

**REGULATION OF GINGIVAL FIBROBLAST GENE EXPRESSION BY LEUKOCYTE
AND PLATELET RICH FIBRIN**

by

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Abstract

Objectives: Leukocyte and Platelet-Rich Fibrin (L-PRF), a new clinically used blood product, is rich in leukocytes and platelets embedded in a high-density fibrin network. L-PRF has shown some clinical promise to promote gingival wound healing. In gingival surgeries where L-PRF is being used, it will be in direct contact with human gingival fibroblasts (HGFs) of the surgical flap. However, little is known about the combined effects of the growth factors and cells present in L-PRF on HGFs. We hypothesized that L-PRF regulates expression of key wound healing genes in HGFs. Thus, our aim was to investigate the effects of soluble biological factors released from L-PRF on HGF gene expression.

Methods: L-PRF membranes were produced from blood samples of six volunteer donors using standard clinical protocol. The membranes were placed into upper compartments of transwell plate inserts (0.4 μm pore size) with confluent layers of HGFs at the bottom of the wells. Thus, only soluble mediators were able to influence HGF gene expression. HGFs with the L-PRF membranes in the wells were incubated for 48h followed by isolation of total RNA for real-time quantitative PCR of wound healing related genes. The levels of selected proteins produced by the cells were analyzed using Western blotting.

Results: Among the 86 genes studied, the expression of 35 genes (41%) was significantly regulated by the L-PRF membranes in HGFs. Replicate membranes from the same donor or between different donors produced similar responses, with membranes from different individuals varying somewhat in magnitude of the response. Among the most regulated genes, matrix

metalloproteinase-1 (MMP-1) and MMP-3 showed several-fold increase by L-PRF-treatment, both at the mRNA and protein level. Angiogenesis related genes, VEGF- α and FGF-2 showed also significant up-regulation in HGFs by the L-PRF-treatment.

Conclusions: The results demonstrate that the L-PRF membranes have a strong and a specific regulatory effect on HGFs that may modulate several aspects of wound healing.

Lay Summary

The aim of this thesis was to investigate the effects of particular blood components found in a concentrated blood product on gum healing. Different laboratory methods were used to assess changes in gene and protein production following exposure to the blood product.

It was found that the production of genes related with gum healing increased as a result of the blood product. Therefore, we concluded that our blood product has a strong effect on gum healing.

Preface

This dissertation is an original intellectual product of the author, Fernanda Barona. University of British Columbia's Research Ethics Board [certificate number H1501881] approved all experimental methods. Dr. Jiarui Bi and Dr. Fernanda Barona performed all experimental manipulation, blood collection and prepared the samples for RT-qPCR and Western blotting. Fernanda Barona prepared the corresponding statistical analysis and Dr. Jiarui Bi prepared the corresponding figures.

