels to fully set and survive the 10% tension force application for the full 72 hours.

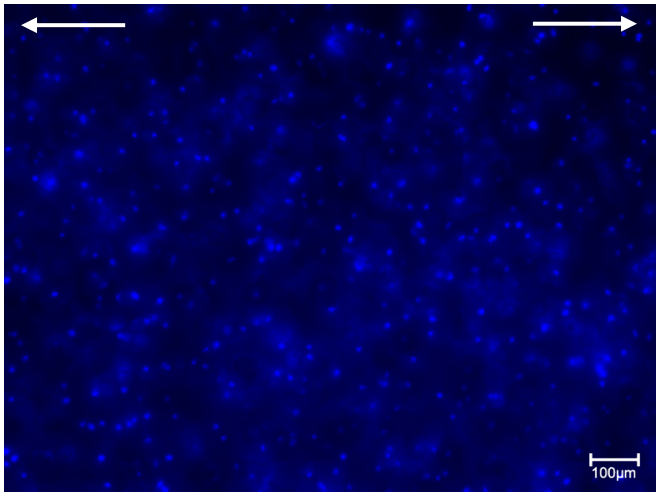
Secondly, the FlexCell®FX-5000 unit was connected to an incubator that did not have a CO_2 supply. In a typical hPDL cell culture using DMEM, 5% CO_2 is added and acts as a buffer allowing the culture medium to maintain a physiologic pH of approximately 7.35 to 7.45 while the cells are growing and passaged[127]. However, adding an external source of CO_2 to the FlexCell®FX-5000 unit was not possible with the current set up. As such, an alternative buffer was required. Initially, DMEM plus a buffer of 25mM HEPES was used for a set of FlexCell®FX-5000 experiments however, imaging revealed that the cells were not viable. Hoechst staining revealed damaged nuclei as seen by their diluted, rounded shape (Figure 8) and Rhodamine Phalloidin staining revealed atypical cell morphology (Figure 9) in comparison to the hPDL cells shown in Figures 5 and 6. Figure 10 overlays both Hoechst and Rhodamine phalloidin stained images.

Additionally, when the TissueTrain® culture plates were inspected visually, the culture medium was a bright pink colour, indicating a pH that was too high and the HEPES buffer was inadequate[127]. Therefore, an alternative cell culture medium/buffering system was used. For this, CO_2-independent medium (GIBCO) – a non-HEPES-containing medium

with a proprietary buffering system – was ordered and an additional FlexCell®FX-5000 tension experiments was completed and the resulting hPDL cells were viable and intact (Data presented in Figures 5, 6 and 7).

In summary, it is essential that a collagen gel of at least 3mg/mL Nutragel® and CO_2-independent medium (GIBCO) is used when completing tension experiments on hPDL cells using the FlexCell®FX-5000 tension system and TissueTrain® culture plates.

a)



b)

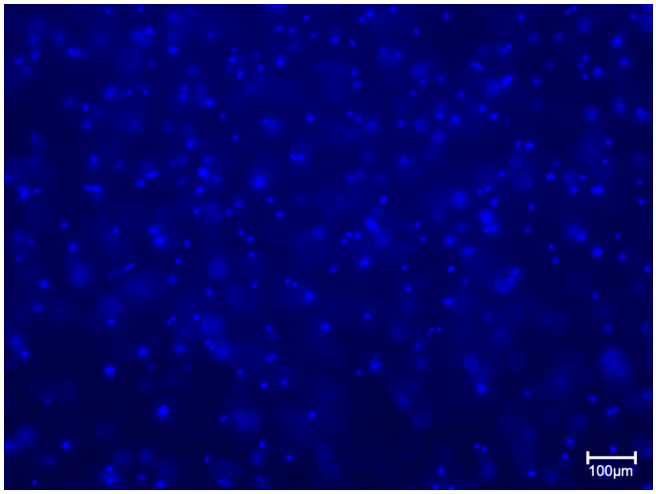
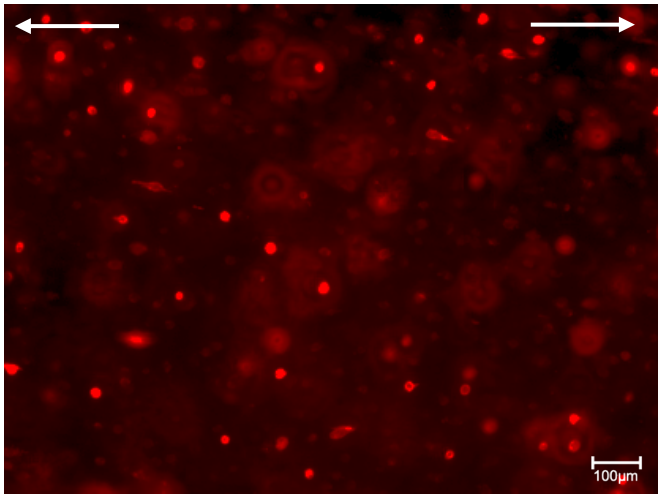


Figure 8: Hoechst staining of 43F (P5) hPDL cells in a 3mg/mL collagen gel after 72 hours with or without 10% cyclical tension force application.

hPDL cells were plated in a TissueTrain® in a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours in DMEM+25mM HEPES with either a 10% cyclical tension force application (a) or a 0% tension application (b) and stained with Hoechst, visualized using the DAPI channel (405nm) at 10x magnification. White arrows represent direction of force application. Hoechst staining demonstrates many damaged nuclei as seen by the diluted, rounded shape.

a)



b)

