Engineering Periodontal Tissue Regeneration with the Use of a Novel Periostin Electrospun Scaffold

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Graduate Program in Biomedical Engineering  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Engineering Science  
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ENGINEERING PERIODONTAL TISSUE REGENERATION WITH THE USE OF A NOVEL PERIOSTIN ELECTROSPUN SCAFFOLD

(Thesis format: Monograph)

by

Kendal Irene Creber

Graduate Program in Biomedical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Engineering Science

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London, Ontario, Canada

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Abstract

Clinical therapies for the treatment of periodontitis are unable to reproducibly stimulate regeneration of the periodontium. We assessed the use of a novel electrospun type I collagen scaffold containing recombinant periostin to stimulate regeneration of the periodontal ligament (PDL) and bone. Human PDL cells demonstrated the ability to form a mineralized matrix \textit{in vitro} and reduced osteogenic potential with increased donor age. \textit{In vitro} analysis indicated scaffolds were biocompatible, however, periostin did not significantly influence adhesion or growth. In the healing of fenestration defects in rats, type I collagen scaffolds (with and without periostin) initially delayed cell infiltration and increased M2 macrophage recruitment, indicating either an elevated healing response or foreign body reaction. The incorporation of periostin into scaffolds had a limited effect on inflammation, collagen synthesis, and bone formation \textit{in vivo}. Further optimization of periostin concentration and/or scaffold design is required to determine whether recombinant periostin influences periodontal healing.

Keywords

Periostin, Periodontal Regeneration, Periodontal Ligament, Electrospun Scaffold, Type I Collagen
Co-Authorship Statement

Thesis was written by K.I. Creber with recommendations, input, and revisions from Dr. D.W. Hamilton.

Experiments were designed by Dr. D.W. Hamilton. Experiments were performed by K.I. Creber with the following exceptions: fenestration defect surgeries were completed by S.S. Kim with assistance from K.I. Creber; scaffolds were developed and fabricated by X. Li (PhD candidate) under the supervision of Dr. J. Guan (Department of Materials and Science Engineering, The Ohio State University).
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**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Antibiotic-Antimycotic</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum album</td>
</tr>
<tr>
<td>CCL2/MCP1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>C-kit/CD117</td>
<td>Stem cell factor receptor</td>
</tr>
<tr>
<td>CXCL8/IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>CXCL10/IP-10</td>
<td>Interferon gamma-induced protein 10</td>
</tr>
<tr>
<td>CXCL11/I-TAC</td>
<td>Interferon-inducible T-cell alpha chemoattractant</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FSP-1</td>
<td>Fibroblast specific protein 1</td>
</tr>
<tr>
<td>GTR</td>
<td>Guided tissue regeneration</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>hPDL</td>
<td>Human periodontal ligament</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin 1 alpha</td>
</tr>
</tbody>
</table>
IL-1β Interleukin 1 beta

IL-1ra Interleukin 1 receptor antagonist

IL-6 Interleukin 6

IL-10 Interleukin 10

IL-12 Interleukin 12

IL-23 Interleukin 23

iNOS Inducible nitric oxide synthase

LOX Lysyl oxidase

LPS Lipopolysaccharide

NO Nitric oxide

PBS Phosphate buffered saline

PDL Periodontal ligament

PFA Paraformaldehyde

PMA Phorbol 12-myristate 13-acetate

pSmad3 Phosphorylated SMAD family member 3

RT-qPCR Quantitative reverse transcription polymerase chain reaction

Runx2 Runt-related transcription factor 2

SD Standard deviation

SEM Scanning electron microscopy

TGF-β Transforming growth factor beta
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
</tbody>
</table>
Chapter 1

1. Introduction

Increasingly prevalent with advancing age, periodontitis is a chronic inflammatory disease that affects the tooth supporting structures. Statistics show that over 25% of adults in Canada have periodontitis and it remains the leading cause of tooth loss due to irreversible bacterial destruction of the periodontium, which consists of the gingiva, cementum, PDL, and alveolar bone (Canadian Health Measures Survey 2010). Although current treatments are able to terminate the progression of periodontitis, the regeneration and restoration of a fully functional periodontium is difficult to achieve on a reproducible basis. It has long been established that cells within the PDL are capable of differentiating into fibroblasts, cementoblasts, and osteoblasts (Melcher 1976), however, the periodontium has a limited capacity for regeneration (Shimono et al. 2003, Chen & Jin 2010). Although guided tissue regeneration (GTR) has demonstrated that tissue regeneration can be improved by controlling cell migration and in particular, impeding the migration of oral epithelium, clinical outcomes remain highly variable (Nyman et al. 1982a, Nyman et al. 1982c). Furthermore, despite significant advances in our understanding of the biology of the periodontium, there is still much debate over the factors that influence its regeneration, particularly when factoring in the variability of individual patients.

1.1 Anatomy and Function of the Periodontium

The periodontium collectively refers to the gingiva, cementum, PDL, and alveolar bone. Under healthy conditions, these specialized tissues function to anchor teeth to the jaw, while facilitating the transmission of the mechanical forces and stresses associated with mastication (Figure 1.1) (Cho & Garant 2000, Nanci & Bosshardt 2006). However, damage to the periodontium, such as that associated with trauma or periodontitis, disrupts the normal occlusal contacts and force distribution, limiting the jaw’s ability to withstand stress. In order to fully understand how the periodontium is affected by disease, it is first crucial to understand the function of each tissue under normal conditions.
Figure 1.1: Anatomy of the periodontium. a) The periodontium collectively refers to the gingiva, cementum, alveolar bone, and PDL. b) The gingiva consists of an epithelium and connective tissue. The junctional epithelium, located at the cemento-enamel junction, is critical in preventing harmful microorganisms from damaging underlying tissues. c,d) The cementum, consisting of acellular and cellular cementum, covers the root surface to facilitate attachment of the tooth to the surrounding alveolar process. The PDL is an unmineralized tissue that has specialized collagen bundles, which anchor into the neighbouring cementum and alveolar bone where they become mineralized and are called Sharpey’s fibres. Diagrams are representative of human periodontium anatomy, histology is Masson’s Trichrome staining of rat maxillary molars.
Figure 1.1: Anatomy of the periodontium.
1.1.1 Gingiva

The gingiva is composed of oral epithelium and underlying connective tissue. The principal role of the gingiva is to protect underlying tissues from infection by acting as a barrier to bacteria. In particular, proper attachment of the gingiva to the tooth at the dento-gingival junction is critical for exclusion of bacteria from the subgingival periodontium. The dento-gingival junction is comprised of sulcular epithelium, which lines the gingival sulcus, and junctional epithelium, which attaches to the cementum at the base of the gingival sulcus extending to the cemento-enamel junction (Figure 1.1b) (Schroeder 1986, Wilson 2006b, Nanci 2013). The connective tissue of the gingiva (lamina propria) is highly collagenous (predominantly type I collagen), containing elastin and oxytalan fibres in addition to vascular and neural elements (Bartold et al. 2000). Collagen fibre bundles within the gingival connective tissue facilitate the attachment of the gingiva to the cementum and periosteum of the alveolar process (Nanci 2013). Together the connective tissue and epithelium of the gingiva protect the cementum, PDL, and alveolar bone from the harsh environment of the oral cavity.

1.1.2 Cementum

The cementum is a mineralized layer of tissue firmly attached to the dentin of teeth, covering the outer surface of the tooth roots. The cementum has a role in the attachment of teeth to the surrounding bone through the PDL; however, it is also associated with tooth movement, repair, and the protection of dentin and pulp (Bosshardt 2005, Nanci 2013). Cementum can be divided into two types, acellular and cellular, both of which are avascular and have a matrix that largely consists of collagen and contains 45% to 50% hydroxyapatite (compared to 50% to 60% for bone and 67% in dentin) (Nanci 2013). As shown in Figure 1.1d, acellular (primary) cementum covers the root from the cemento-enamel junction to the apical third of the root and is primarily involved with the attachment of teeth to the jaw (Bosshardt & Schroeder 1991, Cho & Garant 2000, Goncalves et al. 2005). As the name implies, acellular cementum does not contain cells within the matrix of the tissue, yet cementoblasts are located on the tissue surface. Conversely, cellular (secondary) cementum has a structure similar to bone in that mature
cementoblasts secrete matrix around themselves and mature into cementocytes, which are surrounded by lacunae (Bosshardt & Schroeder 1991). Unlike acellular cementum, cellular cementum has only a minor role in attachment and is mainly associated with tooth movement and repair (Schroeder 1986, Bosshardt & Schroeder 1991, Bosshardt 2005, Wilson 2006b, Nanci 2013). As a whole the cementum is a key tissue in the attachment of teeth, functioning with the PDL and bone to secure teeth and disperse mechanical load resulting from occlusion. Attachment is facilitated by the presence of specialized collagen bundles which originate in the PDL and insert into the cementum where they are either fully or partially mineralized in acellular and cellular cementum, respectively (Nanci & Bosshardt 2006).

1.1.3 Alveolar Bone

The alveolar bone process is the region of the jaw that houses the alveoli (sockets in which the teeth sit). It consists of trabecular bone with an outer (buccal or lingual) cortical plate and an inner alveolar bone that lines the alveoli; the cortical plate and alveolar bone merge at the alveolar crest, as shown in Figure 1.1c (Cho & Garant 2000, Nanci 2013). The inner surface of the alveolar bone (termed bundle bone) is the region in which fibres from the neighbouring PDL insert and become partially mineralized as Sharpey’s fibres. Bundle bone has less intrinsic collagen and a coarse-fibered texture compared to the lamellar bone which constitutes the rest of the alveolar bone (Bosshardt & Schroeder 1991, Cho & Garant 2000, Goncalves et al. 2005, Wilson 2006a, Nanci 2013). As a part of the periodontium, alveolar bone has key roles in the attachment of teeth to the jaw and tooth movement, while the alveolar process as a whole also has a role in mechanical stability and the distribution of occlusal forces.

1.1.4 Periodontal Ligament

The PDL is an unmineralized connective tissue located between the cementum and alveolar bone ranging from 150 to 380 µm wide (Bosshardt & Schroeder 1991, Nanci 2013). The primary function of the PDL is to facilitate the attachment of teeth to the jaw while absorbing the forces of mastication; this is largely achieved by the presence of thick collagen bundles (mainly type I and III) that traverse the width of the PDL and
extend into the cementum and alveolar bone (Lukinmaa & Waltimo 1992, Zhang et al. 1993, Bartold & Narayanan 2006, Nanci 2013). Collagen bundles are formed during tooth development, extending into the cementum and alveolar bone where they become mineralized; these embedded portions of the fibres are called Sharpey’s fibres (Figure 1.1c). The PDL also contains oxytalan fibres which run perpendicular to the collagen bundles; together the fibres form a network which surrounds and supports the tooth (Berkovitz 1990). The PDL also has a sensory role, in particular, receptors within the PDL are essential for proprioception during mastication, enabling proper placement of the jaws and controlling bite force.

The PDL is a unique tissue that exhibits the characteristics of other ligaments and tendons, such as having high matrix turnover and responsiveness to mechanical loading. The PDL is recognized to have one of the fastest collagen turnover rates of any tissue in the body and is constantly undergoing remodelling, especially during tooth eruption and in response to orthodontic movement (Beertsen et al. 2007). Furthermore, the PDL has the capacity to undergo remodelling according to functional demand, such that a decrease in mechanical loading of the tooth will result in a narrowed ligament with thinner collagen bundles, whereas an increase in function will yield thicker collagen bundles and a wider PDL (Esashika et al. 2003, Nanci & Bosshardt 2006). This remodelling response is particularly evident during the development of the PDL as the formation of collagen bundles occurs during tooth eruption but will not thicken until forces are exerted on the tooth (Nanci 2013). It is this sensitive response to mechanotransduction combined with the rapid rate of collagen turnover that makes the PDL such an essential tissue in maintaining the functional attachment of teeth to the jaws.

1.2 Periodontal Disease

Periodontal disease is a term used to refer to diseases that affect any of the tissues of the periodontium (Ranney 1993). The most prevalent forms of periodontal disease are gingivitis and periodontitis; however, other conditions classified as periodontal disease include necrotizing ulcerative periodontal diseases, periodontal abscesses, endodontic lesions, and mucogingival deformities (Armitage 2000, Wiebe & Putnins 2000). Damage to periodontal tissues is most commonly associated with plaque accumulation but can
also occur as a result of systemic diseases that affect the immune system or inflammatory response, such as diabetes, neutropenia, leukemia, and tuberculosis, as well as several genetic disorders (Wiebe & Putnins 2000, Pihlstrom et al. 2005). For the purposes of this thesis, the development of plaque induced chronic periodontitis and its precursor, gingivitis, will be discussed in more detail. For a more comprehensive overview of other periodontal diseases see R. Ranney’s “Classification of periodontal diseases” which discusses the classification criteria outlined in 1989 by the American Academy of Periodontology in addition to several publications which outline the updates made to this classification system in 1999 (Ranney 1993, Armitage 2000, Wiebe & Putnins 2000).

1.2.1 Development of Gingivitis and Chronic Periodontitis

Microorganisms found in the oral cavity naturally form colonies on the tooth surface, establishing a soft layer of biofilm that is easily removed by brushing and flossing. Although, if not removed within 48 hours, these biofilms harden into highly calcified dental calculus (tartar), requiring professional cleaning to be removed (Pihlstrom et al. 2005). The accumulation of dental calculus allows pathogens to release destructive enzymes and activate host immune response, leading to inflammation and bleeding of the gingiva, called gingivitis (Wiebe & Putnins 2000). Plaque-induced gingivitis is the most widespread form of periodontal disease, affecting an estimated 50-90% of the adult population worldwide (Pihlstrom et al. 2005) and at least 32% of adult Canadians (Canadian Health Measures Survey 2010). While the inflammation associated with gingivitis can cause discomfort to the patient, the underlying tissues of the periodontium remain intact and the effects are usually reversible if treated properly.

If gingivitis remains untreated, the build-up of dental calculus will frequently disrupt the attachment of the junctional epithelium to the teeth and expose sub-gingival tissues to bacterial pathogens and the disease is reclassified as periodontitis. Once the sub-gingival tissues are exposed, the host immune response becomes more aggressive and tissue destruction becomes accelerated and irreversible. In the US, over 47% of adults are estimated to have periodontitis; within Canada, 16% of adults have been diagnosed with moderate periodontitis and an additional 4% with advanced periodontitis (Canadian Health Measures Survey 2010, Eke et al. 2012). Without invasive treatment to cease
disease progression, periodontitis causes severe gingival recession and leads to the destruction of the PDL and resorption of the alveolar bone, eventually resulting in tooth loss.

1.2.1.1 Pathogens in Periodontal Inflammation and Activation of Innate Immune Response

The principal bacterial strain associated with plaque-induced periodontal disease is *Porphyromonas gingivalis*, although other pathogens associated with the condition include *Tannerella forsythensis*, *Treponema denticola*, and *Actinobacillus actinomycetemcomitans* (Socransky et al. 1987, Ximénez-Fyvie et al. 2000, Pihlstrom et al. 2005). Microorganisms influence disease progression by producing virulence factors and immunosuppressive factors to interfere with the host defense mechanism (Guthmiller 2002). Furthermore, they stimulate the destruction of tissues directly through the release of collagenases and numerous other hydrolytic, proteolytic, and lipolytic enzymes, while also influencing tissue degradation indirectly by activating host immune response (Pihlstrom et al. 2005, Bascones-Martinez et al. 2009). In particular, lipopolysaccharide (LPS) is a virulence factor in gram-negative bacteria which induces host immune response and is commonly associated with periodontal inflammation (Bascones-Martinez et al. 2009, Li et al. 2012). LPS interacts with toll-like receptors (TLR2, TLR4) to activate a number of signalling cascades which stimulate the innate immune response through the production of inflammatory cytokines (Bascones-Martinez et al. 2009, Li et al. 2012). These interactions between host cells and microbes perpetuate a chronic inflammatory response, which accelerates destruction of the periodontium.

1.2.2 Treatment of Periodontitis

Currently approved treatments for the regeneration of periodontal tissues consist of a number of different strategies to terminate the progression of the disease and help stimulate repair. All treatments involve open-flap debridement procedures to cease disease progression and control the biofilm that is prevalent in the affected tissues. Following debridement, a number of further treatments can be employed such as guided tissue regeneration, bone grafts or implantation of osteoinductive materials, and growth
factor or protein delivery. Detailed descriptions of these treatments will be discussed below.

1.2.2.1 Open Flap Debridement

Open flap debridement is a surgical procedure in which the gingiva is temporarily pulled back to allow for the removal of granulation tissue and bacteria followed by scaling (debridement) and resurfacing of the root surface (Nyman et al. 1982c, Egelberg 1987). Treatment may also include conditioning of the root surface with citric acid to further detoxify the root surface and promote connective tissue attachment (Meyer 1986, Egelberg 1987, Ramseier et al. 2012). The gingiva is then sutured back into place and the tissues are allowed to repair. The principal goal of this treatment is to remove bacteria which has infiltrated the periodontal pocket, preventing further damage to the periodontal tissues and creating an micro-environment which favours tissue repair (Chen et al. 2010). Open flap debridement has been proven to be more effective in reducing pocket probing depth, and gain of clinical attachment level compared to supra-gingival debridement alone, especially in patients with deep periodontal pockets (greater than 6 mm) (Heitz-Mayfield et al. 2002, Van der Weijen & Timmerman 2002). However, full regeneration of the cementum, alveolar bone, and PDL is often difficult to achieve as epithelial cells and gingival fibroblasts will migrate into the periodontal pocket, forming a long junctional epithelium along the root surface, inhibiting regeneration of the cementum and PDL (Nyman et al. 1982c, Bartold et al. 2003, Polimeni et al. 2006). Furthermore, treatment does not usually improve the restoration of lost bone volume (Needleman et al. 2006). Therefore, while open flap debridement will prevent further degradation of tissues, it does not result in the regeneration of a fully functional periodontium and lack of clinical attachment can lead to tooth loss after repair.