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List of Abbreviations

A-PRF: advanced-platelet rich fibrin

ALG9: asparagine-linked glycosylation 9

ALP: alkaline phosphatase

B2M: beta-2-microglobulin

BMSC: bone mesenchymal stem cells

CAF: coronally advanced flap

CTGF: connective tissue growth factor

CX43: Connexin 43

CXCL12: C-X-C motif chemokine ligand 12

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic acid

ECM: extracellular matrix

EDA: extra domain-A fibronectin

EDB: extra domain-B fibronectin

EGF: epidermal growth factor

EGR3: early growth response protein 3

FBS: Fetal bovine serum

FGF: fibroblast growth factor

FGGs: free gingival grafts

FMLP: N-formyl-methionyl-leucyl-phenylalanine

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

GBR: guided bone regeneration

HSP47: heat shock protein 47

IGF: insulin-like growth factor

IL-1: interleukin-1

IL-1 β : interleukin-1 β

IL-2: interleukin-2

IL-4: interleukin-4

IL-6: interleukin-6

KGF: keratinocyte growth factor

L-PRF: leukocyte platelet rich fibrin

L-PRP: leukocyte platelet-rich plasma

LOX: lysyl oxidase

MMP-1: matrix-metalloproteinase-1

MMP-13: matrix-metalloproteinase-13

MMP-3: matrix-metalloproteinase-3

MMP: matrix-metalloproteinase

MSC: mesenchymal stem cells

NAB1: Nuclear polyadenylated RNA-binding protein -1

NAB2: Nuclear polyadenylated RNA-binding protein -2

OFD: open flap debridement

P-PRF: pure platelet-rich fibrin

P-PRP: pure platelet-rich plasma

PBS: Phosphate-buffered saline

PDGF: platelet-derived growth factor

PRF: platelet-rich fibrin

PRP: platelet-rich plasma

PTX: pentoxifylline

RBC: red blood cell

RNA: ribonucleic acid

RT-PCR: reverse transcriptase-polymerase chain reaction

S-PRF: standard platelet rich fibrin

TGF- β 1: transforming growth factor- β 1

TGF- α : transforming growth factor- α

TIMP-2: Tissue inhibitor of metalloproteinase-2

TIMP: Tissue inhibitor of metalloproteinase

TNF- β : tumor necrosis factor - β

TSP: thrombospondin-1

VEGF: vascular endothelial growth factor

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I would like to thank my friends who were kind enough to be volunteers for this project. And lastly, to my family, and my dear boyfriend... for always encourage me to give the best of myself.

Dedication

To God, my dear family and beloved fiancé for all their support through these years.

Chapter 1: Introduction

Leukocyte and platelet rich fibrin (L-PRF) is widely used in dental clinical settings with favorable results reported from case reports suggesting that L-PRF could improve palatal wound healing, periodontal regeneration and healing of extraction sockets¹⁻³. In many of these procedures, L-PRF is placed below gingival flaps. Therefore, the L-PRF membrane will be in direct contact with the gingival fibroblast residing in the connective tissue of the raised flap. Little is known about the effects of the combined growth factors and cells present in L-PRF membranes on gingival fibroblasts. Gingival fibroblasts are unique cells that have special characteristics and may contribute to scarless wound healing. The present study was designed, therefore, to investigate the effects of L-PRF membranes on gingival fibroblasts.

Chapter 2: Function of Gingival Fibroblasts

Fibroblasts are the most popular cells in connective tissues. They participate in regulating several functions like tissue development, organogenesis, hemostasis and maintenance. They also play important roles in various physiological and pathological situations including wound healing, inflammation, fibrosis and cancer⁴.

Gingival fibroblasts have been described before in the literature as having an unique phenotype that shows inter-individual variation⁵. For example, different tissue donors-related factors (age, sex, genetic factors, etc.) may results in qualitative and quantitative changes in fibroblasts subsets gingival tissue. In addition, other factors like local tissue niche, use of medication, injury or pathological processes, or epigenetic factors may cause variability in fibroblast phenotype between different people. Culturing fibroblasts in vitro will further increase this heterogeneity as significant selection of fibroblast subtypes may happen and also other cell types may be present that share properties with fibroblasts⁶. Nevertheless, different studies have shown that human gingival fibroblasts despite the phenotypic variation have overall a high regeneration potential, fetal like phenotype and can regulate inflammation. Due to these properties, gingival fibroblasts may have therapeutic potential^{7,8}. Gingival fibroblasts originate from neural crest and maintain stem cell-like properties such capacity to differentiate to different mesenchymal lineages^{9,10}. In three-dimensional tissue culture, gingival fibroblasts show significantly different gene expression profile from skin fibroblasts¹¹. One of the major differences is that gingival cells express very low level of CD26 that is highly expressed in skin fibroblasts that have a profibrotic phenotype. Compared to skin, oral mucosal gingiva heals faster and with reduced scar formation that may relate to the presence of profibrotic, CD26-

expressing cells in the skin wounds¹¹. Gingival fibroblasts have the capacity to reorganize and remodel ECM that is important for tissue maintenance and wound healing. Interestingly, studies reporting on collagen contraction show ambiguous results between gingival and skin fibroblasts^{12,13}. However, gingival fibroblasts contract and degrade a fibrin lattice faster than dermal fibroblasts due to an increased expression of fibrinolytic tissue plasminogen activator¹³.