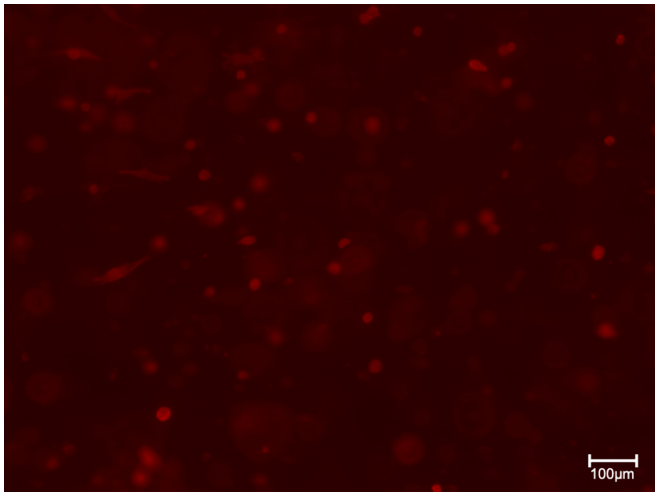
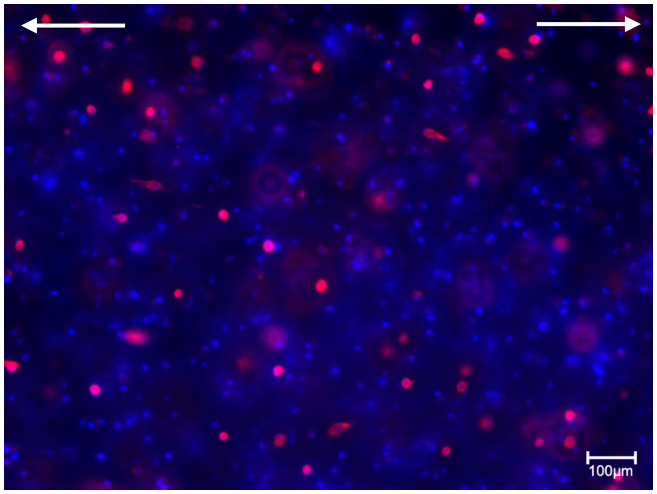


Figure 9: Rhodamine phalloidin staining of 43F (P5) hPDL cells in a 3mg/mL collagen gel after 72 hours with or without 10% cyclical tension force application.

hPDL cells were plated in a TissueTrain® in a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours in DMEM+25mM HEPES with either a 10% cyclical tension force application (a) or a 0% tension force application (b) and stained with Rhodamine phalloidin, visualized using the DsRed channel (558nm) at 10x magnification. White arrows represent direction of force application. Rhodamine phalloidin staining demonstrates hPDL cells have an atypical morphology.

a)



b)

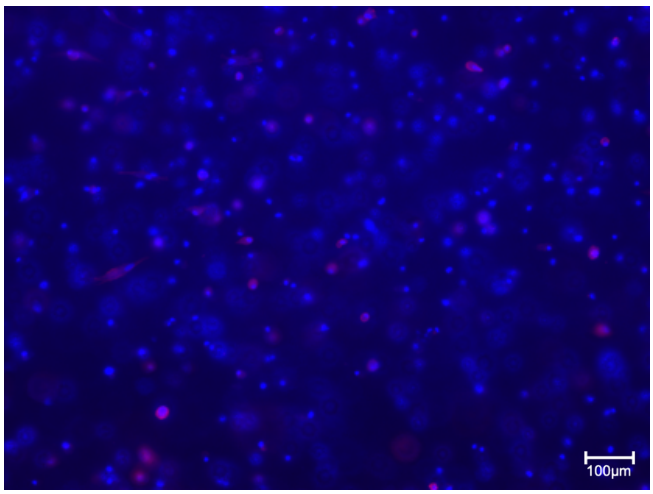


Figure 10: Overlay of Hoechst and Rhodamine phalloidin staining of 43F (P5) hPDL cells in a 3mg/mL collagen gel after 72 hours with or without 10% cyclical tension force application.

hPDL cells were plated in a TissueTrain® in a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours in DMEM+25mM HEPES with either a 10% cyclical tension force application (a) or a 0% tension application (b). White arrows represent direction of force application. The hPDL cells are not viable and are of have an atypical morphology.

4.2 Gene expression in hPDL cells with or without cyclical tension force application

Changes in PSTN, ALP, BSP and CEMP-1 expression was evaluated in three samples of adult hPDL cells (28-year-old male, 28M (P5); 38-year-old female, 38F (P4); 31-year-old male, 31M (P5)) in the presence or absence of cyclical tension force application using the FlexCell®FX-5000 Tension system protocol describe above.

An additional force level and two additional time points were added such that gene expression was evaluated at 0%, 10% and 20% for either 0, 24 or 72 hours. Note that the sample size of this experiment was small and as such the results are presented with each hPDL sample separately using descriptive observations of the data rather than a comprehensive statistical analysis.

4.3 Expression of PSTN in hPDL cells with or without cyclical tension force application

Figure 11 demonstrates the fold change in gene expression of PSTN in the 28M (11a), 38F (11b) and 31M (11c) hPDL cells. Similar to the results previously published by Wilde *et al.,* 2003 and Rangiani *et al,* 2015, PSTN expression increased over time with the application of tension force[95,102]. The results among the three groups were similar for all time points at 0% force application (where minimal changes were observed in PSTN expression) and 10% and 20% force applications (where a general trend of increased PSTN expression over time was noted). The 28M hPDL cells had the greatest fold increase in PSTN expression when compared to the 38F and 31M hPDL cells at both 24 and 72 hours at 20% tension force application. Refer to Appendix C for a table presenting the numerical data visualized on Figure 11.

4.4 Expression of ALP in hPDL cells with or without cyclical tension force application

Figure 12 demonstrates the fold change in gene expression of ALP in the 28M (12a), 38F (12b) and 31M (12c) hPDL cells. Similar to the results previously published by Yamaguchi *et al.,* 1996, ALP expression decreased over time with the application of tension force[54].

The results among the three groups were similar for all time points at 0%, 10% and 20% with an overall trend of decreasing ALP expression over time. The 28M hPDL cells did show an increase in ALP expression from 0 hours to 24 hours at 20% tension force application that did decrease again by 72 hours. A similar trend was seen with the 38F hPDL cells which showed an increase in ALP expression from 0 hours to 24 hours at 10% tension force application that did decrease again by 72 hours. Refer to Appendix C for a table presenting the numerical data visualized on Figure 12.