1.2.2.2 Guided Tissue Regeneration

Designed to prevent the apical migration of oral epithelium during repair following open-flap procedures, GTR is a technique, which involves the use of a barrier membrane, positioned from the outer surface of the remaining alveolar bone to the cervical surface of the tooth. Placement of the membrane prevents bacterial infiltration and inhibits the
migration of gingival oral epithelium, while maintaining space to facilitate the regeneration of subgingival tissues (Ramseier et al. 2012). Originally proposed by Melcher and colleagues in 1976, development of the GTR technique was driven by the idea that progenitor cells in the PDL are capable of regenerating cementum, PDL, and bone if provided the opportunity to migrate into the periodontal pocket before oral epithelium and gingival fibroblasts (Melcher 1976, Nyman et al. 1982a). Membranes were first developed from non-resorbable polymers such as expanded polytetrafluoroethylene (ePTFE, GoreTex®) and required surgical removal of the membrane after repair (Zeichner-David 2006). Today, resorbable GTR membranes are more commonly employed, eliminating the need for a second surgery. Membranes are available in varying porosities and can be made from synthetic materials such as polyglycolic acid or polylactic acid, or natural materials such as collagen (Aurer & Jorgic-Srdjak 2005, Ramseier et al. 2012). GTR membranes have been extremely successful in preventing epithelial downgrowth and have been shown to provide improved healing outcomes compared to open-flap debridement alone (Laurell et al. 1998, Needleman et al. 2006, Ramseier et al. 2012). However, clinical outcomes are still highly variable and rarely result in regeneration of the lost architecture of the periodontium (Laurell et al. 1998, Zeichner-David 2006, Nickles et al. 2009, Chen & Jin 2010).

1.2.2.3 Bone Grafts or Implantation of Osteoinductive Materials

The use of bone grafts or osteogenic materials aims to improve bone regeneration in periodontal therapies and are often used in conjunction with the use of GTR membranes (Baldini et al. 2011). Bone grafts are available from autogenic, allogenic, or xenogenic sources, both of which are used clinically. Autogenic cancellous or cortical bone (harvested from iliac crest, calvaria, or intraoral sites) is considered the gold standard for bone reconstruction as immune response is limited and osteoinduction is more successful than with other grafting materials (Chen & Jin 2010, Clementini et al. 2011, Ramseier et al. 2012). However, autografts require the patient to undergo a separate surgery and expose the patient to potential complications such as donor-site morbidity and infection (Hallman & Thor 2008, Chen et al. 2010). As such, commercially available freeze-dried
bone allografts such as Grafton®, Lifenet®, and Transplant Foundation®, or xenogenic bovine mineral matrixes such as Bio-Oss® and OsteoGraf® are used when autologous sources are unavailable (Baldini et al. 2011, Ramseier et al. 2012). Unfortunately, the processing techniques used to minimize immunogenicity of allogenic and xenogenic bone grafts also reduces the effectiveness of grafts (Bauer & Muschler 2000, Chen & Jin 2010). In recent years, the use of synthetic and natural biomaterials with osteoinductive properties has gained popularity as an alternative to bone grafts. Some of the most common commercially available materials are hydroxyapatite, beta tricalcium phosphate, synthetic polymers, bioactive glass, coral-derived calcium carbonate, and collagen sponges (Chen & Jin 2010, Ramseier et al. 2012). While synthetic materials offer the advantage of tailored degradation time, and can be modified to minimize immune response, they are generally less bioactive and therefore less effective than natural grafts (Chen & Jin 2010). While animal studies have shown that grafts can be successful in improving regeneration in supra-alveolar defects (Polimeni et al. 2006), and there have been reports of reduced probing depth and increased clinical attachment compared to open flap debridement alone (Reynolds et al. 2003, Zeichner-David 2006), clinical outcomes are highly variable. Reviews of clinical outcomes indicate that grafting materials have limited osteoinductive capabilities, frequently resulting in fibrous encapsulation of the graft (Laurell et al. 1998, Bartold et al. 2003).

1.2.2.4 Growth Factor or Protein Delivery

The most recent efforts to advance technology in periodontal therapy involve the application of biologically active proteins (growth factors, matrix proteins) to help induce repair and enhance regeneration of tissues to restore clinical attachment levels. A number of growth factors have been shown to positively influence the proliferation and differentiation of cells from periodontal tissues in vitro and some have shown promising results in animal and human studies. Of particular relevance are platelet-derived growth factor (PDGF), insulin-like growth factor, transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMP -2, -4, -7, -12), and enamel matrix derivative (Bartold et al. 2003, Kaigler et al. 2006, Zeichner-David 2006, Elangovan et al. 2009). While some of these factors have had success in improving tissue regeneration in vivo, results are
variable between patients as delivery remains a challenge as recombinant proteins quickly dilute and become extremely unstable *in vivo* (Bartold et al. 2003, Kaigler et al. 2006, Zeichner-David 2006). One approach to solve the problem of protein instability is gene therapy, which employs vectors to deliver the gene encoding for the protein of interest either directly to cells within the wound or to cells which are subsequently delivered to the wound area (Kaigler et al. 2006, Chen & Jin 2010). While gene therapy is an attractive approach as they can be applied with minimally invasive procedures and allow for longer activity times compared to topical application, the high cost, potential complications, and patient apprehension to gene therapies, make it less feasible for periodontal treatments (Kaigler et al. 2006, Elangovan et al. 2009, Ramseier et al. 2012). Another approach is the use of biodegradable carriers to deliver immobilized growth factors or proteins, allowing for localized delivery, and where required, sustained release of the protein, thus solving many of the problems associated with topical treatments. The use of biodegradable carriers will be discussed in more detail in later sections. A number of carriers have been developed for growth factor or protein delivery. Materials such as hydrogels and beta-tricalcium phosphate have been used in periodontal regeneration (Chen et al. 2010), however, the majority of protein-based therapies for periodontal regeneration are not able to yield consistent and predictable outcomes in a clinical setting (Stavropoulos & Wikesjö 2012).

**1.3 Healing and Regeneration of the Periodontium**

Wound healing of the periodontium generally conforms to the same principles as other tissues, characterized by three overlapping, yet distinct phases 1) inflammation, 2) proliferation, and 3) tissue remodelling. However, in order to regenerate a fully functional periodontium, the healing capacity of all tissues involved (gingiva, cementum, PDL, alveolar bone) must be considered and this varies considerably as will be discussed. First, the basic responses of the three phases will be discussed.
1.3.1 Phases of Wound Healing

1.3.1.1 Inflammation

Immediately after wounding, haemorrhage occurs followed by blood vessel constriction and clot formation. Composed primarily of fibrin, plasma fibronectin, and cellular components such as platelets, the clot serves to cease bleeding, protect injured tissues, and acts as a provisional matrix to facilitate the migration of inflammatory cells into the wound (Grzesik & Narayanan 2002, Polimeni et al. 2006, Guo & DiPietro 2010). Within hours of wounding, the early inflammatory response is initiated as cells within the clot and surrounding tissues secrete growth factors, pro-inflammatory cytokines, and chemokines (Guo & DiPietro 2010, Smith et al. 2014). As a result, neutrophils, macrophages, and lymphocytes are recruited into the clot where they prevent infection through the removal of bacteria, debris, and necrotic tissue (Polimeni et al. 2006). As healing reaches the late inflammatory phase (generally within 3 days of wounding), macrophages transition from a pro-inflammatory (M1) phenotype to a reparative (M2) phenotype (Guo & DiPietro 2010, Davies et al. 2013, Smith et al. 2014). As such, macrophages play a key role in the transition from the inflammatory to the proliferative phase of wound healing, although the two phases overlap considerably.

Macrophage Polarization

Classically activated, M1 macrophages are associated with early immune response and are stimulated by the pro-inflammatory cytokines interferon γ (IFN-γ), LPS, and tumour necrosis factor α (TNF-α) (Mantovani et al. 2004, Benoit et al. 2008, Hao et al. 2012). M1 macrophages are characterized by the production of inducible nitric oxide synthase (iNOS) which catalyzes the conversion of L-arginine into nitric oxide (NO), an agent involved with innate immunity (Bogdan et al. 2000, Coleman 2001). Conversely, M2 or alternatively activated macrophages are associated with the later stages of the immune response, promoting tissue remodelling, repair, and angiogenesis (Mantovani et al. 2004). M2 macrophages are characterized by the production of Arginase I, which catalyzes the conversion of L-arginine into L-ornithine and urea (Mantovani et al. 2004, Munder 2009, Hao et al. 2012). Arginase I promotes repair by reducing the amount of L-arginine available for conversion by iNOS, thus limiting NO production; furthermore, the product
L-ornithine can be further metabolized to form L-proline, a key building block of collagen (Munder 2009). Furthermore, the secretion of cytokines by M2 polarized macrophages reduces inflammation and recruits fibroblasts and endothelial cells to stimulate the formation of granulation tissue and promote angiogenesis.

1.3.1.2 Proliferative Phase

Occurring between 2 and 10 days after wounding, the proliferative phase is characterized by the deposition of granulation tissue and wound contraction (Gurtner et al. 2008, Smith et al. 2014). As discussed, M2 macrophage-associated cytokines induce the migration and proliferation of fibroblasts and endothelial cells. Fibroblasts secrete collagen and other matrix components to produce a collagen-rich (mainly type III collagen) granulation tissue, while endothelial cells participate in angiogenesis (Grzesik & Narayanan 2002, Guo & DiPietro 2010, Smith et al. 2014). Towards the end of the proliferative phase, some fibroblasts within the wound differentiate into myofibroblasts. Characterized by expression of α-smooth muscle actin, myofibroblasts have a contractile network of microfilaments that transmit stress to facilitate wound closure (Gabbiani 2003, Polimeni et al. 2006, Smith et al. 2014). Once the clot has been replaced with granulation tissue and the wound approaches closure, healing enters the tissue remodelling phase.

1.3.1.3 Tissue Remodelling

The tissue remodelling phase of wound healing begins between 2 and 3 weeks and continues for up to a year or more, however timing is highly dependent on wound size and the tissue which is being considered (Gurtner et al. 2008, Smith et al. 2014). Early in the remodelling phase, macrophages, myofibroblasts, and endothelial cells undergo apoptosis and excess capillaries, which were required to promote granulation tissue formation, regress (Guo & DiPietro 2010, Smith et al. 2014). Throughout the remodelling phase, the provisional granulation tissue matrix is replaced with an extracellular matrix composed mainly of type I collagen (Gurtner et al. 2008). Over time, matrix remodelling transforms the provisional matrix to be more analogous to that of the healthy tissue, with a structure more analogous to the healthy tissue and improved mechanical properties (Gurtner et al. 2008).
1.3.2 Healing of Periodontal Tissues

1.3.2.1 Gingiva

Gingival epithelium is recognized to have a high capacity for repair following inflammation or trauma (Bartold et al. 2003). Interestingly, while wound healing in most other tissues (such as skin) results in the formation of scar tissue with inferior mechanical properties compared to native tissue, gingival tissue will regenerate with a functional ECM, including restoration of connective tissue fibre orientation and attachment (Melcher 1976, Gurtner et al. 2008). However, the rapid proliferation and migration of gingival epithelium often prevents functional regeneration of the gingival connective tissue following clinical treatments (Melcher 1985, Needleman et al. 2006). With regards to the influence of the gingiva on healing of the periodontium as a whole, the stability of the gingival flap as a result of suturing has been shown to be of crucial importance in ensuring primary wound closure to protect underlying tissues and allow repair (Wikesjo & Selvig 1999).

1.3.2.2 Cementum

As reviewed by Wikesjo and Selvig, healing at the cementum-PDL interface can result in four possible outcomes 1) encapsulation 2) deposition of new cementum 3) resorption of the root surface followed by deposition of new cementum 4) or ankylosis (Wikesjo & Selvig 1999). Unfortunately, little is understood about the mechanisms that dictate which response will result and clinical success of cementum regeneration is highly variable. In particular, the presence of endotoxins from pathogens associated with periodontitis alters cementum structure and reduces the capacity for regeneration; as such, the more coronal cementum is generally less likely to reform a functional connective tissue attachment (Beertsen et al. 2007). Furthermore, cells originating from the PDL are responsible for the repair of cementum; thus, proximity to the healthy PDL enhances regeneration of the apical portions of cementum (Grzesik & Narayanan 2002, Chen & Jin 2010, Ramsey et al. 2012).
1.3.2.3 Alveolar Process and Alveolar Bone

Regeneration of the alveolar process and alveolar bone is achieved through intramembranous ossification (Melcher 1976). Unlike endochondral ossification, which involves the formation of cartilage as an intermediate, intramembranous ossification involves the division and subsequent differentiation of progenitor cells located within the osteogenic layer of the periosteum (Oryan et al. 2015). Healing of the alveolar process varies slightly from standard intramembranous ossification. While cells from the mucoperiosteum and endosteum (which includes the alveolar bone surface) participate in healing of the alveolar process, progenitor cells originating from the PDL also contribute to repair (Melcher 1976).

1.3.2.4 Periodontal Ligament

Regeneration of the PDL has been regarded by many as the key to successful periodontal regeneration (Meyer 1986, Beertsen et al. 2007). As the principal tissue responsible for the formation of fibrous attachment of cementum to alveolar bone, regeneration of the PDL is of paramount importance. Moreover, progenitor cells originating from the PDL are capable of differentiating into cementoblasts and osteoblasts to contribute to the regeneration of cementum and alveolar bone, respectively (Melcher 1976, Meyer 1986, Polimeni et al. 2006). While the application of GTR membranes to maintain space for PDL cells to repopulate the root surface has had some success in regeneration of a connective tissue attachment, bone will usually regenerate first, resulting in ankylosis. Moreover, the use of bone grafts often inhibits regeneration of the PDL by acting as a physical barrier to cell migration. As such, clinical regeneration of the PDL is unpredictable, and generally results in repair rather than regeneration (Aukhil et al. 1983, Shimono et al. 2003, Beertsen et al. 2007).

1.3.2.5 Periodontal Regeneration

Optimal regeneration of the periodontium has been characterized by many as the restoration of alveolar bone height, the formation of new cementum, and regeneration of the PDL with connective tissue fibre attachment to cementum and alveolar bone (Bartold et al. 2003, Beertsen et al. 2007, Chen et al. 2010). In order to ensure tissue regeneration
rather than repair, all the required cell types and molecular signals must be spatially and temporally available (Grzesik & Narayanan 2002). Periodontal therapies have aimed to control the availability of cells to improve repair (GTR membranes and bone grafting) and more recently, therapies have tried to mimic signals associated with repair processes (growth factor or protein delivery); however, these treatments have still not resulted in reliable, predictable outcomes for periodontal regeneration. There is a need for the development of PDL-targeted therapies to enhance regeneration and restore clinical attachment on a reliable, consistent basis.

1.4 Development of a Scaffold for Periodontal Regeneration

Tissue engineering combines the use of scaffolds, cells, and biologically active molecules to restore, maintain, or improve tissue function (Chan & Leong 2008, O'Brien 2011). With respect to periodontal regeneration a scaffold-based therapy to stimulate regeneration is favourable, as the recruitment of endogenous cells eliminates many of the issues associated with cell-based therapies, such as cell acquisition, host rejection, and patient apprehension (Kaigler et al. 2006, Elangovan et al. 2009, O'Brien 2011, Neel et al. 2014). Specifically, the use of a bioactive scaffold provides the combined benefits of growth factor or protein therapies and biomaterial-based treatments to induce regeneration. The scaffold acts as a temporary ECM, mimicking the structure and functions of the native tissue to provide structural support, facilitate cell growth, signalling, and ultimately guide regeneration of the targeted tissue (Nair & Laurencin 2007, Dhandayuthapani et al. 2011a). These roles are achieved through control of scaffold material, architecture, and bioactivity, where bioactivity can refer to the presence of ligands in the material, topographical features of the scaffold construct, or the incorporation of growth factors, proteins, or drugs (Chan & Leong 2008, O'Brien 2011). Our laboratory focuses on the influence of matricellular proteins on wound healing and we hypothesize that the use of matricellular proteins found in healthy PDL and bone, delivered through a biodegradable scaffold, will stimulate tissue regeneration.
1.4.1 Scaffold Material

Scaffold materials can be classified as synthetic (synthetic polymers, ceramics, glasses) or natural (proteins such as collagen or fibrin, polysaccharides such as chitosan, or decellularized tissue extracts). Synthetic polymers are desirable as they can be manufactured with less batch variability and therefore have more predictable properties. However, synthetic materials often impede cell attachment, requiring additional processing steps or surface treatments to improve adhesion; this also allows for tailorability, such as targeting for the adhesion of specific cell types (Chan & Leong 2008). Natural materials offer the advantage of improved biocompatibility and bioactivity over synthetic materials as they possess cell-binding ligands and are able to more easily be degraded and remodelled (Dhandayuthapani et al. 2011a). The use of natural proteins, such as collagens, which are prominent in the native ECM of most connective tissues, is particularly attractive as cells can degrade and remodel the scaffold easily; moreover, degradation products are less harmful and in some cases directly contribute as building blocks for new tissue formation (Nair & Laurencin 2007, Dhandayuthapani et al. 2011a).