This capability may be important in the context of this thesis, as the L-PRF membrane is made of polymerized fibrin. Gingival fibroblasts also secrete pro-angiogenic vascular endothelial growth factor (VEGF), CXCL12 (stromal cell-derived factor 1 α), CXCL8 (IL-8) and stimulate endothelial cell growth in vitro¹⁴. Regarding VEGF, it is also released from the L-PRF membrane among several other growth factors, including PDGF and TGF- β 1¹⁵. Therefore, it remains unknown how gingival fibroblasts would respond to L-PRF in terms of VEGF expression and angiogenesis.

Gingival fibroblasts are likely to react to the L-PRF membrane during the first two weeks of wound healing as it will be absorbed during that time¹⁶. This timing will coincide with inflammatory and early granulation tissue phases of wound healing. In oral mucosa wounds, the inflammatory response is generally faster and qualitatively different from skin wounds¹⁷.

Gingival fibroblast differs from their dermal counterparts in their response to soluble mediators related with inflammation. For instance, they produce high levels of some matrix metalloproteinases, which could activate or inhibit inflammatory mediators and their inhibitor¹⁸.

Furthermore, they are less receptive to stimulation of IL-1 β expression by bacterial lipopolysaccharide¹⁹.

In summary, gingival fibroblasts possess unique phenotype that may contribute to scarless oral wound healing. The responses of gingival fibroblasts to L-PRF have not been fully

explored. In the present thesis, we will, therefore, investigate how these cells alter their expression of wound healing related genes in the presence of the L-PRF membranes from different individuals.

Chapter 3: Wound Healing

Wound healing is one of the most important processes for the organism often presented in oral cavity, caused by either trauma or surgery. There are several steps involved during wound healing. Firstly, hemostasis starts with the release of epinephrine to attempt to minimize bleeding into the soft tissue. Platelets are the key cells in wound healing due to their essential role in hemostasis. In addition to their function in clotting, platelets are deployed to sites of injury or infection to modulate inflammatory processes through the secretion of growth factors, cytokines like PDGF, VEGF, TGF- β 1, and other inflammatory mediators that participate on healing.

During this innate immune response, inflammatory cells that have been recruited to the wound site to release more chemokines and cytokines, which modulate wound healing outcome²⁰.

The second step is the inflammatory stage, in which leukocytes destroy bacteria and macrophages attempt to clean the wound of cellular debris and prevent infection. Macrophages seem to be particularly critical cells for wound repair due to their capacity for releasing Vascular Endothelial Growth factor (VEGF), Fibroblast Growth Factor (FGF), and Transforming Growth Factor- β 1 (TGF- β 1) that function among the most important regulators of tissue repair. Recent evidence suggests that wound macrophage populations shift over time and cells with different phenotypes coordinate different phases of wound healing. Coinciding the inflammatory stage, the re-epithelialization starts 48-72 hours after injury. Epithelial cells proliferate and migrate over the site to cover the denuded wound to seal the wound from external insults such as bacterial infection²¹. When the wound is sealed, angiogenesis and granulation tissue formation starts. Granulation tissue is rich in immature connective tissue and blood vessels and provides scaffold for complex cellular interactions resulting in the rise of myofibroblasts that produce the

bulk of the granulation tissue matrix. In the maturation stage, myofibroblasts disappear and the connective tissue will be reorganized and remodeled by the fibroblasts for mature tissue with high tensile strength.