4.5 Expression of CEMP-1 in hPDL cells with or without cyclical tension force application

Figure 13 demonstrates the fold change in gene expression of CEMP-1 in the 28M (13a), 38F (13b) and 31M (13c) hPDL cells. Overall, the general trend appears to be such that expression of CEMP-1 decreased over time with or without the application of tension force. The 38F hPDL cells did show a maintenance of CEMP-1 expression levels with the application of either 10% or 20% tension force when compared to hPDL cells without the application of tension (where CEMP-1 expression decreased). Other observations include a slight increase in CEMP-1 expression for the 31M hPDL cells at 24 hours with 20% force application that subsequently decreased at the 72 hour time point. Refer to Appendix C for a table presenting the numerical data visualized on Figure 13.

4.6 Expression of BSP in hPDL cells with or without cyclical tension force application

There is no data to present demonstrating the fold change in gene expression of BSP in the 28M, 38F and 31M hPDL cells. The results from the RTqPCR analysis using the BSP probe either came up as an error or a value of zero.

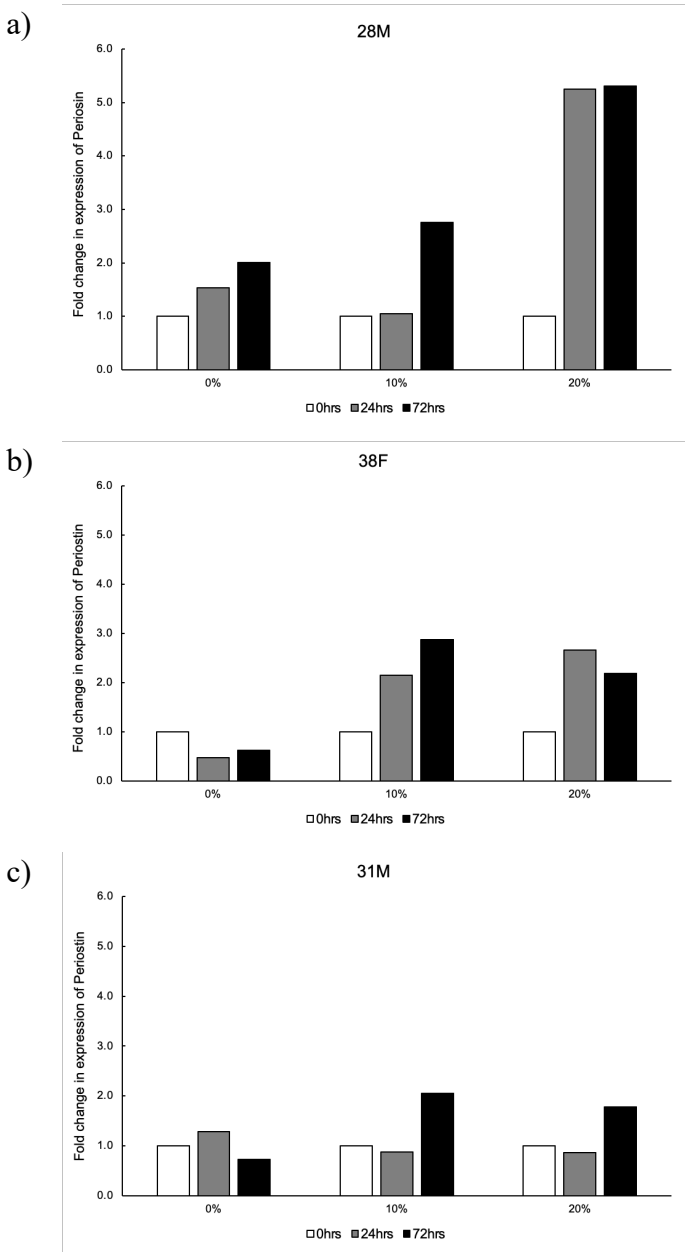


Figure 11: Fold change in PSTN expression over time at various tensile force levels.

hPDL cells isolated from a 28-year-old male, 28M (a), a 38-year-old female, 38F (b) and a 31-year-old male, 31M (c) were plated in triplicate in a TissueTrain® with a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours with either a 0%, 10% or 20% cyclical tension force application. Data was normalized to levels of 18S ribosomal RNA, relative to the value at 0 hours and presented as the mean fold change in expression of PSTN over time for each force level. Expression of PSTN appears to increase when cyclical tension force is applied.

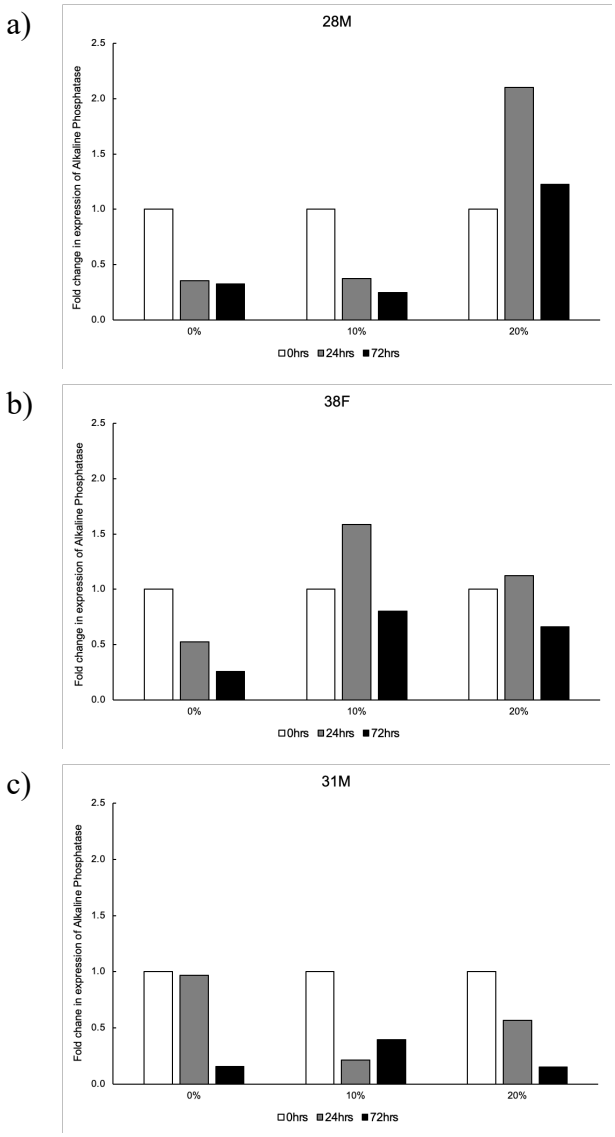


Figure 12: Fold change in ALP expression over time at various tensile force levels.