1.4.1.1 Type I Collagen

Collagens are the major structural component of most connective tissues, providing strength and maintaining the form of tissues. Type I collagen is the most abundant collagen in the body and the principal structural protein of the PDL and bone (Nanci & Bosshardt 2006, Cowin & Doty 2007), making it ideal for use as a scaffolding material. Type I collagen is particularly prominent in tendons, ligaments, skin and bone (Friess 1998). In tendons and ligaments (including the PDL) collagen fibres are aligned in parallel to resist high levels of tensile forces (Hulmes 2008). In bone, type I collagen acts as scaffolding for mineralization to occur and provides mechanical stability after mineralization, improving tensile strength, stiffness, and load bearing properties. Moreover, the gaps between collagen molecules within a fibril act as nucleation sites for the deposition of hydroxyapatite crystals, helping to facilitate the mineralization and development of bone and cementum (Raspanti et al. 2000, Nanci & Bosshardt 2006, Cowin & Doty 2007). Collagens also have a crucial role in signalling and can activate cellular signalling pathways to influence adhesion, growth, differentiation, survival, and
ECM remodelling (Gelse et al. 2003, Hulmes 2008, Yamauchi & Sricholpech 2012). Type I collagen has also been shown to regulate cellular functions through the storage and delivery of growth factors. This ability to bind to and release growth factors and cytokines has prompted the use of collagens as biomaterials for the delivery of therapeutic materials (Gelse et al. 2003).

Type I collagen has been successfully used in a number of biomedical applications, including GTR membranes (Aurer & Jorgic-Srdjak 2005). In addition, methods to isolate and purify collagen from a variety of sources have been optimized to solubilize collagen while leaving the triple helix of molecules intact and subsequent processing produces pure collagen with low immunogenicity (Miller & Rhodes 1982, Friess 1998, Ramshaw et al. 2008, Walters & Stegemann 2014). Therefore, due to its prominent role in the PDL, bone, and as a biomaterial, type I collagen is a promising scaffolding material for the regeneration of the periodontium and was selected for our scaffold.

1.4.2 Scaffold Architecture and Manufacturing

Architecture of the scaffold is a primary concern in dictating cellular response and tissue development, by influencing cell adhesion, migration, and nutrient delivery. Type I collagen has been used as a biomaterial in the form of sheets, tubes, sponges, powders, injectable solutions, and dispersions (Friess 1998, Chattopadhyay & Raines 2014). A number of different methods are available for creating porous collagen-based scaffolds, some of which will be discussed in more detail below.

Freeze-drying or lyophilization is commonly used to produce collagen sponges or hydrogels (Annabi et al. 2010, Dhandayuthapani et al. 2011f). In this process, collagen is suspended in an acidic solvent (usually acetic acid) which is subsequently removed by sublimation to produce a porous collagen structure (Annabi et al. 2010). Specifically, the collagen suspension is frozen to produce ice crystals within the collagen suspension (the size and morphology of which can be controlled by freezing temperature and time), sublimation of the solvent is then achieved under high vacuum while maintaining temperature below the solvent freezing point (Charles 1964, Faraj et al. 2007, Offeddu et al. 2015). The result is a scaffold with interconnected pores. Moreover, scaffold size and
shape can be controlled by performing freeze-drying within a mould to create sheets, disks, or tubes to name a few. Salt/particle leaching incorporates a porogen (in the case of collagen usually salt particles) into a collagen suspension; solidification of the collagen is followed by removal of the porogen by leaching or dissolution to produce a porous collagen scaffold (Annabi et al. 2010, Dhandayuthapani et al. 2011f). Rapid prototyping is an emerging field in the development of scaffolds for tissue engineering and enables the production of complex and precise scaffold architectures on a reproducible basis (Dhandayuthapani et al. 2011f). For example, Kim and colleagues have produced a 3D grid shaped collagen scaffold using a process that combines 3D plotting and freeze-drying (Kim et al. 2009). Electrospinning, which is described in more detail later, involves the formation of collagen fibres to produce a non-woven mesh-type scaffold.

Electrospinning of collagen fibres was selected for the proposed scaffold as it is a very efficient, rapid, and inexpensive method of fabricating biomaterials, factors which are key for manufacturing on a commercial scale (Chew et al. 2006, Kaigler et al. 2006). Furthermore, electrospinning parameters can be easily modified to vary fibre orientation, diameter, porosity, thickness of the construct, and degradation time to closely mimic the native ECM (Chew et al. 2006).

1.4.2.1 Electrospinning

Electrospinning is a process that employs electrostatic force to produce fibres on the nano- or micro-scale from synthetic or natural polymeric solutions. The polymer solution is pumped through a charged capillary to produce fibres, which are collected on a grounded or oppositely charged surface, creating an electric field between the capillary and collecting surface (Walters & Stegemann 2014). The key feature of electrospinning (compared to mechanical spinning methods) is the application of electric charge to the solution. Once a critical voltage is reached, the surface tension of the polymer solution is overcome, forming a Taylor cone at the capillary tip and producing a fine jet (Matthews et al. 2002, Liu et al. 2013). As the charged jet passes through the electric field, the solvent evaporates and bending instability of the jet occurs resulting in the deposition of randomly aligned dry fibres (Reneker et al. 2000, Chew et al. 2006). Collection on a rotating mandrel facilitates the even distribution of fibres to create a multi-layered, non-
woven scaffold. It is possible to produce aligned fibres through a variety of methods, most commonly by increasing rotation speed of the mandrel (Chew et al. 2006, Zhong et al. 2006, Shang et al. 2010, Liu et al. 2013). Fibre diameter and porosity of the scaffold can also be modified by altering the main electrospinning parameters 1) flow rate of solution through the syringe 2) concentration of polymer in solution 3) voltage 4) distance between capillary and collecting surface.

1.4.2.2 Cross-linking

A number of methods are available for cross-linking collagen-based biomaterials, including carbodiimides, glutaraldehydes, glyoxal, genipin, and UV light or gamma radiation to name a few (Friess 1998, Walters & Stegemann 2014). Glutaraldehyde is a commonly accepted method of collagen cross-linking and was selected as it offers quick reaction time, the ability to react with a large number of functional groups, and is relatively cost-effective (Friess 1998, Barnes et al. 2007, Walters & Stegemann 2014). While glutaraldehyde can potentially be cytotoxic (Gough et al. 2002), it is the most commonly used method of chemically cross-linking collagen (Parenteau-Bareil et al. 2010). Moreover, glutaraldehyde has been shown to result in a higher degree of cross-linking, thus imparting increased mechanical strength and slower degradation compared to other methods (Newton et al. 2009). Cross-linking of collagens with glutaraldehyde is a result of reactions between aldehyde groups of glutaraldehyde and the amine groups of lysine or hydroxylysine in collagen to form a Schiff base intermediate, followed by a number of subsequent reaction pathways, which each result in the formation of crosslinks (Damink et al. 1995).

1.4.3 Protein Incorporation

The development of bioactive scaffolds combines tissue engineering with protein delivery to modulate cell behaviour and repair. Scaffolds provide a matrix for cell adhesion and migration, and influence cell alignment and morphology (Chan & Leong 2008). However, the incorporation of proteins can impart more specific responses to stimulate tissue regeneration through integrin binding to activate cell signalling pathways and influence processes such as cell proliferation, differentiation, or cytokine production.
(Morris & Kyriakides 2014). In this thesis, for reasons outlined below, we will increase the activity of electrospun type I collagen scaffolds by incorporating recombinant human periostin into the fibres.

1.4.3.1 Periostin: Structure

The matricellular protein periostin was originally termed osteoblast-specific factor 2 when it was first identified from a murine osteoblastic cell line in 1993, and has since been renamed “periostin” due to localization in the periosteum and PDL (Takeshita et al. 1993, Horiuchi et al. 1999). The 90 kDa, disulfide-linked secreted protein is considered a member of the Fasciclin gene family along with βIG-H3, stabilin-1, and stabilin-2 due to the presence of multiple Fas1 domains (Norris et al. 2007, Kudo 2011). Specifically, periostin’s structure consists of an amino terminal, EMI domain, 4 repeating Fas1 domains, and a C-terminal domain; there is also a heparin binding site at the C-terminal end and, like other members of the fasciclin gene family, it does not have a transmembrane sequence (Takeshita et al. 1993, Horiuchi et al. 1999, Kudo 2011). Periostin is also classified as a Gla-containing protein, resulting from the post-translational modification of glutamic acid residues into γ-carboxyglutamic acid (Gla) by the vitamin K-dependent enzyme γ-carboxylase (Coutu et al. 2008). Other Gla-containing proteins include osteocalcin and matrix Gla protein, both of which have roles in bone formation and mineralization (Coutu et al. 2008). There are at least five known isoforms of human periostin, resulting from alternative splicing events at the C-terminal end (Takeshita et al. 1993, Horiuchi et al. 1999, Yamada et al. 2014). Most recently, Yamada and colleagues have identified a PDL-specific isoform of periostin that has been suggested to have a regulatory effect on differentiation and mineralization in PDL cells (Yamada et al. 2014).

1.4.3.2 Periostin: Functions

Periostin is expressed in a number of tissues during development and in a variety of mature tissues throughout the body (Hamilton 2008, Conway et al. 2013). Expression is generally limited to highly collagenous or fibrotic tissues that are under constant exposure to mechanical stress, such as the PDL and periosteum. This localized expression
indicates a role in mechanotransduction, ECM remodelling, and the modulation of tissue structure in response to changing mechanical demands. Periostin has also been linked to certain pathologies and diseases; notably, periostin is upregulated in inflammation, fibrosis and wound healing and has been shown to influence cell proliferation and survival to accelerate tumour progression; a brief summary is given in Table 1.1.

The expression patterns of periostin in healthy and diseased tissues have indicated roles in processes associated with many aspects of wound healing. A number of *in vitro* studies revealed that periostin activates the AKT cell survival pathway in cancerous and healthy cells, including TNFα/LPS-challenged PDL cells (Bao et al. 2004, Baril et al. 2007, Bakhtyar et al. 2013, Merle et al. 2013, Padial-Molina et al. 2013, Ghatak et al. 2014). Moreover, periostin has been identified as an angiogenic factor in breast and colon cancers and has been shown to promote cell proliferation and migration to advance tumour progression (Bao et al. 2004, Shao et al. 2004, Tai et al. 2004, Baril et al. 2007, Hakuno et al. 2010, Bakhtyar et al. 2013, Ghatak et al. 2014). Stimulation of cell proliferation, migration, and angiogenesis are desirable effects in wound repair and tissue regeneration, as such the use of recombinant periostin could be beneficial in regeneration of the PDL.

Periostin has been shown to bind directly to structural ECM proteins such as collagen I, III, V, fibronectin, tenascin C, and to modulate their organization in a number of tissues, including the PDL (Norris et al. 2007, Bonnet et al. 2009, Takayama & Kudo 2012). Specifically, the EMI domain of periostin can bind to type I collagen and fibronectin, while the Fas1 domains are able to bind to tenascin C (Takayama et al. 2006, Norris et al. 2007, Kii et al. 2010, Kudo 2011). Notably, periostin associates with type I collagen fibrils to facilitate cross-linking and matrix assembly (Norris et al. 2007, Kii et al. 2010, Maruhashi et al. 2010, Sidhu et al. 2010). Moreover, periostin has been shown to enhance collagen expression in some specific instances (Ghatak et al. 2014, Yang et al. 2014). In addition to influencing collagen assembly, periostin can bind to fibronectin and tenascin C to facilitate their incorporation into the ECM (Kii et al. 2010, Maruhashi et al. 2010, Kudo 2011).
Table 1.1: Pathologies and diseases associated with the upregulation of periostin

<table>
<thead>
<tr>
<th>Pathology/Condition</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td><strong>Wounding</strong></td>
<td></td>
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<tr>
<td>Bone fractures</td>
<td>(Nakazawa et al. 2004)</td>
</tr>
<tr>
<td>Skin wounds; excisional, incisional, and cutaneous</td>
<td>(Jackson-Boeters et al. 2009), (Elliott et al. 2012), (Zhou et al. 2010), (Nishiyama et al. 2011)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>(Stanton et al. 2000), (Shimazaki et al. 2008)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>(Norris et al. 2008)</td>
</tr>
<tr>
<td><strong>Fibrotic Conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Bone marrow fibrosis</td>
<td>(Oku et al. 2008)</td>
</tr>
<tr>
<td>Fibrous dysplasia</td>
<td>(Kashima et al. 2009)</td>
</tr>
<tr>
<td>Keloid and hypertrophic scars</td>
<td>(Zhou et al. 2010)</td>
</tr>
<tr>
<td>Bronchial asthma, pulmonary fibrosis</td>
<td>(Takayama et al. 2006), (Uchida et al. 2012), (Roche et al. 1989)</td>
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<tr>
<td>Nifedipine-induce gingival enlargement</td>
<td>(Kim et al. 2013)</td>
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<tr>
<td><strong>Inflammation</strong></td>
<td></td>
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<tr>
<td>Atopic dermatitis</td>
<td>(Masuoka et al. 2012),</td>
</tr>
<tr>
<td>Airway hyperresponsiveness</td>
<td>(Gordon et al. 2011)</td>
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<tr>
<td>Abdominal aortic aneurysm</td>
<td>(Yamashita et al. 2013)</td>
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<tr>
<td>Atherosclerosis</td>
<td>(Hakuno et al. 2010)</td>
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<tr>
<td><strong>Cancer/Tumour Progression</strong></td>
<td></td>
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<tr>
<td>Lung carcinoma</td>
<td>(Sasaki et al. 2001), (Sasaki et al. 2003)</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>(Sasaki et al. 2002)</td>
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<tr>
<td>Ovarian cancer</td>
<td>(Gillan et al. 2002)</td>
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<tr>
<td>Breast cancer</td>
<td>(Sasaki et al. 2003), (Shao et al. 2004)</td>
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<tr>
<td>Colon/colorectal cancer</td>
<td>(Bao et al. 2004), (Tai et al. 2004)</td>
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<tr>
<td>Liver metastasis</td>
<td>(Tai et al. 2004)</td>
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</tbody>
</table>
In addition to modulating ECM synthesis and remodelling, periostin has been linked to early stages of bone formation and repair. Studies with Postn-null mice demonstrated that trabecular bone formation is reduced and adults exhibit dwarfism; moreover, nulls have increased bone damage in response to activity compared to wildtypes, revealing a role of periostin in the development and maintenance of bone (Rios et al. 2005, Bonnet et al. 2009, Bonnet et al. 2013). Furthermore, Bonnet and colleagues used Postn-null mice to show that periostin inhibits Sost (Sclerostin, a protein which inhibits Wnt signalling, and induces osteoblast apoptosis), thus promoting bone formation (Bonnet et al. 2009). Periostin has also been shown to localize to mineralized nodules formed by mesenchymal stromal cells induced to undergo osteogenic differentiation (Coutu et al. 2008), further supporting that periostin is involved with bone formation. Taken together these findings suggest that incorporation of periostin into the scaffold could facilitate assembly and remodelling of the ECM to stimulate wound healing, while also improving bone regeneration.

1.4.3.3 Periostin and the Periodontium

The expression of periostin within the periodontium and examination of its role in the development, maintenance, and repair of periodontal tissues has been extensively investigated to date (Takayama & Kudo 2012). Within the mature periodontium, expression is restricted to the PDL and periosteum of the alveolar bone, with intense staining in Sharpey’s fibres, the principal fibres responsible for tooth attachment (Dangaria et al. 2009, Kashima et al. 2009, Wen et al. 2010). A number of studies have elucidated the role of periostin in the transmission of mechanical forces within the PDL and how it affects tissue structure and maintenance of a functional PDL. In particular, Postn-null mice will develop a widened PDL and periodontitis-like symptoms characterized by root resorption and increased osteoclast activity (Rios et al. 2005, Kii et al. 2010); however, mechanical unloading of teeth will reverse this effect, indicating that periostin expression is required for remodelling of the PDL in response to occlusal loading (Rios et al. 2008). The role of periostin in the PDL is further corroborated with findings from experimental (orthodontic) tooth movement models. In the healthy PDL, periostin expression is upregulated on the compressed side of the PDL during orthodontic
tooth movement (Wilde et al. 2003, Wen et al. 2010, Watanabe et al. 2012). However, nulls exhibited a widened PDL and decreased expression of collagenolytic enzymes normally expressed in the compressed PDL in response to orthodontic movement, designating that periostin has a role in activation of enzymatic remodelling of the PDL in response occlusal loading (Lv et al. 2013). Interestingly, while prominent in the healthy PDL, expression of periostin is significantly decreased in response to periodontal inflammation, further indicating the role periostin in the maintenance of PDL structure and function (Padial-Molina et al. 2012).

1.5 Rationale and Hypothesis

The proposed approach aims to target periodontal regeneration in multiple ways. The use of a scaffold will provide a matrix for PDL cell migration and act as a provisional ECM to accelerate wound healing while the addition of recombinant periostin is expected to stimulate regeneration of the PDL and bone. The specific expression of periostin in the PDL and periosteum, suggests a role in maintaining homeostasis of the PDL and periodontium as a whole (Horiuchi et al. 1999, Kruzynska-Frejtag et al. 2004, Afanador et al. 2005). Moreover, the role of periostin in ECM organization and remodelling in response to mechanical stimuli, suggests that periostin has a key role in maintaining the structural integrity and function of the PDL. Furthermore, expression of periostin during bone development and *in vitro* osteogenesis, suggests a role in bone formation. Decreased expression of periostin in the PDL as a result of periodontal inflammation further elucidates a potential role in maintaining a functional PDL. Therefore, we hypothesized that by re-introducing recombinant human periostin via an electrospun collagen scaffold, periodontal regeneration can be stimulated. Following debridement, the scaffold will be placed on the root surface and within the fenestration defect, in contact with the remaining PDL, cementum and bone, as shown in Figure 1.3.