3.1 Clotting and Inflammation

Hemostasis is the physiological process that preserves the fluidity of the blood and, after injury, minimize blood loss but nonetheless preserves tissue perfusion and stimulates the local repair process. Hence, hemostasis is a balance between clot formation and clot destruction and any alteration of this balance leads to either hyper-coagulation and thrombosis or hypocoagulation and hemorrhage. Blood clotting following extravasation is initiated by the surface activation of Hageman factor that leads to activation of the entire clotting cascade²².

Furthermore, blood clotting is enhanced by tissue pro-coagulant factors released from damaged cells, surface membrane coagulation factors and specific phospholipids expressed on activated platelets and endothelial cells²³. Succeeding injury, the exposure of the sub endothelial tissue and types IV and V collagen will stimulate the binding and aggregation of platelets.

Following activation, platelets will secrete adhesive proteins such as fibronectin, fibrinogen, thrombospondin and von Will brand factor VIII that act as ligands for platelet aggregation²⁴. In addition, platelets release other soluble mediators such as serotonin, platelet derived growth factor (PDGF), transforming growth factor $-\alpha$ (TGF- α), factor V, 1,2-hydroxy eicosanoid acid, platelet-activating factor and thromboxane A₂, which promote vasoconstriction, additional platelet aggregation and release of other growth factors²². After activation, platelet will undergo structural and functional changes, such as acquiring a spherical shape with spinous pseudopod projections that promotes increased aggregation with other platelets. The activated platelets undertake a re-organization of their membrane phospholipids, which will allow the

interaction of clotting factors V and X²². This interaction will increase the production of thrombin, which catalyzes the formation of fibrin from fibrinogen and forms a meshwork around the aggregated platelets²⁵. This platelet and fibrin-rich meshwork will act as a clot and fill in the wounded tissue, preventing further bleeding and bacterial infection. Furthermore, this clot will act as a provisional matrix for cell migration. When L-PRF membranes are produced from donor's blood, the clotting starts with Factor XII reacting with the negative surface of the collection vial. This leads to platelet activation and creation of fibrin as described above but under the influence of the centrifugation force (see below).

During wound healing or after injury, it is important that clot formation remain limited to the injured area. Several factors participate in termination of clotting including the production of prostacyclin that inhibits platelets aggregation, the binding of antithrombin to thrombin, and the production of protein C, which is an enzyme that degrades clotting factor V and VIII. In fibrinolysis, plasminogen is converted to plasmin by tissue plasminogen activator which process initiates clot lysis²⁶⁻²⁸.

The inflammatory phase of wound healing can be divided into early and late inflammatory phases. Early phase starts immediately upon tissue injury starting with the damage of blood vessels and the extravasation of blood components including circulating neutrophils that reach the wounded site to start the early inflammatory phase. Neutrophils clean the wounded area by phagocytosis, enzymatic and oxygen radical-mediated mechanisms. They are recruited to the wounded site by chemoattractants including fibrinopeptides, complement components C5a and C3a, bacterial products, leukotriene B₄, PDGF, platelet activated factor and platelet factor 4. Several of these chemoattractants also support the adhesion of neutrophils to endothelial cells by increasing the expression of CD11/CD18 complex²⁹. Influenced by concentration gradient of

chemoattractants, the neutrophils begin the process of diapedesis through the endothelial cells to the inflammation site. Neutrophil activation by chemoattractants also stimulate the release of collagenase and elastase, which further facilitate cell penetration through the blood vessel basement membranes²². Diapedesis is also enhanced by increased capillary permeability caused by numerous vasodilating agents such as serotonin, bradykinin, arachidonic acid metabolites and histamine. The early inflammatory phase usually lasts for about three days during which time neutrophils become trapped within the wound clot and dehydrated tissue. This eschar sloughs off during tissue repair. Whichever neutrophils remaining within sustainable tissue will be phagocytosed by macrophages during the late inflammatory phase³⁰.