hPDL cells isolated from a 28-year-old male, 28M (a), a 38-year-old female, 38F (b) and a 31-year-old male, 31M (c) were plated in triplicate in a TissueTrain® with a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours with either a 0%, 10% or 20% cyclical tension force application. Data was normalized to levels of 18S ribosomal RNA, relative to the value at 0 hours and presented as the mean fold change in expression of ALP over time for each force level. In general, the expression of ALP decreases over time when cyclical tension force is applied. However, for both the 28M hPDL cells at 20% and 38F hPDL cells at 10% tension force application, the expression of ALP does appear to increase at the 24 hour time point prior to decreasing by the 72 hour time point.

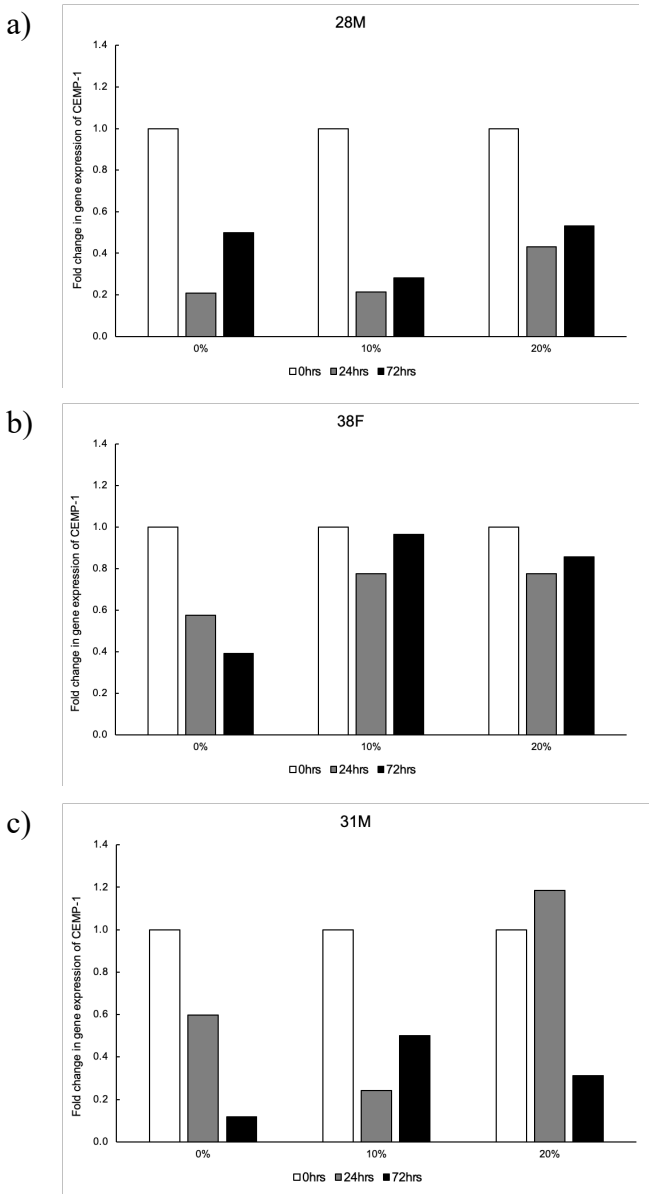


Figure 13: Fold change in CEMP-1 expression over time at various tensile force levels.

hPDL cells isolated from a 28-year-old male, 28M (a), a 38-year-old female, 38F (b) and a 31-year-old male, 31M (c) were plated in triplicate in a TissueTrain® with a 3mg/mL collagen gel and incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours with either a 0%, 10% or 20% cyclical tension force application. Data was normalized to levels of 18S ribosomal RNA, relative to the value at 0 hours and presented as the mean fold change in expression of CEMP-1 over time for each force level. In general, expression of CEMP-1 appears to decrease over time with or without the application of tension force.

Chapter 5 – Discussion

5.1 Objective 1 – To establish and validate a 3D culture method for application of tension to human PDL cells in a 3-Dimensional collagen gel

A protocol for applying tension to hPDL cells using the FlexCell®FX-5000 Tension system was developed. After some troubleshooting various different culture media and concentrations of collagen, it was determined that a CO_2-independent medium (GIBCO) and a concentration of 3mg/mL of collagen and was necessary to maintain TissueTrain® integrity and cell viability throughout the experiment. Fluorescent microscopy was used to confirm that cells were still viable after 72 hours of 10% tension force application, and that the cells appeared qualitatively more aligned in the direction of force application (as shown in Figures 3, 4 and 5).

For this experiment, a cyclical loading protocol was used (5 second stretch followed by a 5 second relax) to replicate how a tooth might experience force during OTM. For example, when using sliding mechanics, teeth are "walked" along the continuous arch wire in a mesio-distal direction via a series of cyclical force applications[111]. Tooth movement starts with friction alone as the crown tips after which elastic binding is added when the tooth tips enough to bring the corners of the bracket into contact with the wire[111]. As root movement catches up with the crown, elastic binding is reduced and the tooth is back to experiencing friction alone[111].