**Hypothesis:**

The use of an electrospun type I collagen scaffold containing recombinant periostin will increase adhesion, proliferation, and matrix deposition by hPDL cells *in vitro* and will
recruit and activate PDL cells *in vivo* to stimulate the regeneration of the PDL and alveolar bone.

**The specific objectives of this thesis were:**

1. **Characterization of hPDL Cells:** To further elucidate the unique properties of human PDL (hPDL) cells, focusing on their ability to form a mineralized matrix *in vitro*.

2. **Cellular Response to the Electrospun Scaffolds *in vitro***: To assess cellular response to the electrospun type I collagen and recombinant periostin scaffold *in vitro*.

3. **Influence of the Electrospun Scaffolds on Fenestration Defect Healing:**
   Assessment of the electrospun type I collagen and recombinant periostin scaffold on early healing of periodontal fenestration defects.
Figure 1.3: Electrospun type I collagen and periostin scaffold for periodontal regeneration. a) Periodontitis is characterized by inflammation of the gingiva, root resorption, and deterioration of cementum and PDL. b) Open flap procedure to debride root surface. c) Electrospun type I collagen scaffold containing recombinant periostin positioned along the root surface to stimulate regeneration of the PDL. A GTR membrane can be used in combination with the scaffold. d) Scaffold is expected to enhance regeneration of the PDL and periodontium as a whole.
Figure 1.2: Electrospun type I collagen and periostin scaffold for periodontal regeneration.
Chapter 2

2. Methodology

2.1 Scaffold Fabrication

Scaffolds were developed in collaboration with Dr. J. Guan (The Ohio State University) and the scaffolds used in this thesis were made by X. Li (PhD candidate) under the supervision of Dr. J. Guan. The collagen and periostin/collagen scaffolds were fabricated using a conventional electrospinning apparatus, shown schematically in Figure 2.1. Type I collagen (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to produce a 15% w/v mixture. 3 mL of the collagen mixture was combined with 20 µL of human recombinant periostin (R&D Systems, Minneapolis, MN, USA) in phosphate buffered saline (PBS) at a concentration of 0.1% w/v. Collagen (control) scaffolds, contained 20 µL of 0.1% w/v bovine serum albumin (BSA) in PBS instead.

The collagen and periostin solution was loaded into a syringe and injected at a feed rate of 1 mL/h through a charged capillary (+15 kV) to produce uniform fibres which were collected on a rotating mandrel charged to -10 kV. During this process, the solvent evaporated, producing a type I collagen and periostin scaffold mat with a weight ratio of 1:22500 periostin to collagen. The electrospun scaffold, shown in Figure 2.2, was composed of randomly oriented type I collagen fibres between 2-7 µm in diameter and the scaffold was approximately 30 µm thick. Cross-linking of the scaffold was achieved by submerging the scaffold mat in a 5% glutaraldehyde-ethanol solution for 30 minutes, followed by several washes in anhydrous ethanol to remove any traces of glutaraldehyde.

For experiments, circular pieces of scaffold, 6 mm in diameter, were created with a biopsy punch to ensure uniform size. Scaffolds were then decontaminated in 70% ethanol for 30 minutes, followed by 3 rinses in sterile, deionized water. Decontaminated scaffolds were stored in sterile, deionized water for up to 24 hours prior to use in experiments.
Figure 2.1: Electrospinning of collagen and periostin/collagen scaffolds. A syringe pump was used to feed the electrospinning solution through a charged capillary (+15 KV) to produce fibres, which were collected on a rotating mandrel (-10 KV). The scaffold mat was then submerged in a 5% glutaraldehyde in ethanol solution for 30 minutes and rinsed thoroughly in 100% ethanol. A biopsy punch is used to produce uniform size scaffold pieces, which were then decontaminated in 70% ethanol.
Figure 2.1: Electrospinning of collagen and periostin/collagen scaffolds.
Figure 2.2: Characterization of electrospun collagen and periostin/collagen scaffolds. Scanning electron microscopy was used to characterize the a) collagen scaffold b) periostin/collagen scaffold. Both scaffolds were composed of randomly aligned fibres approximately 2-7 µm in diameter (as indicated between arrows in a,b). c) Representative image, showing that scaffolds are approximately 30 µm thick (collagen scaffold shown).
Figure 2.2: Characterization of electrospun collagen and periostin/collagen scaffolds.
2.2 Isolation of Human Periodontal Ligament Cells

Human periodontal ligament (hPDL) cells were isolated from samples of healthy periodontal ligament obtained from patients between 20 and 65 years of age who underwent routine extractions at the Oral Surgery Clinic at The University of Western Ontario. The use of this tissue was under informed patient consent and in accordance with the guidelines of the University's Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB; Appendix A). hPDL cells were isolated using an explant technique as described previously by Somerman and colleagues (Somerman et al. 1988). Briefly, tissue was removed from the middle third of the root surface, rinsed several times with Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 4.5 g/mL D-glucose (high glucose) and cultured for several weeks until outgrown cells reached confluence. Cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% AA (Antibiotic-Antimycotic; 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 mg/mL amphotericin B; Gibco, Grand Island, NY, USA). Cultures were maintained in a 5% CO₂ atmosphere with 100% relative humidity at 37°C. For passaging and use in experiments, cells were detached using a 0.05% trypsin and 0.2g/L EDTA-4Na solution. hPDL cells were used between passages 1 and 7 for experiments.

2.3 hPDL Cell Characterization: Mineralization Assays

Cells used for osteogenic differentiation experiments were analyzed by immunocytochemistry, Alizarin Red S staining, and quantitative reverse transcription polymerase chain reaction (RT-qPCR), were seeded at a density of 200,000 cells per well of a 6-well plate. Cells were serum-starved prior to experiments for 24 hours in MEM Alpha (Minimal Essential Medium Alpha; Gibco, Grand Island, NY, USA) supplemented with 1% AA. To induce osteogenic differentiation, cells were cultured in MEM Alpha supplemented with 10% FBS, 1% AA, containing 14.58 mM Beta Glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) and 0.284 mM L-ascorbic acid (Sigma-Aldrich, St.
Louis, MO, USA), control samples were cultured in MEM Alpha with 10% FBS and 1% AA, and media was changed every 2-3 days.

2.3.1 Alizarin Red S Staining

Calcium deposition was visualized with Alizarin Red S staining and elution. Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 5 minutes, rinsed with PBS 3 times, followed by thorough washing with deionized water and were dried prior to staining. A 1% (w/v) solution of Alizarin Red S (Fisher Scientific, Waltham, MA, USA) was applied to each sample for 30 minutes followed by several rinses with deionized water to remove excess dye. Matrix calcification was quantified by extracting the dye. A solution of 0.5N HCl in 5% Sodium Dodecyl Sulfate (SDS; Sigma-Aldrich, St. Louis, MO, USA) was applied to dried samples for 10 minutes and absorbance was measured at 415 nm with a Safire microplate reader (Tecan Group Ltd, Maennedorf, Switzerland).

2.3.2 Quantitative Reverse Transcription Polymerase Chain Reaction Analysis (RT-qPCR)

Total RNA was extracted using Trizol® reagent (Ambion, Life Technologies, Carlsbad, CA, USA). A reaction volume of 15 µL, containing 25 ng RNA was amplified using qScript™ XLT One-Step RT-qPCR ToughMix® (Quanta BioSciences Inc., Gaithersburg, MD, USA). Taqman® (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) primers and probes were used for alkaline phosphatase (ALPL, Hs01029144_m1), osteocalcin (BGLAP, Hs00609452_g1), bone sialoprotein (IBSP, Hs00173720), runt-related transcription factor 2 (RUNX2, Hs00231692_m1), and cementum protein 1 (CEMP1, Hs04185363_s1), normalized to eukaryotic 18s (18s, 4354930E). RT-qPCR reactions were performed using the Prism 7900 HT Sequence Detector (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and analyzed with SDS v2.1 software.

2.4 Cellular Response to the Electrospun Scaffolds in vitro: Experiments with hPDL Cells

For adhesion, proliferation, and matrix deposition experiments on the electrospun scaffolds, cells were serum-starved for 24 hours prior to commencement of the
experiment in low glucose (1g/mL) DMEM with 0.5% AA. During experiments, cells were seeded in high glucose DMEM, supplemented with 10% FBS, 1%AA, and 50 ug/mL L-Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) with media changes every 2-3 days. For matrix deposition experiments, cells were seeded on 6 mm diameter scaffolds at a density of 50,000 cells per scaffold. Seeding densities for adhesion and proliferation specified within experiment description.

2.4.1 CyQUANT® Assay for Adhesion and Proliferation

For adhesion assays, cells were seeded in 96 well plates and 6mm diameter scaffolds at a density of 20,000 cells per well. 6 hours after seeding, non-adherent cells were removed by 3 rinses with warm, sterile, PBS before freezing plates at -80°C. To assess total cell number, cells were also seeded on a separate plate, pelleted within the plate, and media was removed prior to freezing.

For proliferation assays, cells were seeded at a density of 5,000 cells per well (low seeding density, to facilitate proliferation) in 96 well plates and on 6 mm diameter scaffolds. At the time of seeding, a microcentrifuge tube containing 500,000 cells was centrifuged and frozen at -80°C to create a standard curve. At 1, 3, 7 and 10 days post-seeding, non-adherent cells were removed by 3 rinses with warm, sterile, PBS and plates were frozen at -80°C.

Cell adhesion and proliferation was assessed with the CyQUANT® GR fluorescent dye (Molecular Probes, Life Technologies, Carlsbad, CA, USA) to quantify cell number based on DNA content, according to the manufacturer’s protocol. Briefly, samples were thawed and incubated with 250 µL CyQUANT® GR dye and cell lysis buffer solution for 10 minutes while vortexing frequently, standard curve samples were created using the cell pellet frozen at the time of seeding. 200 µL of each sample was transferred to a new 96 well plate and fluorescence was measured (480 nm excitation and 520 nm emission) with a Safire microplate reader (Tecan Group Ltd, Maennedorf, Switzerland).
2.4.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to assess cell adhesion to scaffolds. hPDL cells were seeded on 6 mm diameter scaffold punches at a density of 5,000 cells/scaffold. At 0.5, 1, 2, and 6 hours post-seeding, cells were fixed in 3% glutaraldehyde in 0.1M phosphate buffer at room temperature for 1 hour, followed by 3 rinses in 0.1M phosphate buffer. Samples were immersed in 1% osmium tetroxide in 0.1M phosphate buffer for 1 hour. After rinsing 3 times in 0.1M phosphate buffer, samples were dehydrated in a graded series of ethanol. Critical point drying was achieved with a Samdri PVT-3B critical point dryer (Tousimis, Rockville, MD, USA) and samples were subsequently sputter coated with gold/palladium using a Hummer VI sputter coater (Anatech USA, Union City, CA, USA). Samples were imaged with the Hitachi 3400-N Variable Pressure Scanning Electron Microscope (Hitachi, Tokyo, Japan).

2.5 Cellular Response to the Electrospun Scaffolds *in vitro*: THP-1 Monocyte Culture Conditions and Cytokine Array Analysis

Cytokine array experiments were performed with a human monocytic leukemia cell line, THP-1 (TIB-202; ATCC Manassas, VA, USA). Cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated FBS, 1% AA and 0.05 mM 2-mercaptoethanol (Bio-File, Hercules, CA, USA). Cultures were maintained in a 5% CO₂ atmosphere with 100% relative humidity at 37 °C.

Prior to seeding, coated plates and scaffolds were prepared. To coat plates, wells were incubated at 4°C overnight with bovine type I collagen (Nutragen; Advanced BioMatrix, Carlsbad, CA, USA) at 180 ug/mL in PBS, with the periostin/collagen condition also containing recombinant human periostin (R&D Systems, Minneapolis, MN, USA) at a concentration of 10 ug/mL. The coating solution was then removed, rinsed with PBS once, and incubated in sterile, 1% BSA for 1-2 hours at 37°C. BSA was removed and plate was rinsed once with PBS prior to seeding with cells. Collagen and periostin/collagen scaffolds were cut into 20 mm by 20 mm squares for use in cytokine array experiments. Scaffolds were then decontaminated in 70% ethanol for 30 minutes,
followed by 3 rinses in sterile, deionized water. Decontaminated scaffolds were stored in sterile, deionized water for up to 24 hours prior to use in experiment.

For use in cytokine array experiment, cells were activated 72 hours prior to experiments by treatment with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 50 ng/mL of media, added every 24 hours. Cells were serum-starved 24 hours prior to seeding for experiments. Cells were removed from tissue culture flasks with Accutase™ cell detachment solution (Millipore, Bellerica, MA, USA) and seeded at a concentration of 175,000 cells per well (12-well plate) in RPMI 1640 medium with 0.5% AA and 0.05 mM 2-mercaptoethanol. Throughout the course of the experiment, cytokines were added every 24 hours. Recombinant human interferon gamma (IFN-γ; PeproTech, Rocky Hill, NJ, USA) and recombinant human interleukin 4 (IL-4; PeproTech, Rocky Hill, NJ, USA) were added to a concentration of 10 ng/mL. Media was changed after 48 hours and cell culture supernatant was collected at 72 hours post-seeding.

The detection of cytokines in cell culture supernatant was achieved with the use of a Proteome Profiler™ Human Cytokine Array Kit, Panel A (R&D Systems, Minneapolis, MN, USA). Cell culture supernatants were removed from each sample and particulates were removed by centrifugation. Samples were prepared as specified by the manufacturer’s protocol adapted for the LI-COR® system, which uses IRDye® 800CW Streptavidin and LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA) and analyzed with Image Studio Software (LI-COR, Lincoln, NE, USA).

### 2.6 Immunocytochemistry

Samples which were analyzed with immunofluorescent labeling were fixed in 4% PFA in PBS for 5-10 minutes at room temperature and permeabilized with 0.1% Triton-X 100 in PBS for 5 minutes. After rinsing in PBS, samples were blocked in 1% BSA (Fisher Scientific, Waltham, MA, USA) for 30 minutes at room temperature. Primary antibodies (Table 2.1) were diluted in 1% BSA at 1:100 dilution and incubated overnight (16 – 21 hours) at 4°C. Samples were rinsed 3 times in PBS, and blocked with 1% BSA for 5 minutes. Appropriately conjugated secondary antibodies were diluted 1:200 in 1% BSA,
added to samples and incubated at room temperature for 90 minutes. Samples were rinsed and cell nuclei were stained with Hoecsht dye prior to imaging. Samples were mounted with Immu-Mount (Fisher Scientific, Waltham, MA, USA) aqueous mounting medium and imaged with a ZEISS Axio Imager.M2m upright microscope (ZEISS, Oberkochen, Germany) equipped with Zen Pro 2012 software. Negative controls for non-specific staining were performed with the omission of primary antibodies. Images for negative controls from immunocytochemistry are shown in Appendix B.

Table 2.1: Primary antibodies used in immunocytochemistry

<table>
<thead>
<tr>
<th>Primary Antibody Target</th>
<th>Product Number and Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-kit/CD117 (stem cell factor receptor)</td>
<td>MS-289-P Thermo Fisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>(0.N.297) sc-71113 Santa Cruz Biotechnology, Dallas, TX, USA</td>
</tr>
<tr>
<td>FSP-1 (fibroblast specific protein 1)</td>
<td>07-2274 Millipore, Bellerica, MA, USA</td>
</tr>
<tr>
<td>pSmad3 (phosphorylated SMAD family member 3)</td>
<td>Ab52903 Abcam, Cambridge, England</td>
</tr>
<tr>
<td>Runx2 (runt-related transcription factor 2)</td>
<td>(M70) sc-10758 Santa Cruz Biotechnology, Dallas, TX, USA</td>
</tr>
<tr>
<td>Stro-1</td>
<td>MAB1038 R&amp;D Systems, Minneapolis, MN, USA</td>
</tr>
<tr>
<td>Scleraxis (SCXA)</td>
<td>Ab129822 Abcam, Cambridge, England</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>(300-3) sc-13578 Santa Cruz Biotechnology, Dallas, TX, USA</td>
</tr>
</tbody>
</table>

2.7 Fenestration Defect Model

All animal procedures were in accordance with protocols that had been approved by the University Council on Animal Care at The University of Western Ontario (Appendix A). Fenestration defects were created in the alveolar bone of Wistar rats, aged between 13 and 18 weeks (250-400 g). Animals were anesthetised with ketamine (75 mg/kg) and xylazine (10 mg/kg). Two defects were created in each rat (one per side), on the lingual
Figure 2.3: Fenestration defect surgery in maxillary molars of rats. A gingival flap was created in the palatal gingiva to expose alveolar bone. A ¼ round bur was used to create a fenestration defect in the bone to create a defect approximately 1.8mm long and 0.5mm wide, the defect depth was determined by feel to remove the full width of alveolar bone and periodontal ligament, keeping the roots intact.
Figure 2.3: Fenestration defect surgery in maxillary molars of rats.
surface of maxillary molars (see Table 2.2 for conditions and animal numbers).