The late inflammatory phase lasts for 7 days overlapping with the early phase. Monocytes/macrophages are the leading cell type. There are two types of macrophages that both differentiate from blood monocytes, namely inflammatory M1 and reparative M2 macrophages³¹. Inflammatory macrophages (M1) are stimulated by TLR-ligands, IFN- γ , TNF- α . They express high levels of TH1 pro-inflammatory cytokines (IL-2, IL23) and promote inflammation³². Reparative macrophages (M2) are stimulated by IL-4, IL-13, IL-10 and fibrillary collagen. Furthermore, they express high levels of TH2 cytokines and TGF- β , PDGF and VEG¹⁰. M2 macrophages also promote ECM accumulation, wound healing and may also contribute to fibrosis and scar formation. Transition and balance of pro-inflammatory macrophages to reparative macrophages will determine the tissue response after wounding. Macrophages arrive after the neutrophils and are attracted to the site by chemoattractants like collagen fibers, fibronectin, elastin, enzymatically active thrombin and TGF- β ^{33,34}. The transformation of monocytes into macrophages happens by interaction with the wound extracellular matrix²². The main function of M1 macrophages is to debride the tissue through phagocytosis. Phagocytosis is

stimulated by the communication of the macrophages with the extracellular matrix proteins through integrin receptors. M2 macrophages have important roles with growth factor and cytokine release. They are considered the major secretory cells producing many powerful cytokines such as colony stimulating factor-1, TNF- α , TGF- β , TGF- α , FGF, IL-1. These macrophage derived factors are necessary for the initiation of new tissue formation in wounds. By the release of soluble mediators, they regulate the phenotype and function of other mesenchymal and inflammatory cells²⁰.

3.2 Re-epithelialization and Granulation Tissue Formation

Wound re-epithelialization happens within hours after injury. Migrating epithelial cells begin to migrate over the site to cover the denuded wound²¹. In the later stages of re-epithelialization, proliferation of the epithelial cells will occur to provide an increased number of cells to cover the wound²¹. The basal epithelial cells will respond to the wounding by dissolving their hemidesmosomal complexes that will release them from the basement membrane. Furthermore, the epithelial cells will lose intercellular desmosomal attachments and form peripheral cytoplasmic actin filaments which will allow cell movement^{35,36}. In order to migrate keratinocytes express matrix metalloproteinases (MMP's) which have the capacity to degrade extracellular matrix components in order to facilitate migration below or through the clot. Other group of cells like macrophages, fibroblasts and epidermal cells can produce MMP's which collaborate in the degradation of fibrin, fibronectin and collagen³⁷. MMP's plays an important role in re-epithelialization and several studies have shown that blocking MMP's prevents keratinocytes migration. Furthermore, epithelial cells express urokinase-type plasminogen activator that activates plasmin to facilitate the degradation of the fibrin-fibronectin

clot³⁸. The migration of epithelial cells through a provisional wound matrix composed mainly of fibrin, fibronectin, and vitronectin is highly regulated by integrin cell-surface receptors. Integrins are cell-surface transmembrane $\alpha\beta$ heterodimers that are responsible for most cell-extracellular matrix interactions in keratinocytes¹⁷. They have several functions including regulation of cellular functions during development, differentiation and the immune response³⁹.

During wound healing, there is a change in the expression of existing epithelial integrins to allow migration through a provisional wound matrix²⁰. From underneath of the migrating keratinocytes, common basement membrane components such as collagen type IV and VII, heparan sulphate proteoglycan and laminin-1 are replaced by fibronectin, tenascin-C and laminin-5⁴⁰. Fibronectin is essential in the formation of the primary clot and for many cellular functions during re-epithelialization and granulation tissue formation. Cellular EDA fibronectin is expressed by migrating keratinocytes¹⁷. Laminin-5 provides migratory and stopping signals for migrating cells, depending on its proteolytic processing⁴¹. Finally, Tenascin-C is also expressed during re-epithelialization of wounds⁴⁰. It is believed to function as modulation of cell adhesion during the migratory stage.