Note that it was hypothesized that the hPDL cells would appear aligned perpendicular to the direction of force application as this was the trend seen by Oortgiesen *et al.,* 2012. In their experiment, mouse PDL fibroblasts were seeded in a 3-dimensional collagen gel after which an 8% tension force load was applied[122]. After 24 hours of stretching, the PDL fibroblasts were elongated and oriented perpendicular to the direction of tension force application[122]. However, when evaluating the cell alignment in the hPDL cells using the

FlexCell®FX-5000 system, the cells appeared more aligned parallel to the direction of force application. This discrepancy could be due to various reasons.

Firstly, the cell type used by Oortgiesen *et al.,* 2012 was rodent PDL fibroblasts while the cells used in this experiment were derived from human PDL. Additionally, the device used to apply the tension forces were different. Oortgiesen *et al.,* 2012 used a moving bracket and coverslip to apply a purely linear force. However, in this experiment, the FlexCell®FX-5000 system applies the tension force using a vacuum that stretches the collagen gel down and around the loading post producing equibiaxial (or circumferential) strain. Interestingly, the alignment was more prominent in the peripheral of the gels for both the rodent PDL fibroblasts and the hPDL cells[122].

5.1.1 Limitations of the study and suggestions for future studies

The first limitation of this portion of the study is reflected by the age of the hPDL cells used. It is known that the ability of PDL tissue to regenerate form and function declines with age[128,129]. The cells used for this fluorescent microscopy experiment were from a 43-year-old female. Orthodontics is more commonly performed in adolescents than adults as seen from a survey conducted by the American Association of Orthodontics in 2010 whereby 27% of orthodontic patients were adults[130]. As such, whereas PDL cells from a patient of 43-years-old might be acceptable to study periodontal disease, it is preferable that PDL cells from a younger donor be used in the field of orthodontic research. It is suggested that this florescent microscopy protocol be repeated on hPDL cells from a younger donor to evaluate whether age has an effect on the PDL cell's ability to respond to tensile strain.

Secondly, while the hPDL cells were plated in triplicate on the TissueTrain® for each experimental condition, this experiment was only completed with one set of hPDL cells (43F, P6). Repeating the experiment with other hPDL cells would strengthen the findings and perhaps pick up on some trends that are were not perceived on one set alone.

Finally, the fluorescent microscopy images are a 2-dimensional representation of a 3-dimentional object. Sectioning the gel after fixation prior to imaging may have given a more accurate representation of how the cells were aligned with regards to force application.

5.2 Objective 2 – To evaluate CEMP-1 gene expression during cyclical tensile force

The protocol outlined and discussed in Objective 1 was used to evaluate gene expression in hPDL cells with or without the application of tension. While CEMP-1 was the original focus of this research project, this protocol was also used to evaluate additional genes (PSTN, ALP and BSP) with known responses to tension strain to ensure the protocol developed was valid. Of importance, since only three different hPDL samples were used (28M (P5), 38F (P4), 31M (P5)) results are discussed in terms of qualitative observations rather than quantification of values and statistics.

5.2.1 Periostin expression increases with the application of tension

Periostin is highly expressed in the periodontal ligament, is required for wound healing and remodeling and is required to maintain PDL integrity during mechanical loading[101,131]. Additionally, it has been shown that periostin is upregulated in the compression zone relative to the tension zone during orthodontic tooth movement[95]. In other words, periostin was upregulated in both zones of compression and tension however it was upregulated to a greater degree in the zone of compression relative to the zone of tension[95].

As a general trend, it is shown that periostin levels increase with the application of tension. This is in alignment with the previous research by Wilde *et al.,* 2003. This effect was seen most prominently in the 28M samples with an approximately 5.5-fold increase in PSTN expression at both 24 and 72 hours with 20% tension force application. The maximum fold

increase in PSTN expression for the other two samples (38F and 31M) was no greater than 3-fold.

Perhaps this increased fold change in expression of PSTN in 28M hPDL cells was due to the fact that these hPDL cells came from the youngest donor (28-year-old male). Given that periostin is involved in maintaining PDL integrity during mechanical loading and that the ability to regenerate the PDL decreases with age, it follows that the hPDL cells from the youngest donor would have the highest PSTN expression[128,129].

5.2.2 Alkaline phosphatase expression decreases with the application of tension

Alkaline phosphatase is a protein enzyme involved in mineralization and calcification of hard tissue and is a biomarker of osteoblast activity and is also known to be modulated by orthodontic forces[54]. For their experiment, Yamaguchi *et al.,* 1996 cultured human PDL cells in a tissue culture plate with a flexible bottom and applied tension using a unit similar to the FlexCell®FX-5000 to show that ALP expression decreased over the course of 5 days in a magnitude-dependent manner.

As expected and similar to the findings from Yamaguchi *et al.,* 1996, the general trend presented in this research project shows ALP expression decreasing over time. However, individual conditions strayed away from this trend. For example, the 28M hPDL cells showed an increase in ALP expression from 0 hours to 24 hours at 20% tension force application that decreased by 72 hours. A similar trend was seen with the 38F hPDL cells which showed an increase in ALP expression from 0 hours to 24 hours at 10% tension force application that decreased by 72 hours).

Of importance, it does appear that ALP expression was also shown to decrease over time without any force application (0% tension force). Given that hypoxia has been shown to decrease ALP expression, perhaps the cells in the middle of the 3-dimensional collagen gels were not receiving adequate oxygenation, leading to the decrease in ALP

expression[132]. However, if the cells in the middle of the collagen gels were experiencing hypoxia, then this would be the case for all the gels, not just the samples without any force application. Subsequently, determining if the decrease in ALP expression was due to hypoxia or tension would not be possible.

An alternative explanation for the trends seen with ALP expression could be due to the fact that only three hPDL samples were tested. It is likely that ALP expression at 0% force level is relatively unchanged over time but too few samples were evaluated to pick up on a specific trend or complete any statistics.

5.2.3 Expression of bone sialoprotein was not evaluated

As mentioned in the results section, there was no data to present regarding BSP expression in either of the 28M, 38F and 31M hPDL cells under any condition. The results from the RTqPCR analysis using the BSP probe either came up as an error or a value of zero.