**Table 2.2: Fenestration defect surgery experimental conditions.**

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Litter Age</th>
<th>Left Maxillary Molars</th>
<th>Right Maxillary Molars</th>
<th>Litter Age</th>
<th>Left Maxillary Molars</th>
<th>Right Maxillary Molars</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Litter 1 18 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Empty</td>
<td>Litter 2 16 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Empty</td>
</tr>
<tr>
<td>1</td>
<td>Litter 1 18 wks.</td>
<td>Collagen Scaffold</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Litter 2 16 wks.</td>
<td>Collagen Scaffold</td>
<td>Periostin/ Collagen Scaffold</td>
</tr>
<tr>
<td>2</td>
<td>Litter 3 15 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Empty</td>
<td>Litter 3 15 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Empty</td>
</tr>
<tr>
<td>3</td>
<td>Litter 4 14 wks.</td>
<td>Collagen Scaffold</td>
<td>Empty</td>
<td>Litter 4 14 wks.</td>
<td>Collagen Scaffold</td>
<td>Empty</td>
</tr>
<tr>
<td>3</td>
<td>Litter 5 13 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Collagen Scaffold</td>
<td>Litter 4 14 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Collagen Scaffold</td>
</tr>
<tr>
<td>4</td>
<td>Litter 6 13 wks.</td>
<td>Empty</td>
<td>Collagen Scaffold</td>
<td>Litter 6 13 wks.</td>
<td>Empty</td>
<td>Collagen Scaffold</td>
</tr>
<tr>
<td>4</td>
<td>Litter 6 13 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Empty</td>
<td>Litter 6 13 wks.</td>
<td>Periostin/ Collagen Scaffold</td>
<td>Empty</td>
</tr>
<tr>
<td>TOTAL</td>
<td>Empty: 4</td>
<td>Collagen Scaffold: 5</td>
<td>Periostin/Collagen Scaffold: 6</td>
<td>Empty: 3</td>
<td>Collagen Scaffold: 4</td>
<td>Periostin/Collagen Scaffold: 4</td>
</tr>
</tbody>
</table>

* Greyed conditions were excluded from analysis due to errors in defect placement, size, or surgical complications

The gingival attachment was disrupted at the tooth surface and pulled away to create a gingival flap and exposing the lingual surface of the alveolar bone. To create the fenestration defect, a no. ¼ round bur (0.5 mm in diameter) was used to drill three holes: at the center of the second molar, between the first and second molar, and between the
second and third molar. These holes were connected with a 0.7 mm diameter tapered fissure bur to create a defect approximately 1.8 mm long and 0.5 mm wide, as depicted in Figure 2.3; defect depth was determined by feel, such that the alveolar bone and periodontal ligament were removed while the roots remained intact. Bone fragments were removed with sterile saline and the defect area was dried thoroughly. Four 3 mm diameter pieces of collagen or collagen/periostin scaffolds were placed into the surgically created bone defects with empty defects as controls. The gingival flap was replaced and reattached with Vetbond™ tissue adhesive (3M, St. Paul, MN, USA) and a small amount of 0.12% chlorohexadine was applied to the surgical area. Anaesthesia was reversed with atipamezole (10 mg/kg) and analgesic (buprenorphine, 0.05 mg/kg) was administered subcutaneously, twice daily for 72 hours following surgery. Animals were fed powdered food following surgery. Animals were euthanized at 3 or 14 days after surgery with CO₂ to collect samples for histological analysis.

2.7.1 Histological Analysis

Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 48 hours. Following fixation, tissues were treated Cal-Ex™ decalcifier (Fisher Scientific, Waltham, MA, USA) for 48 hours at room temperature. Tissues were embedded in paraffin, cut in 5 µm thick mesiodistal sections, and mounted on glass slides. Sections were deparaffinized in xylene, rehydrated and stained with hematoxylin and eosin (H&E) and Masson’s Trichrome.

For immunohistochemistry, sections were deparaffinized in xylene and rehydrated followed by blocking of exogenous peroxidase activity with 3% hydrogen peroxide in absolute methanol for 5 minutes. Heat-mediated antigen retrieval was performed with a decloaking chamber (Biocare Medical, Concord, CA, USA); sections were heated to 100°C while immersed in 10 mM sodium citrate buffer at pH 6 with 0.05% Tween. Non-specific antibody interactions were blocked with 10% horse serum in PBS incubated at room temperature for 30 minutes. Primary antibodies against Arginase I (V-20, sc-18354, Santa Cruz Biotechnology, Dallas, TX, USA) were diluted 1:100 in 10% horse serum and incubated overnight at 4°C. After rinsing in PBS, sections were treated with ImmPRESS HRP Anti-Goat Ig peroxidase reagent (Vector Laboratories, Burlingame CA, USA) for
30 min at room temperature. Staining was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame CA, USA) in H₂O₂ and counterstained with hematoxylin. Negative controls for non-specific staining were performed with the omission of primary antibodies. Images for negative controls from immunohistochemistry are shown in Appendix B. Staining was visualized with a Leica DM1000 (Leica Microsystems, Wetzlar, Germany) equipped with Leica Application Suite V3.8 software.

2.8 Statistical Analysis

P-values less than 0.05 were considered significant and multiplicity-adjusted p-values were reported, when applicable. Statistical analysis were performed with Prism 6.0 for Mac Os X (GraphPad Software, San Diego, CA, USA).

2.8.1 Objective 1: Characterization of hPDL cells

Data from individual patients are shown separately and presented as a mean ± SD of one experiment with three internal replicates, unless specified otherwise. Quantification of calcium content with Alizarin Red S staining was analyzed with two-way ANOVA and Tukey post-test for multiple comparisons. Regression analysis of calcium content with patient age was completed with linear regression and F-test for significance of non-zero slope. Gene expression analysis was analyzed with two-way ANOVA, using Bonferroni post-test for multiple comparisons between treatments within each time point.

2.8.2 Objective 2: Cellular response to electrospun scaffolds in vitro

Data is presented as a mean ± SD of triplicate experiments (each with three internal replicates, unless specified otherwise). Cytokine array data was analyzed with a matched one-way ANOVA and Tukey post-test for multiple comparisons. Quantification of cell adhesion was analyzed with one-way ANOVA and Bonferroni post-test for multiple comparisons. Proliferation assay data was analyzed with a two-way, matched ANOVA and Bonferroni post-test for multiple comparisons between treatments within each time point.
Chapter 3

3. Results

3.1 Objective 1: Characterization of hPDL cells

3.1.1 Expression of markers associated with fibroblasts, osteoblasts, and progenitor cells by hPDL cells

As hPDL cells have been shown to express many specific markers associated with fibroblasts, progenitor cells, and mineralizing osteoblasts, we analyzed the phenotype of the cells isolated in our laboratory using a subset of markers. Immunocytochemistry was used to visualize the presence of specified proteins in isolated hPDL cells, shown in Figure 3.1. All isolated cells expressed both the fibroblast marker FSP-1 (fibroblast specific protein 1) and the pre-osteoblast associated transcription factor Runx2 (runt-related transcription factor 2). hPDL cells were also immunoreactive for C-kit/CD117 (stem cell factor receptor) and Stro-1, markers associated with hematopoietic, and mesenchymal progenitor populations, respectively. Immunocytochemistry also confirmed the presence of pSmad3 (phosphorylated SMAD family member 3) and the tendon/ligament-specific transcription factor Scleraxis in hPDL cells.

3.1.2 Ability of hPDL cells to form a mineralized matrix and express markers associated with osteogenic differentiation

3.1.2.1 Alizarin Red S Staining

Initial assessment of the osteogenic potential of hPDL cells was examined with Alizarin Red S staining to visualize and quantify calcium deposition in response to culture with osteogenic media. Cells were isolated from patients between 20 and 65 years of age (median age 42), shown in Figure 3.2a. In all patients, culturing in control (unsupplemented) media did not significantly influence the amount of matrix-bound calcium, while culture in osteogenic media induced deposition of a mineralized matrix, to varying degrees depending on patient age. In all patients, calcium content at 4 weeks was significantly different from the control condition (p<0.01), however, at 2 weeks a significant difference (p<0.001) was only observed in M20. In younger patients (M20
Figure 3.1: Staining of hPDL cells for various cell type markers. a,b) hPDL cells stained positive for the cell marker FSP-1 (fibroblast specific protein 1) and pre-osteoblast marker Runx2 (runt-related transcription factor 2). c) pSmad3 (phosphorylated Smad3), which is associated with undifferentiated mesenchymal cells was also present. d,e) hPDL cells also stained positive for the stem cell markers C-kit /CD117 (stem cell factor receptor) and Stro-1. f) The tendon-specific transcription factor Scleraxis was also expressed by hPDL cells. Blue: nuclei (DAPI), Red: specified protein. Inset shows black and white image of red channel. Images are representative of 3 independent experiments (each with 3 internal replicates). Scale bar: 50µm.
Figure 3.1: Staining of hPDL cells for various cell type markers.
Figure 3.2: Alizarin Red S staining of calcium in hPDL cells during osteogenic differentiation. a) Alizarin Red S staining was used to visualize calcium (stained red) deposited by cells at 2 and 4 weeks post-seeding. Dye was subsequently eluted to quantify calcium content. Data for each patient is shown separately and presented as mean ± SD of 3 internal replicates from one experiment (except patient F31, 2 internal replicates); patient gender, age, and cell passage is designated under graphs. Two-way ANOVA with Tukey post-test for multiple comparisons was completed and multiplicity-adjusted p-values are reported. * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001. Regression analysis at b) 2 weeks and c) 4 weeks indicated that calcium deposition was negatively correlated with age at 2 and 4 weeks in response to osteogenic media ($r^2=0.8208$, $r^2=0.8282$; p<0.05), while deposition of calcium in control media was not significantly related to age.
Figure 3.2: Alizarin Red S staining of calcium in hPDL cells during osteogenic differentiation.
and M31) the amount of calcium was increased by 47- and 34-fold, respectively, at 4 weeks in osteogenic media (compared to control media at 2 weeks). Whereas cells from older patients (F42, M55, F65) showed less than a 7-fold increase in calcium content at 4 weeks under the same conditions. Regression analysis (Figure 3.2b,c) confirmed that calcium deposition in response to culture in osteogenic media was negatively correlated with age, this effect was significant at 2 weeks ($r^2=0.8208$, $F_{(1,3)}=13.74$, $p=0.0341$) and 4 weeks ($r^2=0.8282$, $F_{(1,3)}=14.46$, $p=0.0319$). Calcium deposition following culture of hPDL cells in control media was not significantly affected by patient age at either 2 weeks ($r^2=0.0001$, $F_{(1,3)}=0.0003$, $p=0.9856$) or 4 weeks ($r^2=0.3476$, $F_{(1,3)}=1.598$, $p=0.2954$).

### 3.1.2.2 Gene Expression

Gene expression in response to osteogenic culture conditions was examined in cells isolated from three patients: M20, F39, F65. Gene expression in cells from M20 and F39 were consistent with osteogenic differentiation, while F65 did not appear to respond to osteogenic stimuli based on gene expression data. In cells from patient M20 (Figure 3.3a), culture in osteogenic media resulted in gene expression characteristic of osteogenic differentiation. $ALPL$ expression was suppressed at 4 and 6 weeks ($p<0.05$, $p<0.01$), while $BGLAP$ expression was simultaneously increased ($p<0.05$, $p<0.0001$). Expression of $RUNX2$ was decreased at 2 weeks ($p<0.01$) and $CEMP1$ was decreased at 6 weeks ($p<0.001$). Gene expression in cells from donor F39 (Figure 3.3b) cultured in osteogenic media had reduced $ALPL$ at weeks 2, 4, and 6 ($p<0.0001$) compared to controls, while $BGLAP$ expression was increased at 4 and 6 weeks ($p<0.01$, $p<0.0001$), indicating osteogenic differentiation. $RUNX2$ and $CEMP1$ expression was more variable in response to osteogenic stimuli. $RUNX2$ was upregulated at 2 weeks and subsequently downregulated at 6 weeks ($p<0.001$), while expression of $CEMP1$ was upregulated at 2 and 6 weeks ($p<0.0001$). Conversely, in the older patient (F65, Figure 3.3c), osteogenic inductive media had little influence on gene expression. However, $ALPL$ expression was significantly lower ($p<0.05$) at 6 weeks compared to control and $BGLAP$ expression was decreased at 4 weeks ($p<0.001$).
Figure 3.3: Gene expression of hPDL cells during osteogenic differentiation. Expression of *ALPL* (alkaline phosphatase), *BGLAP* (osteocalcin), *RUNX2* (runt-related transcription factor 2), *CEMP1* (cementum protein 1) was assessed in hPDL cells that were induced to undergo osteogenic differentiation (osteogenic media) compared to controls (control media) at 0, 2, 4, and 6 weeks. a) Cells from patient M20 showed gene expression typical of osteogenic differentiation, indicating mature osteoblast phenotype at 4 and 6 weeks as *ALPL* expression decreased and *BGLAP* increased relative to controls. Expression of *CEMP1* at 6 weeks was significantly lower in osteogenic condition. b) Cells from patient F39 showed a decrease in expression of *ALPL* at 2, 4, and 6 weeks and expression of BGLAP at 4 and 6 weeks in response to osteogenic stimuli. *RUNX2* and *CEMP1* expression was variable between control and osteogenic conditions. c) Gene expression of cells from patient F65 had minimal response to osteogenic stimuli. In the osteogenic condition *ALPL* expression was significantly lower at 6 weeks and BGLAP expression was decreased at 4 weeks compared to controls. *RUNX2* and *CEMP1* expression was not significantly influenced by osteogenic media. Data for each patient is presented as mean ± SD of 3 internal replicates from one experiment. Two-way ANOVA, Bonferroni post-test for multiple comparisons within time points, multiplicity-adjusted p-values reported. * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.
Figure 3.3: Gene expression of hPDL cells during osteogenic differentiation.
3.1.2.3 RUNX2 Staining

Immunofluorescent staining was used to assess nuclear translocation of the transcription factor Runx2 in hPDL cells. Cells from two of the three patients, F29 and M46 (Figure 3.4, Figure 3.6), had the same temporal and localized expression of Runx2; at 2 weeks, Runx2 immunoreactivity was undetected, while expression in cells cultured under osteogenic conditions was restricted to nuclei. In these patients at 4 weeks, there was weak nuclear staining of cells in control cultures, while immunoreactivity in the osteogenic conditions was localized to both nuclei and cytoplasm. Cells from patient F42 (Figure 3.5) also labeled positively for Runx2; however, cells cultured under control and osteogenic conditions exhibited similar staining. At 2 weeks, Runx2 was labeled in nuclei and the cytoplasm, while only present in nuclei at 4 weeks.

3.2 Objective 2: Cellular Response to the Electrospun Scaffolds in vitro

3.2.1 Influence of collagen and perioestin/collagen scaffolds on cytokine production by activated THP-1 monocytes

Cytokine production from activated THP-1 monocytes between 48 and 72 hours was quantified to assess if macrophage polarization was influenced by collagen or perioestin/collagen scaffolds (shown in Figure 3.7). As controls for loss of protein function due to electrospinning, cell response to collagen and perioestin/collagen coatings were also assessed. IFN-γ and IL-4 conditions acted as positive controls for M1 and M2 polarization, respectively. The secretion of cytokines associated with M1 macrophage polarization was not significantly influenced by either of the coatings or scaffold conditions (Figure 3.7a). Although the effect was not significant, the perioestin/collagen coating resulted in a slight increase in production of IL-1β and TNFα. Furthermore, the M1 and M2 controls did not effect the production of M1-associated cytokines compared to controls; although levels of CCL2, CXCL8, and CXCL10 were slightly elevated in the IFN-γ condition and CCL2 was increased in the IL-4 condition, the difference was not significant.
Figure 3.4: Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient F29. hPDL cells were immunoreactive for Runx2 protein in response to osteogenic media; at 2 weeks, expression was localized to the nuclei, while expression was observed in both nuclei and cytoplasm at 4 weeks. Control cells did not stain positively for Runx2 at 2 weeks, weak staining was observed at 4 weeks. Representative images of one experiment, with 3 internal replicates, scale bar: 50µm.
Figure 3.4: Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient F29.
Figure 3.5: Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient F42. Cells at 2 weeks were immunoreactive for Runx2 in both control and osteogenic conditions; staining in control was in cytoplasm and nuclei, while staining was mainly within nuclei in the osteogenic condition. At 4 weeks, both control and osteogenic conditions were immunoreactive for Runx2 within nuclei. Representative images of one experiment, with 3 internal replicates, scale bar: 50µm.
Figure 3.5: Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient F42.
Figure 3.6: Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient M46. At 2 weeks, control cells were not immunoreactive for Runx2 and in the osteogenic condition expression was localized to the nuclei. At 4 weeks, cells in the control condition were weakly immunoreactive for Runx2 within nuclei, while expression was observed in both nuclei and cytoplasm in the osteogenic condition. Representative images of one experiment, with 3 internal replicates, scale bar: 50µm.
Figure 3.6: Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient M46.
Figure 3.7: Cytokine secretion by activated THP-1 monocytes. Relative levels of cytokines present in cell culture supernants between 48 and 72 hours of culture, compared to control. a) M1 polarization-associated cytokines do not differ significantly from control despite the addition of recombinant IFN-γ or IL-4. The presence of collagen coating, periostin/collagen coating, collagen scaffold, or periostin/collagen scaffold did not influence cytokine production either. b) Cytokines characteristic of M2 polarized macrophages. There was no significant difference in levels of IL-1ra and IL-10 produced in response to IFN-γ, IL-4, coatings, or scaffolds. Data is presented as mean ± SD of data from 3 experiments (each with 2 internal replicates). Matched one-way ANOVA, Tukey post-test for multiple comparisons.
Figure 3.7: Cytokine secretion by activated THP-1 monocytes.
Production of the M2-associated cytokines IL-1ra and IL-10 were not influenced by the presence of collagen or periostin/collagen scaffolds either (Figure 3.7b). Although levels of IL-1ra were slightly elevated on both coatings, the IFN-γ, and IL-4 conditions the difference was not significant (matched one-way ANOVA, p>0.05).