Granulation tissue formation will begin to form beneath the epithelium four days after injury to act as a temporary barrier against external environment²². Clinically this tissue bleeds easily and have a granular look⁴². It is primarily composed by inflammatory cells mainly macrophages (see above), newly formed and sprouting blood vessels, fibroblasts and loose connective tissue. As mentioned above, M2 macrophages provide a continuous source of cytokines essential for fibroplasia and angiogenesis²¹. Fibroplasia is a mixture of fibroblasts and extracellular matrix (ECM). Fibroplasia stimulus arises from chemotactic, mitotic and modulatory effects of cytokines, which are mainly released from macrophages and platelets.

Fibroblasts themselves are also capable of producing cytokines and these cells respond in an autocrine manner⁴³. Some of the key cytokines involved in fibroplasia include PDGF, TGF- α , TGF- β , connective tissue growth factor (CTGF), and basic fibroblast growth factor (b-FGF).

3.3 Angiogenesis and Remodeling

The process that involves formation of new blood vessels is angiogenesis, and is essential for the formation of granulation tissue. The migration and mitogenesis of the endothelial cells in conjunction with an appropriate extracellular matrix is needed for angiogenesis²¹. There are several molecules with angiogenic capacities including vascular endothelial growth factor (VEGF), TGF- β 1, angiotropin, angiogenin, angiopoietin, interleukin-8, thrombospondin, and lactic acid^{22,44,45}. All these molecules promote angiogenesis by stimulating the production of b-FGF or VEGF by macrophages or endothelial cells. Both molecules are important in the process of wound repair and granulation tissue formation⁴⁶. In addition to these factors, the presence of an appropriate extracellular matrix is needed. Proliferating endothelial cells adjacent to and within wounds express fibronectin receptors⁴⁷ and, therefore, a perivascular matrix rich in fibronectin may promote endothelial cell movement into the wound⁴⁸. Sequence of events leading to angiogenesis have been hypothesized in the literature⁴⁹. Tissue injury causes hypoxia leading to release of angiogenic factors such as bFGF and VEGF. The production of plasminogen activator from endothelial cells will convert plasminogen into plasmin. Afterwards, plasmin and activated collagenase will digest the basement membrane. This event will then allow endothelial cells to migrate into the wound and create new blood vessels.

The process of extracellular matrix remodeling, cell maturation, and cell apoptosis generate the last phase of wound healing known as tissue remodeling. This phase overlaps with granulation tissue formation, for example granulation tissue formation might be happening in the middle of the wound while tissue remodeling happens in the edges of it²². In late granulation tissue, resident fibroblasts and other cell types in the wound differentiate to myofibroblasts in the presence of fibronectin and TGF- β ¹⁵⁰. This phenotype is characterized by abundant cytoplasmic microfilament bundles of α -smooth muscle actin⁵¹. Approximately by two weeks 70% of fibroblasts in mature granulation tissue express α -smooth muscle actin⁵¹. Myofibroblasts are responsible for deposition remodeling of the main components of the wound extracellular matrix.

The attachment of myofibroblast to fibronectin and collagen by various integrins permit contraction by the myofibroblasts to be transmitted across the wound. Wound contraction by myofibroblast occurs between days seven and fourteen following injury and is stimulated by TGF- β and PDGF⁵². In the end of the healing, myofibroblasts are eliminated from wounds by apoptosis and replaced by resident fibroblasts in a process that is not well documented but may relate to local tissue environment and pericellular environment^{21,53}. Collagen remodeling stimulated by TGF- β 1 and tension occurs during the transition from granulation tissue to healed tissue or scar²¹. Tension mediated from the matrix seems to regulate the balance between production vs degradation. For instance, collagen gels under tension exhibit a relatively high protein synthesis rate⁵⁴, while stress relaxation down-regulates collagen type I expression and up-regulates MMP-1 and MMP-13 expression⁵⁵. Healing wounds will gain about 20% of its strength by the end of the third week of healing²¹. After this time, wound will accumulate collagen and increased tensile strength at a slower rate. Additional increase in tensile strength has more to do with collagen remodelling, formation of larger collagen bundles and alteration of