Given that all the genes (PSTN, ALP, BSP, CEMP-1 and 18S) were run on the same set of plates under the same RTqPCR experimental conditions, it can be presumed the issue was with the probe, not the experimental technique. Perhaps the probe vial became contaminated or exposed to too much light thus reducing its effectiveness.

5.2.4 Cementum Protein-1 expression decreases with the application of tension

Given that CEMP-1 is expressed in the PDL cementoblastic cell layer and PDL cell populations, has been shown to enhance cementoblast differentiation and is postulated to have a key role in cementum repair, it was hypothesized that CEMP-1 expression would increase with the application of tension, similar to how osteoblastic activity increases in the zone of tension during orthodontic tooth movement[113,133,134].

However, contrary to the hypothesis, the general trend appears to be such that expression of CEMP-1 decreased over time with or without the application of tension force. This was the case for both the 28M and 31M hPDL samples for most time points at all force levels. Note that there was a relative increase in CEMP-1 expression (or perhaps more of a maintenance of CEMP-1 levels) in the 31M hPDL cells at the 24 hour time point at 20% tension force application that subsequently decreased by the 72 hour time point.

Interestingly, the 38F hPDL cells did appear to show a maintenance of CEMP-1 expression levels with the application of either 10% or 20% tension force over time when compared to no application of tension force whereby CEMP-1 expression decreased. Combining this information with the fact that there was a relative increase (or maintenance) of CEMP-1 levels at the 24 hour time point in the 31M cells at 20% force application, perhaps there was simply not enough samples evaluated to distinguish this specific expression trend for CEMP-1 when under tensile force.

If the true trend is that of the one seen in the 38F hPDL cells (maintenance of CEMP-1 expression with the application of tension force) and knowing that CEMP-1 has a key role in cementogenesis and cementum repair, then perhaps the hPDL cells only need to maintain their CEMP-1 expression while undergoing mechanical loading (to "protect" the cementum). Whereas if there is no mechanical loading, the cells do not need to maintain their CEMP-1 expression. Understanding that this is pure conjecture without any scientific basis, the inconsistent data with regards to CEMP-1 expression is more realistically due to small sample size.

5.2.4 Limitations of study and suggestions for future studies

5.2.4.1 Small and limited sample size

As already mentioned, the most significant flaw with the data presented in this study is the small samples sizes for both the fluorescent microscopy and gene expression assays. In particular, a sample size of three was not adequate to elucidate specific trends seen in the differential gene expression with or without tension force application. There was simply

not enough data to complete a statistical analysis and as such, evaluation of the data was purely qualitative. However, this does not mean that the data presented is of no value.

Now that a protocol to apply tension to hPDL cells has been developed (Objective 1), more hPDL samples can be easily evaluated and added to the data presented, eventually acquiring enough data for a statistical analysis. Furthermore, once more hPDL samples are added, other factors such as effect of age or gender on expression of PSTN, ALP, CEMP-1 (and BSP) can be evaluated.

For example, this study used hPDL cells from relatively older donors (no younger than 28 years) whereas 73% of orthodontic patients are adolescents[135]. Therefore, the data presented in this study might not be as relevant to younger patients undergoing orthodontic treatment. Additionally, since the protocol involves isolating total mRNA, it would be relatively simple to evaluate other genes of interest using RTqPCR as it would entail simply purchasing different probes of interest.

5.2.4.2 Comparison of gene expression during tension and compression

Once the gene expression profiles of hPDL cells under tension using the protocol described in this study has been more thoroughly investigated, it would be interesting to see how gene expression changes when the cells are under compression.

For example, it is known that PSTN expression increases in both the tension and compression zones. However, expression does increase more in the zone of compression relative to the zone of tension so perhaps other genes are similarly regulated[95]. FlexCell® International Corporation does offer another unit (FlexCell®FX-5000C) with BioPress™ culture plates which applies positive air pressure to compress cells and can be programmed to mimic *in vivo* conditions.

5.2.4.3 Force levels of the FlexCell®X-5000 Tension system

Another limitation to this study is that the amount of tension applied to the hPDL cell samples with the FlexCell®FX-5000 Tension system is gauged in terms of percent force.

For example, the condition used in this experiment include 0%, 10% and 20% force. This amount of force is related to the proposed percent increase in length of cells in an equibiaxial direction during strain rather than an actual amount calculated in Newtons (N) or gram-force (gm). For example, tipping of a tooth requires 35-60gm whereas only 10-20gm is required to intrude a tooth[1].

Given that a force gauge is not a component of the FlexCell®FX-500 Tension system unit, it is not known how much force was applied and whether the amount of force applied during these experiments is clinically relevant. Perhaps a device could be fabricated to determine the precise amount of force generated by the FlexCell®FX-5000 Tension system so that a clinically relevant tension amount of force can be used.

5.2.4.4 Confirmation that gene expression activity relates to protein activity

By using RTqPCR to evaluate expression of PSTN, ALP and CEMP-1, this only provides information with regards to the amount of that specific mRNA present in the cells but does not provide any information with regards to whether or not the mRNA becomes a fully formed and functional product.

As such, once the gene expression profiles have been more conclusively elucidated by adding more hPDL samples, it is recommended that a Western Blot analysis be performed on the hPDL cell samples with or without application of tension to correlate the amount of mRNA expression with amount of resulting protein.

5.2.4.5 Power failure during mRNA isolation

As mentioned previously, there was a building-wide power failure during the mRNA isolation of samples from the 31M hPDL cells. As such, the majority of 31M hPDL samples were on the RNeasy Mini Kit (QIAGEN) column for over one hour rather than the recommended 2 minutes. This affected the overall yield of the mRNA for those samples and perhaps may have skewed the data if mRNA corresponding to PSTN, ALP and CEMP-1 was destroyed.