3.2.2 Adhesion of hPDL cells to collagen and periostin/collagen scaffolds

Adhesion of hPDL cells to the electrospun scaffolds was quantified at 6 hours post-seeding, shown in Figure 3.8. Adhesion to tissue culture plastic was significantly higher than either of the scaffolds (p<0.05), however, no significant difference in attachment was seen between the collagen only scaffold (49.87% ± 28.80%) and the periostin/collagen scaffold (60.30% ± 17.06%). We next investigated hPDL spreading and morphology on collagen and periostin/collagen scaffolds. Cells attached within 15 minutes of seeding (not shown) and within 30 minutes cells had spread and extended lamellipodia on both the collagen and periostin/collagen scaffolds (Figure 3.9a,b). At 1 hour, cells seeded on the collagen scaffold had filopodial extensions to the scaffold fibres. While cells on the periostin/collagen scaffold were observed to penetrate and migrate beneath the surface fibres at 1 hour, filopodia were not observed until 2 hours (Figure 3.9c-f). At 6 hours, cells had spread within the scaffold pores, attaching along the fibres.

3.2.3 Proliferation of hPDL cells on collagen and periostin/collagen scaffolds

hPDL cell number on the collagen and periostin/collagen scaffolds was assessed up to 10 days as a measure of biocompatibility (Figure 3.10). Proliferation of hPDL cells was highest on tissue culture plastic. While cell numbers on the collagen scaffold was not significantly different from the control, growth on the periostin/collagen scaffold was significantly lower than tissue culture plastic at 4, 7, and 10 days (p<0.01). Moreover, the addition of periostin to the scaffold resulted in significantly lower cell number at 7 and 10 days (p<0.05).
Figure 3.8: Adhesion of hPDL cells to collagen and periostin/collagen scaffolds.

Adhesion of hPDL cells to the electrospun scaffolds was assessed at 6 hours post-seeding. Adhesion on both the collagen scaffold and periostin/collagen scaffold was significantly lower than tissue culture plastic control. Data is presented as mean ± SD of 3 independent experiments (each with 3 internal replicates). Matched one-way ANOVA, Bonferroni post-test for multiple comparisons, * p < 0.05.
Figure 3.8: Adhesion of hPDL cells to collagen and periostin/collagen scaffolds.
Figure 3.9: SEM images of hPDL cell adhesion to collagen and periostin/collagen scaffolds. Attachment of hPDL cells to the electrospun scaffolds was visualized with scanning electron microscopy. Cells attach within 15 minutes of seeding (not shown). a,b) At 30 minutes, cells have spread on the scaffold surface and lamellipodia are observed (arrows). c,d,e,f) Filopodia extend from hPDL cells on the collagen scaffold as early as 1 hour post-seeding and on the periostin scaffold at 2 hours (arrow). g,h) hPDL cells are spread on both scaffolds at 6 hours post-seeding.
Figure 3.9: SEM images of hPDL cell adhesion to collagen and periostin/collagen scaffolds.
Figure 3.10: Proliferation of hPDL cells on collagen and periostin/collagen scaffolds.

Proliferation of hPDL cells seeded on collagen and periostin/collagen scaffolds was assessed at 1, 4, 7, 10 days. Both scaffolds supported the proliferation of hPDL cells, although with lower cell numbers than tissue culture plastic. Cells seeded on the periostin/collagen scaffold had a significantly lower cell number compared to the control at 4, 7, and 10 days and significantly lower than collagen scaffold at 7 days. Data is presented as mean ± SD of 3 independent experiments (each with 3 internal replicates). Matched two-way ANOVA, Bonferroni post-test for multiple comparisons, multiplicity-adjusted p-values reported, * = p < 0.05, ** = p < 0.01.
Figure 3.10: Proliferation of hPDL cells on collagen and periostin/collagen scaffolds.
3.2.4 Extracellular matrix deposition by hPDL cells on collagen and periostin/collagen scaffolds

At 3 and 7 days post-seeding, extracellular fibronectin immunoreactivity was observed on the surface of both the collagen and periostin/collagen scaffolds (Figure 3.11). The amount of fibronectin appeared to increase from 3 to 7 days; however, there was no observable difference in the levels of fibronectin secreted by cells seeded on the periostin/collagen scaffolds compared to collagen only scaffolds. Synthesis of tenascin C by hPDL cells was also assessed on the scaffolds, shown in Figure 3.12. Immunoreactivity for tenascin C was observed on both the collagen and periostin/collagen scaffolds seeded with hPDL cells. At 3 and 7 days, periostin/collagen scaffolds appeared to have increased immunoreactivity for tenascin C compared to collagen only scaffolds.

3.3 Objective 3: Influence of the Electrospun Scaffolds on Fenestration Defect Healing

Figure 3.13 shows the area of alveolar bone that was removed during surgery and designates the region of interest, which is shown in subsequent figures. At 3 days following surgery, scaffolds were visible within the defect region and cells had infiltrated defects in all conditions as shown in Figure 3.14. While the empty defects were densely populated with inflammatory cells at 3 days, cell penetration into the defect site was reduced in the presence of scaffolds. In both the collagen and periostin/collagen scaffold conditions, spaces between folds of the scaffolds were observed that were not occupied with cells (as indicated by black arrowheads in Figure 3.14). However, where gaps were present in the scaffold, cells migrated between the folds, as indicated by green arrows in Figure 3.14d.

To further characterize cell populations in the defect at 3 days, sections were stained for the M2 macrophage marker Arginase I (shown in Figure 3.15). In the empty defect, staining was weaker than in either scaffold condition and labeled cells were observed throughout the defect region. Conversely, Arginase I positive cells in the scaffold
Figure 3.11: Deposition of fibronectin by hPDL cells on collagen and periostin/collagen scaffolds. Deposition of fibronectin by hPDL cells cultured on collagen and periostin/collagen scaffolds was visualized with immunocytochemistry, fibronectin (green), nuclei (blue). Cells seeded on both scaffolds secreted fibronectin within 3 days. There was no observable difference in the amount of fibronectin or fibre organization between the two scaffolds. Images are representative of 3 independent experiments (each with 3 internal replicates). Scale bar: 50 µm.
Figure 3.11: Deposition of fibronectin by hPDL cells on collagen and periostin/collagen scaffolds.
Figure 3.12: Deposition of tenascin C by hPDL cells on collagen and periostin/collagen scaffolds. Deposition of tenascin C by hPDL cells cultured on collagen and periostin/collagen scaffolds was visualized with immunocytochemistry, tenascin C (green), nuclei (blue). a,b) At 3 days, tenascin C deposition was increased on periostin/collagen scaffold compared to collagen only scaffold. c,d) At 7 days, cells seeded on periostin/collagen scaffold appeared to secrete more tenascin C compared to cells on collagen scaffold. Images are representative of 3 independent experiments (each with 3 internal replicates). Scale bar: 50 µm.
Figure 3.12: Deposition of tenascin C by hPDL cells on collagen and periostin/collagen scaffolds.
Figure 3.13: Location of fenestration defect and region of interest used in analysis. The trajectory of the drill used to created fenestration defect is indicated by green lines. Black box denotes the region of interest, which was used for analysis and shown in subsequent figures. T = Tooth, PDL = Periodontal Ligament, AB = Alveolar Bone, G = Gingiva.
Figure 3.13: Location of fenestration defect and region of interest used in analysis.
Figure 3.14: Masson’s Trichrome staining of fenestration defects at 3 days following surgery a,b) In the empty defect, cells had fully infiltrated the defect region. c,d,e,f) In the collagen and periostin/collagen scaffold conditions, scaffolds were visible and cells had infiltrated the region surrounding the scaffolds. In some areas, cells can be seen migrating between the folds of the scaffolds, as indicated by green arrows. However, if the scaffold is folded to inhibit cell infiltration, empty spaces are observed (black arrowheads). Images are representative of staining from at least 3 independent experiments with 2 sections per sample; empty: n=4, collagen scaffold: n=5, periostin/collagen scaffold: n=6. T=Tooth, PDL=Periodontal Ligament, G=Gingiva, AB=Alveolar Bone S=Scaffold.
Figure 3.14: Masson’s Trichrome staining of fenestration defects at 3 days following surgery.
Figure 3.15: Staining of Arginase I in fenestration defects at 3 days. a,b,c) Arginase I positive cells in the empty defect were stained more weakly than in scaffold conditions. Staining was observed throughout the defect. d,e,f) In the collagen scaffold condition, Arginase I positive cells were observed in the areas around the scaffold. Black arrowheads denote the interface between scaffolds and Arginase I positive cells, while red arrows indicate the migration of Arginase I positive cells between the folds of the scaffold. Arginase I positive cells were observed within the collagen scaffold, indicated by green arrowheads. g,h,i) Staining of Arginase I positive cells in the periostin/collagen scaffold condition was similar to the collagen scaffold. Cells were localized around the scaffold (black arrowheads) and, where applicable, migrated between the folds of the scaffolds (red arrows). Images are representative of staining from at least 3 independent experiments with 2 sections per sample; empty: n=4, collagen scaffold: n=5, periostin/collagen scaffold: n=6. T=Tooth, PDL=Periodontal Ligament, G=Gingiva, AB=Alveolar Bone, S=Scaffold.
Figure 3.15: Staining of Arginase I in fenestration defects at 3 days.
conditions were restricted to areas surrounding the scaffolds. As indicated by black arrowheads in Figure 3.15, Arginase I positive cells migrated towards the scaffold, yet there was a boundary between cells directly adjacent to the scaffolds and Arginase I positive cells. Cells were observed to migrate between the folds of scaffolds as indicated by red arrows and, in some cases, Arginase I positive cells were seen within the scaffold (Figure 3.15f).

At 14 days, the gingival epithelium and connective tissue were regenerated in all conditions. Cells had fully infiltrated the fenestration region and had begun to synthesize extracellular matrix; furthermore, both the collagen and periostin/collagen scaffolds were incorporated into the wound and were not discernable from newly formed tissue (Figure 3.16, Figure 3.17, Figure 3.18). Collagen (stained blue) was present in all conditions (Figure 3.16); however, there was increased collagen in the empty defect compared to the scaffold conditions. As shown in Figure 3.17, there was inflammation around the remaining alveolar ridge in all conditions; however, in collagen and periostin/collagen scaffold conditions, inflammation was considerably higher and extended into the fenestration area. In the collagen and periostin/collagen scaffold conditions, a distinct interface was observed between the cells infiltrating the defect region from the gingiva (black arrows) and the inflammatory cells in the scaffold region (green arrowheads), shown in Figure 3.17c,d,e,f. Some level of PDL regeneration was observed in all conditions, shown in Figure 3.18. Cells were observed to migrate into the defect region from the apical portion of the remaining PDL (indicated by black/white arrows), with increased cell migration in the collagen and periostin/collagen scaffold conditions. In some cases, regions of bone on the edges of the defect were less dense, and more cellular than mature bone and characteristic of either newly formed bone, or bone which is being resorbed (green arrowheads in Figure 3.18b).
Figure 3.16: Masson’s Trichrome staining at 14 days to visualize collagen deposition within the defect region. a,d,f) In empty, collagen scaffold, periostin/collagen scaffold conditions the gingiva is healed, collagen (blue) is observed in all defects. Inflammation is observed around the remaining alveolar bone in all conditions (nuclei stained red/pink); in the scaffold conditions, inflammation extends into the fenestration region as well. Black boxes denote areas shown in higher magnification. b,e,g) Within the fenestration region, the empty defect had substantial collagen deposition compared to collagen scaffold and periostin/collagen scaffold conditions. c) In some cases, areas of new bone were observed in the empty defect. Images are representative of staining from at least 3 independent experiments with 2 sections per sample; empty: n=3, collagen scaffold: n=4, periostin/collagen scaffold: n=4. T=Tooth, PDL=Periodontal Ligament, G=Gingiva, AB=Alveolar Bone.
Figure 3.16: Masson’s Trichrome staining at 14 days to visualize collagen deposition within the defect region.
Figure 3.17: Masson’s Trichrome staining at 14 days; infiltration of gingival fibroblasts. Cells from are observed to migrate from the gingiva into the fenestration defect region, as indicated by black arrows. a,b) In the empty defects, gingival fibroblasts migrated into the defect. Inflammatory cells were present around the alveolar bone, yet only extended partially into the defect region. c,d,e,f) In the collagen scaffold and periostin/collagen scaffold conditions, there is a distinct interface between cells from the gingiva (black arrows) and inflammatory cells in the defect (green arrows). Images are representative of staining from at least 3 independent experiments with 2 sections per sample; empty: n=3, collagen scaffold: n=4, periostin/collagen scaffold: n=4. T=Tooth, PDL=Periodontal Ligament, G=Gingiva, AB=Alveolar Bone.
Figure 3.17: Masson’s Trichrome staining at 14 days; infiltration of gingival fibroblasts.
Figure 3.18: Masson’s Trichrome staining at 14 days; infiltration of cells from PDL.

a,c,e) Empty, collagen scaffold, and periostin/collagen scaffold conditions showed some level of PDL regeneration. Black boxes denote areas shown in higher magnification.

b,d,f) Cells from the PDL are observed to migrate into the fenestration defect region (indicated by black/white arrows) from the apical portion of the remaining PDL. Areas of bone resorption or new bone formation were observed on the edges of the defect (green arrowheads). Images are representative of staining from at least 3 independent experiments with 2 sections per sample; empty: n=3, collagen scaffold: n=4, periostin/collagen scaffold: n=4. T=Tooth, PDL=Periodontal Ligament, G=Gingiva, AB=Alveolar Bone.
Figure 3.18: Masson’s Trichrome staining at 14 days, infiltration of cells from PDL.
Chapter 4

4. Discussion

Clinical therapies that reproducibly stimulate regeneration of the periodontium have yet to be developed and the factors that contribute to regeneration and cause the high variability observed between patients is poorly understood (Bartold et al. 2003, Chen et al. 2010). We have focused on the influence of the matricellular protein periostin due to its localized expression within the PDL and periosteum of bone (Horiuchi et al. 1999) and its due to its downregulation in response to periodontal inflammation (Padial-Molina et al. 2012). Furthermore, periostin has roles in ECM synthesis, organization, remodelling (Takayama & Kudo 2012, Conway et al. 2013) and is expressed during bone development and healing (Hamilton 2008, Merle & Garnero 2012, Takayama & Kudo 2012); suggesting, overall that re-introduction of recombinant periostin into periodontal defects could stimulate regeneration of both the PDL and bone. An electrospun type I collagen scaffold acted as a provisional matrix substitute to facilitate healing and deliver recombinant periostin into the wound.

4.1 Objective 1: Characterization of hPDL Cells

Several studies have shown that PDL cells are key to regeneration of periodontal defects (Aukhil et al. 1987), although this is a particularly heterogeneous cell population (Marchesan et al. 2011). We therefore, we first performed characterization of isolated cells to assess the phenotypic properties of hPDL cells. Cells derived from the PDL have been previously shown to express a number of cell markers associated with fibroblasts, osteoblasts, stem cells, and ligament/tendon specific proteins; including FSP-1 (Inanc et al. 2006), Runx2 (Saito et al. 2002, Wang et al. 2011, Hakki et al. 2015), pSmad3 (Ling et al. 2009), C-kit/CD117 (Nagatomo et al. 2006, Kawanabe et al. 2010), Stro-1 (Seo et al. 2004, Nagatomo et al. 2006, Xu et al. 2009), and Scleraxis (Seo et al. 2004, Shi et al. 2005, Itaya et al. 2009). Moreover, it has been previously shown that hPDL cell populations possess characteristics of mesenchymal stem cells, namely the ability to
differentiate into cementoblasts, osteoblasts, or adipocytes \textit{in vitro} (Gould et al. 1980, McCulloch & Bordin 1991, Seo et al. 2004, Gay et al. 2007, Wu et al. 2009). While our findings corroborate the current understanding of hPDL cells, we also demonstrate a high level of variability in osteogenic potential between patients depending on age and suggest further consideration of how these variations \textit{in vitro} may influence experimental analyses and translate to clinical outcomes.

Immunocytochemistry was used to detect the fibroblastic/mesenchymal cell marker FSP-1 and confirmed that mesenchymal cells were isolated from the PDL without contamination from epithelial cells, which do not express this protein (Strutz et al. 1995). To exclude the possibility of contamination from other cells of mesenchymal origin in neighbouring tissues, the tendon/ligament specific transcription factor Scleraxis was also examined. All cells expressed Scleraxis, as is consistent with the literature and confirming the cells had an appropriate ligament phenotype (Seo et al. 2004, Fujii et al. 2008, Itaya et al. 2009, Inoue et al. 2011). While hPDL cells exhibit a fibroblastic phenotype, cells were also immunoreactive for the transcription factor Runx2. As Runx2 is associated with immature pre-osteoblasts (Komori 2009), expression supports that hPDL cells are not in a fully differentiated osteoblastic state, while retaining the potential to differentiate into mature osteoblasts. Moreover, hPDL cells were immunoreactive for pSmad3; while expressed by many cell types, including most mesenchymal cells, TGF-β activated Smad3 (pSmad3) has been shown to act as a transcriptional repressor to inhibit the differentiation of mesenchymal cells into adipogenic, myogenic, chondrogenic, and osteoblastic cells (Alliston et al. 2001, ten Dijke & Heldin 2007). As such, expression of pSmad3 and Runx2 could be reflective of the current understanding that hPDL cells are not fully differentiated towards a specific cell type (Wilde et al. 2003, Seo et al. 2004, Kawanabe et al. 2010).