5.2.4.6 CEMP-1 is only found in humans

CEMP-1 is a cementum molecule that is only found in humans meaning that there is no animal model[113,134]. This means that in order to study this novel protein, PDL cells must be extracted from human donor samples which increases the complexity of ethics approval and decreases the number of samples available to study.

Contrarily, Periostin is found in mice and its role in wound healing can be evaluated in depth by taking advantage of the murine model. For example, mice were engineered to lack the periostin gene (*Postn*$^{-/-}$) and were shown to have a significantly reduced percent of wound closure when compared to mice that had the periostin gene (*Postn*$^{+/+}$)[136]. This provides extremely strong evidence for the importance of Periostin during wound healing. Such a study is not possible with CEMP-1.

5.3 Clinical relevance

While the research presented in this study does not have a direct clinical relevance, it has opened doors for future research that might eventually help guide how orthodontic treatment is completed. For example, once the gene expression profile of CEMP-1 has been elucidated and its protein function more thoroughly evaluated, perhaps genetic testing might help determine whether or not a patient will be more susceptible to external root resorption during orthodontic treatment.

If it is determined that CEMP-1 does have a cementum-protective effect as proposed and discussed in this study, then perhaps individuals with a reduced level of CEMP-1 expression be identified and their orthodontic treatment modified with slower tooth movement and using less force to decrease the likelihood of root resorption. However, as with most studies, many additional studies are required to clarify these recommendations.

Chapter 6 – Conclusions

Objective 1 – To establish and validate a 3D culture method for application of tension to human PDL cells in a 3-Dimensional collagen gel

- The FlexCell®FX-5000 Tension system protocol developed using CO_2-independent medium (GIBCO) and 3mg/mL collagen is a valid method to evaluate gene expression in human PDL ligament cells.

Objective 2 – To evaluate CEMP-1 gene expression during cyclical tensile force

- The control genes performed as expected. Periostin expression increased with the application of tension and alkaline phosphatase expression decreased with the application of tension.
- In certain samples, CEMP-1 expression appeared to decrease over time regardless of amount of force application and was not dependent on time; however, in other samples, CEMP-1 expression also appeared to be maintained with the application of tension force.
- Given the small sample size, statistical analysis was not performed and as such only qualitative observations were made. Repeating the experiment with more sets of human PDL cells (preferably from younger donors) is required to make quantitative observations of any statistically significant trends.

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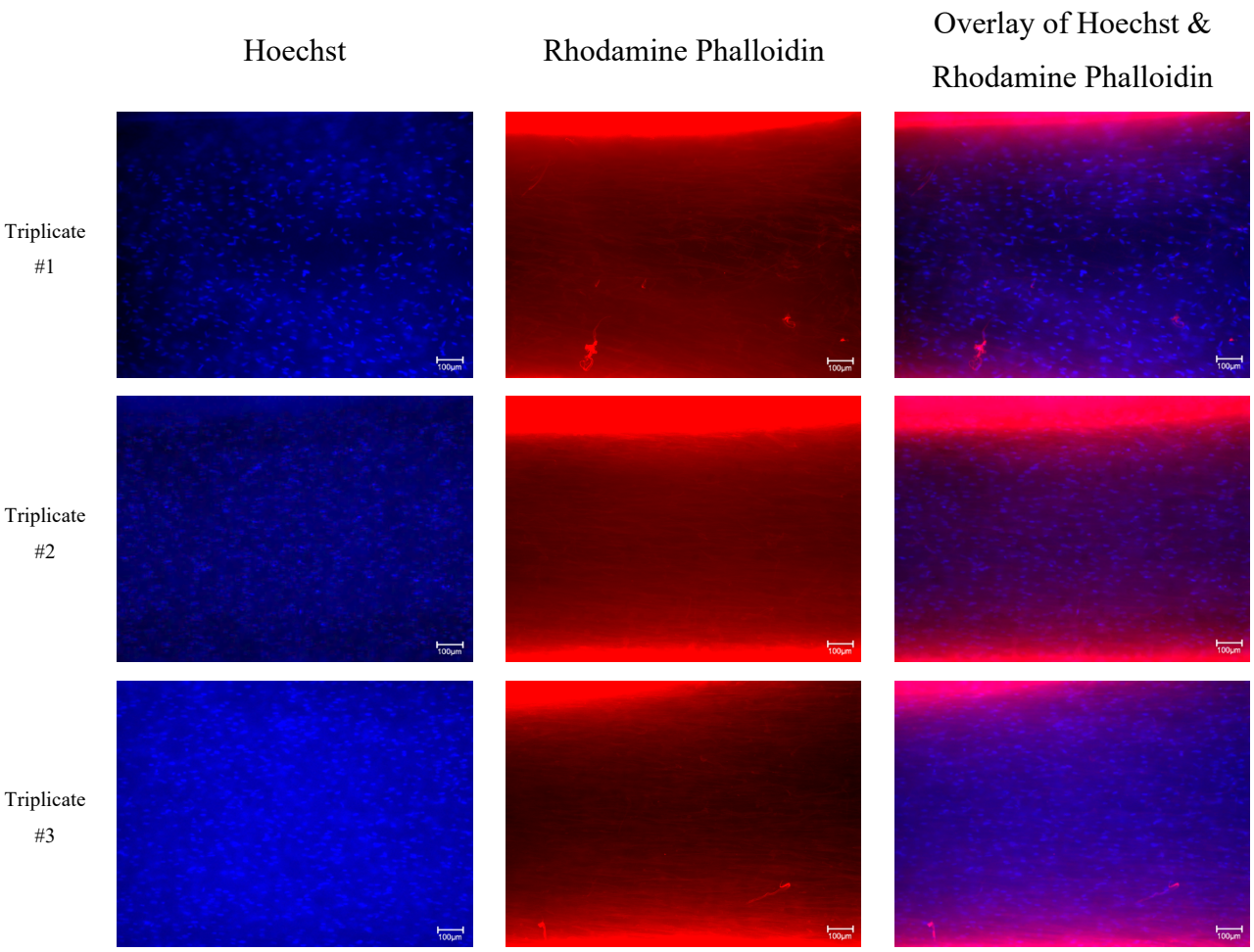
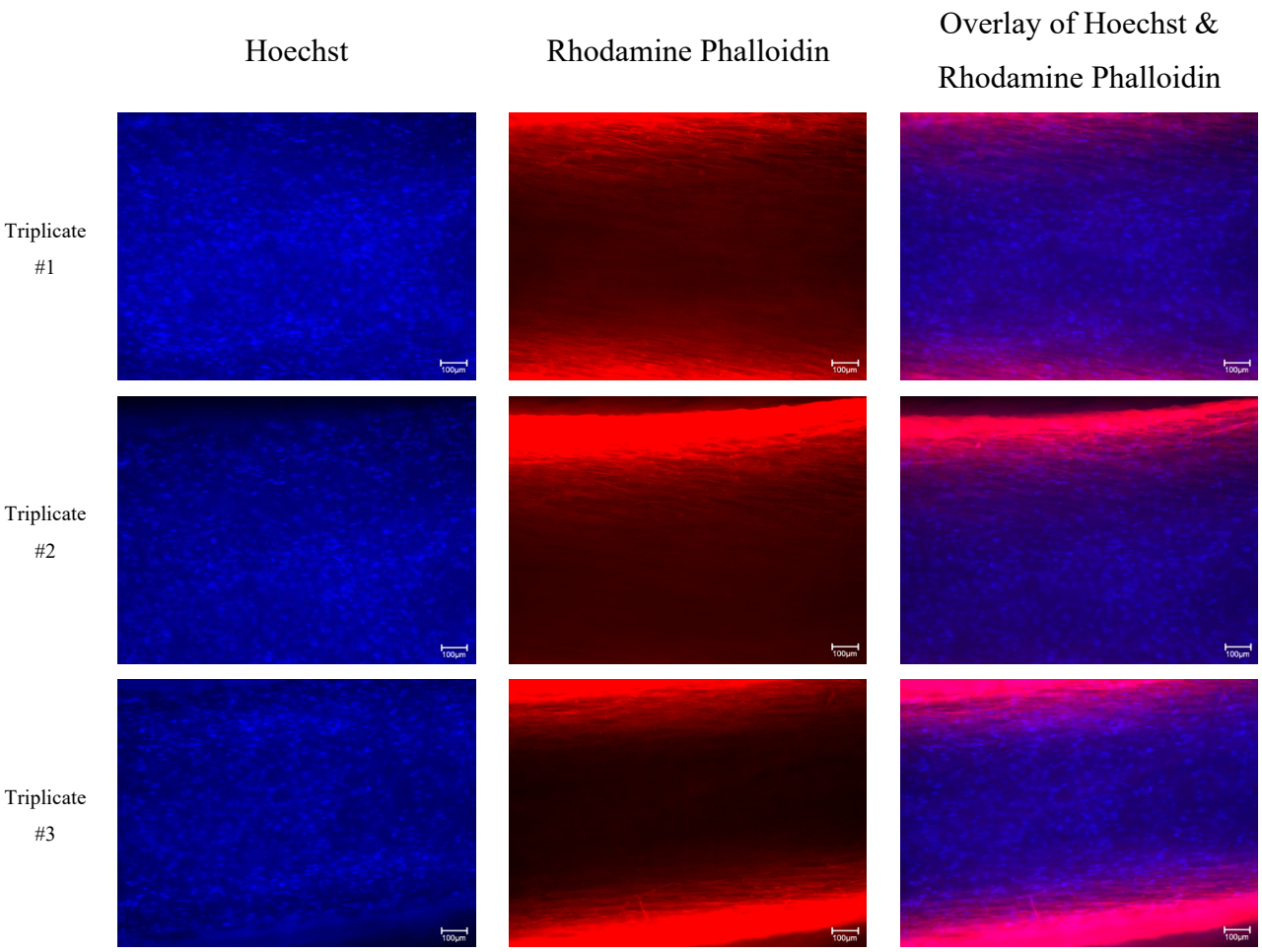
Appendices

Appendix A: Representative images of triplicate hPDL cell samples isolated from a 43-year-old female without application of cyclical tension force incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours.



Appendix B: Representative images of triplicate hPDL cell samples isolated from a 43-year-old female with the application of 10% cyclical tensile force incubated at 37°C in a humidified atmosphere without CO_2 for 72 hours.



Appendix C: Numerical data represented by graphs in Figures 4, 5 and 6

Data presented was normalized to levels of 18S ribosomal RNA, relative to the value at 0 hours and presented as the mean fold change in expression of either PSTN, ALP and CEMP-1 over time for each force level.

		PSTN			ALP			CEMP-1		
		0%	**10%**	**20%**	**0%**	**10%**	**20%**	**0%**	**10%**	**20%**
28M	**0hrs**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	24hrs	1.53	1.05	5.25	0.36	0.37	2.10	0.21	0.22	0.43
	72hrs	2.01	2.76	5.31	0.32	0.25	1.23	0.50	0.28	0.53
38F	**0hrs**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	24hrs	0.47	2.15	2.66	0.52	1.59	1.12	0.57	0.78	0.78
	72hrs	0.62	2.87	2.18	0.26	0.80	0.66	0.39	0.97	0.86
31M	**0hrs**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	24hrs	1.29	0.88	0.87	0.97	0.22	0.56	0.60	0.24	1.19
	72hrs	0.72	2.06	1.78	0.15	0.40	0.15	0.12	0.50	0.31

Curriculum Vitae

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Honours and Awards:	Lindsey O. Kesling Orthodontic Resident Scholarship Award 2020
	John and Nancy Murray Prize 2020
	American Academy of Implant Dentistry Dental Student Award 2015

BC Society of Orthodontists Charles C Craig Memorial Scholarship
2014

Related Work Experience

1 Dental Unit Detachment Petawawa
Petawawa, Ontario, Canada
2015-2018

1 Dental Unit Detachment Ottawa at Montfort Hospital
Ottawa, Ontario, Canada
2018-2019