All isolated cells also expressed the proteins C-kit/CD117 and Stro-1, which are markers for hematopoietic (Andre et al. 1989) and mesenchymal (Simmons & Torok-Storb 1991) progenitor cells, respectively. Previous reports, discussed in more detail below, indicate that C-kit and Stro-1 positive cells each represent small subpopulations of cells in the PDL; however, our findings indicate that nearly all isolated cells were immunoreactive
for each protein. While C-kit has traditionally been associated with hematopoietic cells and even regarded as a negative marker of mesenchymal cells (Kolf et al. 2007), recent reports have indicated that it is expressed by subpopulations (on average less than 1%) of mesenchymal cells, including cells derived from the PDL (Gagari et al. 2006, Nagatomo et al. 2006, Suphanantachat et al. 2014). Stro-1 was originally identified as a cell surface antigen in progenitor cell populations within the bone marrow (Simmons & Torok-Storb 1991) and a number of studies have shown that Stro-1 positive cells are present in isolated hPDL cells, representing between 2.6% to 33.5% of the total population (Seo et al. 2004, Nagatomo et al. 2006, Itaya et al. 2009, Wu et al. 2009, Xu et al. 2009, Kawanabe et al. 2010, El Fattah et al. 2011). While our findings reveal a cell phenotype that is consistent with the literature, high levels of C-kit and Stro-1 positive cells has not been previously reported. It should be noted that prior studies have mainly examined C-kit and Stro-1 populations from freshly isolated cells by flow cytometry and the findings presented here were from explant cultures up to passage 7. As the explant isolation technique used is based on cell migration and proliferation during tissue outgrowth (Freshney 2006), it is possible that isolation is selectively promoting the growth of a subpopulation of hPDL cells. While the influence of culture on expression of C-kit and Stro-1 in hPDL cells has not been widely examined in hPDL cells, previous reports have shown both down- and up-regulation of Stro-1 in various cell types in response to culture (Simmons & Torok-Storb 1991, Lallier & Spencer 2006, Itaya et al. 2009, Marchesan et al. 2011). The isolation of hPDL cells using an explant culture technique is commonly accepted to yield fibroblast-like populations, which our analysis supported (Somerman et al. 1988, Palaiologou et al. 2001, Marchesan et al. 2011). Furthermore, cells were shown to be immunoreactive for FSP-1, Runx2, pSmad3, C-kit, Stro-1, and Scleraxis proteins.

To further characterize hPDL cells and examine their potential contribution to the formation of bone, osteogenic differentiation of hPDL cells was assessed. Alizarin Red S staining for calcium confirmed that the isolated hPDL cells readily produced a mineralized matrix in response to osteogenic culture conditions, as is consistent with the literature (Arceo et al. 1991, Wu et al. 2009). Of potential significance, is that the level of mineralization varied significantly between patients and regression analysis revealed that the amount of calcium deposited was inversely related to donor age. This trend
corroborates findings by Zhang and colleagues, who demonstrated that decreased osteogenic differentiation in cells derived from older donors based on the area of mineralized nodules, alkaline phosphatase activity, and gene expression of osteocalcin, osteopontin, and bone sialoprotein (Zhang et al. 2012).

Diminished response to osteogenic stimuli with increased patient age was also reflected in our analysis of genes associated with osteogenic differentiation. Cells isolated from younger patients (M20, F39) showed a gene expression pattern that was characteristic of osteogenic differentiation and osteoblast maturation, while cells from F65 did not show significant differences in gene expression compared to the control condition. Specifically, in M20 and F39, expression of the early osteogenic marker alkaline phosphatase (ALPL) was suppressed as early as 2 weeks after osteogenic induction, with simultaneous increase in osteocalcin (BGLAP), a marker of late-stage mineralization and osteoblast maturation (Hauschka et al. 1989, Yamamoto et al. 2014). Conversely, in F65, alkaline phosphatase expression did not diminish before 6 weeks and no corresponding upregulation of osteocalcin occurred, indicating a delayed response to osteogenic stimuli (Golub & Boesze-Battaglia 2007).

While expression of CEMP1 is not specific to cementoblasts, it has been shown to be downregulated in hPDL cells induced to undergo osteogenic differentiation (Komaki et al. 2011). In M20 our results were consistent with the findings of Komaki and colleagues, although gene expression in cells from F39, contradicted the literature. As such, our results clearly show that there is variability in the expression of CEMP1 by hPDL cells during osteogenic induction. While the factors that dictate these changes have not been identified in this current study, it does merit further investigation.

RUNX2 gene expression did not appear to reflect osteogenic differentiation in any patients. While the role of RUNX2 in osteogenic differentiation has been well characterized (Karsenty 2009, Komori 2009), there are a number of studies which have indicated that mRNA levels of RUNX2 are a poor indicator of activity (Xiao 1998, Shui et al. 2003, Kirkham & Cartmell 2007, Miron et al. 2010, Prowse et al. 2013). As Runx2 protein is expressed at basal levels within the cells, nuclear translocation of Runx2 is a
significantly better measure of activity in response to osteogenic induction. Immunoreactivity of Runx2 reflected that osteogenic potential was variable across patients. For patients F29 and M46 (Figure 3.4, Figure 3.6), the induction of osteogenesis resulted in nuclear translocation of Runx2 at 2 weeks followed by cytoplasmic localization at 4 weeks, which is indicative of osteoblast maturation (Karsenty 2009). However, in patient F42 (Figure 3.5) Runx2 persisted within nuclei at 4 weeks, indicating immature or pre-osteoblasts, as Runx2 is associated with the inhibition of osteoblast maturation (Komori 2006). Results from osteogenic differentiation and mineralization assays are consistent with our current understanding that hPDL cells are capable of differentiating into osteoblast-like cells and forming mineralized nodules *in vitro* (Arceo et al. 1991, Ivanovski et al. 2001, Lekic et al. 2001, Gay et al. 2007, Wu et al. 2009).

In conclusion, the PDL cells used in this study express markers that are consistent with the classification of hPDL cells in the literature. While hPDL cells are known to produce a mineralized matrix in response to osteogenic induction, we show that patient age is significantly and inversely correlated to the osteogenic potential of isolated cells *in vitro*. While hPDL cells are commonly used for *in vitro* analysis, the influence of culture on cell phenotype and osteogenic potential has not been widely examined. The findings presented here suggest that additional investigation of the behaviour and properties of hPDL cells that have been maintained in culture is still needed and the influence of isolation on cell population should be considered when designing *in vitro* analyses. Moreover, the finding that patient age is inversely related to osteogenic potential of hPDL cells *in vitro* suggests that further investigation into the influence of age on mineralization and bone repair *in vivo* be examined. Further understanding of the factors responsible for diminished response to osteogenic stimuli and the identification of markers to better predict this variability could prove invaluable in tailoring clinical treatments for individual patients.

**4.2 Objective 2: Cellular Response to the Electrospun Scaffolds *in vitro***

In our first assessment of cellular response to the scaffolds, we examined the response of human macrophages to collagen and peristatin/collagen scaffolds *in vitro*. For this
preliminary experiment, collagen and periostin/collagen coatings were also assessed to determine if the proteins themselves had any influence on early immune response. Periostin has been shown to be upregulated in several inflammatory diseases such as bronchial asthma, skin inflammation, and atherosclerosis (Liu et al. 2014). Furthermore, it is known that periostin expression in vascular smooth muscle cells acts in a cycle to sustain inflammation in abdominal aortic aneurysms (Masuoka et al. 2012, Yamashita et al. 2013). As such, cytokine production by PMA-differentiated THP-1 macrophages was quantified to examine whether the presence of recombinant periostin in either the scaffolds or protein coatings influenced production of cytokines associated with Th1 or Th2 inflammatory responses.

Both the collagen and periostin/collagen coatings and scaffolds did not have a significant effect on the production of cytokines associated with either M1 or M2 macrophage polarization between 48 and 72 hours. However, PMA induced differentiation of THP-1 monocytes is known to induce differentiation into macrophages that more closely resemble M2 polarization (Tjiu et al. 2008, Ma et al. 2010, Laskar et al. 2013). As such, it is possible that the scaffolds had no influence on production of M2-associated cytokines because the M2 polarized state is being maintained. Scaffolds did not significantly influence the production of M1-associated cytokines either; further supporting that macrophages could be being maintained in a M2 polarized state. It is also possible that recombinant periostin has no direct effect on macrophage polarization. As an example, Gordon and colleagues investigated the influence of periostin on T regulatory cell differentiation (Gordon et al. 2011). Interestingly, while recombinant periostin did not influence differentiation, co-culture with airway epithelial cells over-expressing periostin did. As such, it is possible that while recombinant periostin alone is not sufficient to influence macrophage polarization, it could influence other cells to release growth factors that induce polarization when introduced in vivo. Moreover, periostin is suggested to amplify its own expression as binding of periostin to integrins activates Focal adhesion kinase to induce expression of periostin (Hakuno et al. 2010, Wen et al. 2010, Li et al. 2011, Yamashita et al. 2013, Li et al. 2014). Therefore, while recombinant periostin may not directly influence macrophage polarization, it could act on fibroblasts within the
defect to express periostin, which may subsequently influence macrophage polarization \textit{in vivo}.

Adhesion to scaffolds is crucial for wound healing processes \textit{in vivo} and an indicator of biocompatibility \textit{in vitro}. Homology with Fasciclin I and \( \beta \)IG-H3 suggested that periostin acts as an adhesive molecule (Takeshita et al. 1993, Horiuchi et al. 1999). However, recombinant periostin has been shown to have varying effects on adhesion (Gillan et al. 2002, Baril et al. 2007, Hwang et al. 2014). In our laboratory, we have previously shown that the adhesion of dermal fibroblasts is reduced on recombinant periostin coated surfaces (Elliott et al. 2012). Similarly, when incorporated into the periostin/collagen scaffold, periostin did not have significant influence on PDL cell adhesion when incorporated into an electrospun collagen scaffold (periostin/collagen scaffold). With regards, to inducing repair \textit{in vivo}, the ability of cells to interact with the scaffold fibres is important for modulating cellular response; moreover, surface topography has an important role in dictating cell morphology alignment, and migration (Hamilton et al. 2010, Dhandayuthapani et al. 2011a). SEM showed that hPDL cells seeded on both scaffolds had attached to and spread on the scaffolds within 1 and 2 hours post-seeding. Combined, adhesion assay and SEM data suggest that the collagen and periostin/collagen scaffolds are able to support the adhesion of hPDL cells, although the presence of recombinant periostin does not have a significant effect on adhesion compared to the collagen only scaffold.

As an important factor in wound healing and to further assess biocompatibility, cell growth of hPDL cells \textit{in vitro} was assessed. CyQUANT® assays showed that cell numbers on both scaffolds was lower than the tissue culture plastic control at all time points. Decreased growth on scaffolds can likely be attributed to lower stiffness compared to tissue culture plastic. The influence of stiffness on cell proliferation has been widely examined in a number of cell types, indicating that proliferation is elevated on stiffer substrates (Wells 2008, Hadjipanayi et al. 2009, Oleg V Semenov & Andreas 2009, Skotak et al. 2010, Edalat et al. 2011). Furthermore, surface topography of the scaffold or integrin binding with the electrospun collagen fibres could be influencing proliferation by inducing cells to differentiate (Hamilton et al. 2010, Roca-Cusachs et al.
Similarly, recombinant periostin could be inducing hPDL cells to undergo osteogenic differentiation, resulting in the reduced rate of proliferation on periostin/collagen scaffold compared to the collagen only scaffold. However, further studies, directly investigating the differentiation of cells in relation to proliferation are needed to confirm this hypothesis.

Fibronectin and tenascin C are two ECM proteins that are prominent in the native PDL and involved in granulation tissue formation during healing (Berkovitz 1990, Zhang et al. 1993, Nanci & Bosshardt 2006, Glim et al. 2013, Oryan et al. 2015). Although the addition of recombinant periostin to the electrospun collagen scaffold did not appear to effect on the amount of fibronectin synthesized, results indicated that both scaffolds are able to support hPDL cells in the production of an ECM containing fibronectin. Moreover, fibronectin has a vital role in wound repair and is a key component in granulation tissue while also having roles in the regulation of cell adhesion, proliferation, and migration (To & Midwood 2011). As such, incorporation of fibronectin into the matrix is a strong indication that the scaffold is able to support the synthesis of matrix during early wound healing in vivo. Immunolabelling of tenascin C indicated that the presence of recombinant periostin in the scaffold increased deposition. This result is consistent with previous findings that periostin has been shown to bind with and promote the incorporation of tenascin C into the ECM (Kii et al. 2010). Although further studies would be required to determine the influence of increased synthesis of tenascin C on periodontal healing, it has been shown to increase cell migration and is associated with ECM production in the proliferative and remodelling phases of wound repair (Midwood & Orend 2009, Kudo 2011). Production of fibronectin and tenascin C on the scaffolds in vitro indicates that they could have a positive response during wound healing processes once implanted.

Overall, in vitro analysis indicated that scaffolds did not induce a negative cellular response and thus suitable for testing in vivo. Neither scaffolds nor protein coatings induced the production of pro-inflammatory (M1 macrophage associated) cytokines by PMA-differentiated THP-1 cells. Adhesion and proliferation of hPDL cells were lower on both scaffolds compared to tissue culture plastic, which can be attributed to topographical
features of the electrospun fibres and the increased stiffness of the tissue culture plastic. While recombinant periostin did not appear to influence proliferation, hPDL cells demonstrated the ability to grow on both the collagen and periostin/collagen scaffolds up to 10 days. Moreover, synthesis of fibronectin and tenascin C demonstrates the ability to synthesize ECM proteins \textit{in vitro}. Altogether, this series of \textit{in vitro} experiments supported use of both the collagen and periostin/collagen scaffolds in preliminary \textit{in vivo} experiments.

**4.3 Objective 3: Influence of the Electrospun Scaffolds on Fenestration Defect Healing**

\textit{In vivo} experiments assessed the effect of collagen and periostin/collagen scaffolds on periodontal regeneration, using a fenestration defect model in the alveolar bone of the maxillary molars of rats. In this study, repair was examined histologically during the inflammatory phase (3 days) to assess immune response to the scaffolds and the influence of scaffolds on cell recruitment following wounding. Examination of the early remodelling phase (14 days) evaluated matrix synthesis, remodelling of the scaffold, and possible bone formation.

At 3 days post-wounding, histological analysis confirmed that fenestration defects were properly created and that each scaffold was retained within the defects following replacement of the gingival flap. The empty defect was fully infiltrated with inflammatory cells at 3 days. While cells in both the collagen and periostin/collagen scaffold conditions were present between some folds of the scaffolds (Figure 3.14d,f), in general, the inflammatory cells were present on the outside of the scaffold in apposition to the gingival connective tissue. This is suggestive that the scaffolds may have acted as a physical barrier to cell migration into the defect region at 3 days. Previous reports have shown that biomaterial density and porosity influence cell infiltration rates and bone regeneration in periodontal defects. For example, CaCO$_3$ particles or freeze-dried demineralized bone fragments in supraalveolar bone defects are obstructive at the early stages of bone repair, resulting in the formation of dense connective tissue or bone around the material (Caplanis et al. 1998, Wikesjo et al. 1998, Trombelli et al. 1999). Our results suggest that electrospun collagen with or without periostin may not have sufficient
porosity to allow early inflammatory cell infiltration. However, the exact role of inflammatory cells in bone, PDL and gingival regeneration is not well understood.

The finding that both scaffold conditions had increased levels of M2 macrophages at 3 days compared to empty defects, could suggest that the inflammatory response is delayed as a result of scaffolds impeding cell infiltration. While tissue-resident macrophages contribute to repair, macrophages are also recruited from the surrounding vasculature; therefore, scaffolds could be stimulating the recruitment of M2 macrophages. Based on our data, two scenarios are possible: 1) scaffolds could be recruiting M2 macrophages as a part of the healing response or 2) a foreign body reaction to the scaffolds could be inducing increased Th2-associated inflammation.

In normal healing of skin wounds, M2 macrophages facilitate the transition from the inflammatory to the proliferative phase through the secretion of anti-inflammatory cytokines such as IL-10, TGF-β, and Arginase I (Hao et al. 2012). Moreover, M2 macrophages have roles in the recruitment of fibroblasts and endothelial cells to promote ECM synthesis, remodelling, and angiogenesis (Mantovani et al. 2004). However, the role of M2 macrophages in repair has mainly been determined in skin and has not yet been characterized in the periodontium. Healing of the gingiva, for example, is known to vary considerably from skin healing with accelerated matrix formation and minimal scarring (Glim et al. 2013). Furthermore, in our fenestration defect model, healing of both soft and hard connective tissues must be considered and the implications of M2 macrophages on this process are not known.

Alternatively, interactions with the scaffolds could be elevating a Th2 immune response as a part of the immune system’s foreign body reaction. Foreign body reaction is a typical response to the implantation of biomaterials and it has been shown to result in increased Th2-associated inflammation (Allman et al. 2001, Lu et al. 2004). At 3 days we show that scaffold conditions had an increased number of M2 macrophages (Figure 3.14), and at 14 days defect regions had increased inflammation and less collagen synthesis compared to empty defects (Figure 3.16). Our results could be consistent with a foreign body reaction to biomaterials, which is characterized by a prolonged, chronic inflammatory phase with
an increased number of macrophages, giant cell formation, and in some cases resulting in fibrous encapsulation of the material (Anderson 2001, Anderson et al. 2008, van Putten et al. 2013). While periostin has been implicated with Th2-associated inflammation (Izuhara et al. 2014, Liu et al. 2014), our results indicate that it did not influence the inflammatory response at 3 days when compared to collagen only scaffold condition. In summary, we show increased recruitment of M2 macrophages in response to the scaffolds. However, whether this is a part of regenerative healing response or elevated inflammation from a foreign body reaction is not clear from the current study.

At 14 days, Masson’s Trichrome staining showed evidence that fibroblastic cells from both the gingiva and PDL migrated towards the bone defect with evident deposition of collagen. As PDL cells have been shown to be required for regeneration of multiple tissues within the periodontium (PDL, cementum, bone) (Aukhil et al. 1987, Polimeni et al. 2006), their migration is beneficial in healing and could contribute to regeneration of bone at later time points. In the empty defect, collagen appears to have been primarily synthesized by cells that migrated from the gingiva and, to a lesser extent, by cells from the PDL. Conversely, in both scaffold conditions there appeared to be less collagen overall than in empty defects; however, an increased number of cells migrated from the PDL into the bone defect. Reduced collagen in response to scaffolds is likely a result of the delayed cell infiltration and increased inflammatory response, which was observed at 3 days. While scaffold conditions had reduced infiltration of cells from the gingiva at 14 days and less collagen synthesis; it appears that scaffolds may be facilitating or even inducing the migration of PDL cells. However, additional time points examining the remodelling phase of healing and matrix mineralization are required to fully evaluate the influence of both collagen and periostin/collagen scaffolds on regeneration of the PDL, bone, and gingiva.

Our findings indicate that the addition of recombinant periostin to scaffolds did not result in an observable difference in bone formation at 14 days. Periostin was originally identified a murine osteoblastic cell line (Takeshita et al. 1993) and studies with Pstn-null mice have shown that is has a crucial role in the development, maintenance, and repair of bone (Rios et al. 2005, Bonnet et al. 2009, Bonnet et al. 2013). It has also been shown
that periostin is upregulated in endochondral bone formation during the early stages of callus formation, and in intramembranous ossification; however, expression is restricted to the periosteum and undifferentiated mesenchymal cells rather than mature osteoblasts and bone matrix (Nakazawa et al. 2004, Kashima et al. 2009). Furthermore, expression of periostin mRNA is high during the early stages of osteoblast differentiation in vitro, yet downregulated once matrix mineralization occurs (Merle et al. 2013). This could suggest that periostin is implicated in matrix synthesis but may not be involved with matrix mineralization. Based on the current study, we cannot make any conclusions about the influence of periostin on bone formation in fenestration defects, however the potential inhibitory role of periostin in mineralization is worth examining in the future. While not directly related to periodontal healing, Pstn-null mice exhibit ectopic mineralization of aortic valves through suppression of Notch signalling (a negative regulator of osteoblast maturation) (Tkatchenko et al. 2009, Mead & Yutzey 2012). Moreover, periostin has been shown to be have a role in the localization of Notch1 to the cell surface within the PDL (Tanabe et al. 2010). Future studies will investigate the influence of periostin on bone formation in fenestration defects at later time points (28 and 56 days).

Overall, our analysis of fenestration defect healing suggests that the electrospun type I collagen scaffold (with or without recombinant periostin) delays cell infiltration at 3 days, is associated with increased inflammation, and reduced collagen synthesis at 14 days compared to an empty defect. Increased recruitment of M2 macrophages in both the scaffold conditions could be an indicator of healing response or evidence of foreign body reaction; however, this is not evident based on the current study and requires further examination. At 14 days, scaffold conditions exhibited more inflammatory cells than the empty defect, and appeared to have more cells migrating from the PDL, which could contribute to regeneration at later time points. In conclusion, optimization of the scaffold porosity is required to improve cell infiltration and additional time points are needed to fully evaluate its influence on regeneration.
Chapter 5

5. Conclusion and Summary

We have confirmed that hPDL cells represent a unique cell population that express markers characteristic of fibroblastic, osteoblastic, and ligamentous cell types. Moreover, it was established that the rate of *in vitro* mineralization by hPDL cells is inversely related to donor age. Scaffolds induced a positive cellular response *in vitro* by assessment of cell adhesion, proliferation, and synthesis of the ECM proteins fibronectin and tenascin C. *In vitro*, neither collagen nor periostin/collagen in the form of coatings or electrospun scaffolds induced the production of inflammatory cytokines. *In vivo*, both the collagen and periostin/collagen scaffolds impeded cell infiltration and increased recruitment of M2 macrophages, which could indicate a foreign body reaction or could be associated with healing. The current study indicated that recombinant periostin in an electrospun type I collagen scaffold had only a minor effect on inflammation, collagen synthesis, or bone formation in the healing of fenestration defects. Overall, results indicate that optimization of scaffold design and increased concentrations of recombinant periostin are required to fully assess its influence on healing of fenestration defects.

5.1 Future Directions

5.1.1 Effect of Periostin Concentration on Cellular Response and Scaffold Optimization

The data in this thesis suggests that the presence of recombinant periostin in an electrospun collagen type I scaffold does not promote adhesion or proliferation of hPDL cells, nor does it have any significant effect on cytokine production by macrophages *in vitro*. This is supported by our *in vivo* data, which based on our analysis to date, suggests that periostin/collagen scaffolds have a minimal influence on inflammation, matrix synthesis, and bone formation *in vivo*. While this is the first study to investigate the delivery of recombinant periostin into fenestration defects, the results were unexpected based on previous research that implicates periostin with inflammation (Izuhara et al. 2014), the synthesis and organization of ECM proteins (collagens, fibronectin, tenascin...
Our laboratory has previously demonstrated that the delivery of periostin/collagen scaffolds into excisional skin wounds recovers the phenotype of periostin deletion in mice (Elliott et al. 2012), such that we can conclude that the scaffolds are bioactive. However, while our results show that the periostin/collagen scaffold did not appreciably modulate the healing of fenestration defects at 3 and 14 days compared to collagen scaffolds, only one concentration of periostin was tested. It is therefore possible that the amount of periostin is not sufficient to have an effect on healing in the periodontium or that the electrospun type I collagen scaffold may not be an appropriate vehicle for delivery. In future studies, we will increase the concentration of periostin in the scaffolds, as well as investigate the use of alternate scaffold architectures and/or materials.

In our study, it was also observed that by day 14, the scaffold could not been discerned from the tissue, either suggesting the scaffold had been incorporated into the tissue, or that it was degraded. In future studies, we will pre-label the scaffold using a fluorescent NHS (N-Hydroxysuccinimide) ester, which reacts with amines, to assess localization of scaffolds within the defects at each time point. This will be important in establishing the potential relationship between the scaffold and areas of inflammation within the tissue. Should we find that the scaffold is completely degraded by day 14, it is likely that we would need to incorporate synthetic polymers into the scaffold to increase the biomechanical stability and reduce degradation time of the scaffolds (Karageorgiou & Kaplan 2005). Synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA), poly(ester-urethane)urea (PEUU), or polycapralactone (PCL) can be electrospun to produce a porous scaffold with a slower degradation in vivo compared to the type I collagen scaffold. Furthermore, as our results indicated that the type I collagen scaffold impeded cell infiltration, increasing the porosity of the scaffold through variations in spinning parameters, may be required. It is has been previously characterized that pore sizes less than 50 to 100 µm are insufficient to support mineralization of osteoblasts and that larger (300 to 400 µm) pores are required for capillary ingrowth to facilitate nutrient and oxygen transport for the formation of new bone in vivo (Hulbert et al. 1970,
After scaffold modification, it will be necessary to comprehensively assess the effects of the scaffolds on cell phenotype in vitro prior to in vivo implantation. As controls for the electrospinning process, collagen and periostin/collagen coated surfaces would be included in future in vitro analysis. Particular attention will be made to how changing the concentration of recombinant periostin and scaffold porosity alters the production of cytokines associated with M1 and M2 macrophages by PMA-differentiated THP-1 cells. Future projects will also investigate how changing the scaffold properties (porosity, pore size, fibre diameter, material) influences adhesion, proliferation, matrix synthesis and differentiation of hPDL cell, osteoblast, and gingival fibroblasts. Adhesion and proliferation will be assessed using the CyQUANT® assay to assess cell number based on DNA content. Matrix synthesis will be evaluated using RT-qPCR to detect relative mRNA levels of the ECM proteins fibronectin, tenascin C, biglycan, decorin, versican, and type I collagen. Protein production will be assessed using western blotting and immunocytochemistry with antibodies specific for each ECM protein. Osteogenic differentiation and matrix mineralization will be assessed in cells cultured in osteoinductive media on both coatings and scaffolds. Alizarin Red S staining, and mRNA expression of ALPL (alkaline phosphatase), BGLAP (osteocalcin), IBSP (bone sialoprotein), OSX (osterix) will also be examined.

5.1.2 Influence of Periostin/Collagen Scaffolds on Cell Phenotype and Temporal Tissue Regeneration in vivo

To further quantify the influence of periostin/collagen scaffolds on the healing of PDL and bone in experimentally created fenestration defects further investigations are required. Within this thesis, we have shown a limited effect of the scaffolds at a microscopic level, but periostin is known to modulate many molecular processes that we have not yet investigated; many of which relate to wound healing. With respect to overall experimental design, it will be necessary to expand the animal numbers to include surgeries that allow examination of the healing response at 5, 7, 28, and 56 days.
As we are investigating the healing of many tissues located in one compartment (periodontium), understanding how the scaffolds influence the overall inflammatory condition of the tissues will be of great importance. While the data in this thesis examined the relative numbers of Arginase I positive macrophages between experimental conditions, this polarization of macrophages can be associated with either a healing response (Smith et al. 2014) or a foreign body reaction (van Putten et al. 2013). Given that more Arginase I positive macrophages were observed in the presence of the scaffolds, eliminating a foreign body reaction is of paramount importance. Therefore, it will be necessary to identify all inflammatory cell populations within the wounds at 3 and 14 days. Specifically, neutrophils and M1 polarized macrophages will be identified by staining for the neutrophil-specific protease neutrophil elastase (Mocsai 2013) and the M1 macrophage associated enzyme iNOS (Mantovani et al. 2004, Kadl et al. 2010). Continued presence of neutrophils can be associated with either infection or non-specific inflammation (Dovi et al. 2004, Koh & DiPietro 2011). Therefore, the relative levels of neutrophils, M1 macrophages, and M2 macrophages within the wound will be examined histologically.

Continued presence of monocytic cells also plays a significant role in bone repair and regeneration. Monocytes fuse to form osteoclasts and bone resorption occurs during the early stages of wound healing (Oryan et al. 2015) as well as during foreign body response (Anderson 2001). Additionally, periostin has been shown to decrease osteoclast activity through inhibition of Sost (Sclerostin) (Bonnet et al. 2009). Future studies will focus on whether the presence of periostin in a scaffold influences osteoclast activity, which we will assess using tartrate-resistant acid phosphatase staining on tissue sections.

With respect to healing post-inflammation, much further analysis is required. As stated earlier, denuding the PDL inhibits tissue regeneration (Aukhil et al. 1987), suggesting that these cells are crucial for bone formation and PDL regeneration. Understanding which cell populations enter the defects and interact with the scaffold will be of paramount importance in assessing tissue regeneration. In oral pathologies including peripheral ossifying fibromas that involve ectopic mineralization in the gingiva, PDL cells are considered to be a cell type likely underlying this pathology. We have shown
that cells surrounding the ossifying lesion are Stro-1 positive (Appendix C), suggesting that Stro-1 positive PDL cells could be a prominent cell type in pathology and repair. While using lineage-tracing mice would be the ideal model to assess the role of individual cell populations, mice are too small to perform the fenestration defect surgery described in this thesis. However, as cells were observed to migrate from the gingiva and PDL into the defect, staining of Stro-1 and Scleraxis could be potentially used to distinguish fibroblastic cells from the PDL against cells from the gingiva within the defects, which will be completed in future studies.

Angiogenesis is also an important process in wound healing and regeneration (Madeddu 2005, Gurtner et al. 2008), particularly in periodontal defects where the microvasculature is often disrupted. Therefore, we will investigate how the scaffolds influence angiogenesis post wounding by labeling sections with alpha smooth muscle actin, vascular endothelial growth factor, and CD31/PECAM-1.

Finally, quantification of bone regeneration in a temporal manner within the same animal would be an important step to achieve. To do this, micro CT imaging will be used in combination with histological analysis in additional animals. Furthermore, by injecting tetracycline into the animals new bone formation can be labelled, allowing for the rate of bone formation to be calculated. In combination with Micro CT data, this will give a comprehensive analysis of how the scaffolds influence periodontal regeneration.

5.2 Limitations

The fenestration defect model only allows for a maximum of two surgical sites per animal. As such, it was not possible to assess healing in response to all treatments within the same animal (empty defect, collagen scaffold, periostin/collagen scaffold). To account for this, we used sex-matched littermates in each experiment to minimize variations in healing between animals and pairing of conditions within animals was randomized. The time points examined in this thesis in vivo was limited by the number of animals and future studies will require significantly more animals. Therefore, the time point at 3 days was selected to examine inflammatory response while 14 days was selected to examine matrix synthesis and possible bone formation.
5.3 Summary

This thesis examined the use of an electrospun type I collagen and periostin scaffold to stimulate periodontal regeneration, by assessing cellular response in vitro, and in the healing of rat fenestration defects in vivo. Characterization of hPDL cells confirmed the expression of markers characteristic of fibroblastic, osteoblastic, and ligamentous cell types. Moreover, cells demonstrated the ability to form a mineralized matrix in vitro and showed a negative correlation between osteogenic potential of hPDL cells and donor age. Biocompatibility of scaffolds was indicated with hPDL cells, which were able to adhere, proliferate, and secrete matrix on scaffolds. Furthermore, scaffolds did not significantly effect cytokine production by macrophages. In vivo, scaffolds initially delayed cell infiltration and increased M2 macrophage recruitment, indicating either an elevated healing response or foreign body reaction. The incorporation of periostin into scaffolds had a limited effect on inflammation, collagen synthesis, and bone formation in vivo. Overall, results presented within this thesis suggest that examination of increased concentrations of periostin and optimization of scaffold design are required to conclusively determine the influence of recombinant periostin on periodontal healing.
Bibliography


Appendix A: Human Ethics Approval and Animal Protocol
Office of Research Ethics
The University of Western Ontario
Room 00045 Dental Sciences Building, London, ON, Canada N6A 5C1
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Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D. W. Hamilton
Revision Number: 13937E
Review Date: February 14, 2008
Review Level: Expedited

Protocol Title: Influence of substratum topography on human gingival cell physiology in vitro

Department and Institution: Dentistry, University of Western Ontario

Sponsor:
Ethics Approval Date: February 14, 2008
Expiry Date: December 31, 2012
Documents Reviewed and Approved: Letter of Information and Consent
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB’s as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB’s periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:
- a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
- b) all adverse and unexpected experiences or events that are both serious and unexpected;
- c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald

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UWO HSREB Ethics Approval - Revision
V.2007-10-12 (p41) [ApprovalNotice/HSREB_REV]
13937E

Figure A-1: Human Research Ethics Approval
Figure A- 2: Animal Use and Protocol Approval
Appendix B: Negative Controls of ICC and IHC Staining
Figure B-3: No primary antibody negative control for staining of hPDL cells for various cell type markers. Scale bar: 50 µm
Figure B-4: No primary antibody negative control for Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient F29. Scale bar: 50 µm
Figure B-5: No primary antibody negative control for Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient F42. Scale bar: 50 µm
Figure B-6: No primary antibody negative control for Runx2 localization in hPDL cells induced to undergo osteogenic differentiation, patient M46. Scale bar: 50 µm
Figure B-7: No primary antibody negative control for deposition of fibronectin by hPDL cells on collagen and periostin/collagen scaffolds. Scale bar: 50 µm
Figure B-8: No primary antibody negative control for deposition of tenascin C by hPDL cells on collagen and periostin/collagen scaffolds. Scale bar: 50 µm
Figure B-9: No primary antibody negative control for Arginase I staining in fenestration defects at 3 days.
Appendix C: Stro-1 Immunohistochemistry in Peripheral Ossifying Fibromas
Figure C-1: Stro-1 localized to calcified regions of peripheral ossifying fibromas.
Peripheral ossifying fibromas exhibit ectopic calcification. Stro-1 immunoreactivity was localized on the periphery of osteoids. Representative images of biopsies from 3 patients, corresponding negative controls shown to right. OS=Osteoid.
Figure C-1: Stro-1 localized to calcified regions of peripheral ossifying fibromas.
# Curriculum Vitae

## Kendal Creber

**Post-secondary Education and Degrees:**

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<tr>
<th>Degree</th>
<th>Institution</th>
<th>Location</th>
<th>Years</th>
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<tr>
<td>Master of Engineering Science (Biomedical Engineering)</td>
<td>The University of Western Ontario</td>
<td>London, Ontario, Canada</td>
<td>2012-2015</td>
</tr>
<tr>
<td>Bachelor of Engineering Science (Chemical Engineering)</td>
<td>The University of Western Ontario</td>
<td>London, Ontario, Canada</td>
<td>2008-2012</td>
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**Honours and Awards:**

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<tr>
<td>CADR-NCOHR Student Research Award</td>
<td>2015</td>
</tr>
<tr>
<td>2nd Place Senior Basic Science Category</td>
<td>2015</td>
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<tr>
<td>Chemical Engineering Capstone Design Competition</td>
<td>2nd Place</td>
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**Related Work Experience:**

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<th>Years</th>
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<tr>
<td>Teaching Assistant</td>
<td>The University of Western Ontario</td>
<td>2012 – 2014</td>
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**Publications:**


**Presentations and Conferences Attended:**

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<tr>
<td>IADR General Session and Exhibition</td>
<td>Boston, MA, USA</td>
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<tr>
<td>31st Annual Meeting of the Canadian Biomaterials Society</td>
<td>Halifax, NS, Canada</td>
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<tr>
<td>London Health Research Day</td>
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