intermolecular cross-links rather than increased collagen deposition⁵⁶. In the end, wound healing in the gingiva results almost perfect histological healing although it is not clear whether wounds in gingiva ever heal completely at molecular level⁵⁷. In skin, however, deeper or wider wounds heal by scarring characterized by disorganized collagen in the extracellular matrix and hypercellularity with the presence of residual myofibroblasts.

Chapter 4: Platelet Concentrates

Platelets concentrates designated to reinforce the natural process of healing have been studied over three decades. These preparations have been promoted for surgical or wounded site in order to stimulate and improve healing⁵⁸. The benefits of platelet concentrates are their autologous nature, easy collection, easy chair side preparation, and clinical application without potential risks associated with allogenic products. Currently, various preparations of platelet concentrates are being applied in sports medicine⁵⁹, dental and maxillofacial surgery and veterinary medicine.

Platelet concentrates could be used either topical or as local infiltrations for many clinical purposes. They are obtained, mostly through centrifugation, after different processing of the donor's blood sample. The main objective is to remove elements like red blood cells considered as not usable and to collect other elements like platelets, leukocytes, fibrinogen/fibrin and other forms of circulating cells. There is some confusion with the existing literature regarding the preparation and efficacy of these products. There are several techniques to produce platelet concentrates resulting in different final preparations. In addition, the unclear terminology to classify and describe the many different variations of platelet concentrates in publications makes it difficult to compare them to each other and determine the efficacy.

4.1 History

Platelet concentrates were initially termed as fibrin glues that improve skin wound healing in a rat model⁶⁰. In 1978, new terms were proposed for the use of blood extracts such as "Platelet fibrinogen-thrombin mixtures" or "Gelatin Platelet". These products comprised a consistent number of platelets inside the final fibrin preparation. The main idea was to strengthen

the fibrin gel and combine the healing properties of platelets and fibrin. These techniques were the first platelet rich gels that were used in several branches of medicine and in oral surgery⁶¹.

The field developed slowly until 1998 when platelet rich concentrates with growth factors were introduced to improve regeneration in medical fields⁶². Afterwards, several investigations were published on the topic and confusion about the terminology started to evolve. All these products were then called platelet rich plasma (PRP), without considering their true composition. Furthermore, another form of platelet concentrates was advanced in France and termed Platelet-Rich Fibrin (PRF), because of the robust fibrin gel polymerization of the preparation⁵⁹. This technique had a completely different configuration than PRPs and it was named a “second-generation” platelet concentrate, while this term is perhaps not adequate taking into account the long history of evolution of the platelet concentrates. Later on, the second most important evolution of the terminology occurred. Platelet concentrates were additionally linked with different forms of circulating cells, especially leukocytes^{63,64}. It is interesting to see how the evolution of these concentrates influenced research in this field. Evolving from interest about the unique fibrin matrix, later to the healing properties of the platelets and lastly to the influence of the growth factors and circulating cells for tissue regeneration^{63,64}.

4.2 Classification

The current classification was described by Dohan. This classification separated the products following at least two key parameters, namely cell content and fibrin construction. Based on this classification, four families of platelet products were proposed; Pure Platelet Rich Plasma (P-PRP), Leucocyte and Platelet Rich Plasma (L-PRP), Pure Platelet Rich Fibrin (P-PRF) and Leucocyte and Platelet Rich Fibrin (L-PRF)⁵⁹

Pure Platelet- Rich Plasma (P-PRP)

P-PRP products are preparations without leukocytes and with a low-density fibrin network after platelet activation. All these preparations can be used as liquid solutions or activated gel forms. Therefore, P-PRP can be injected⁶⁵. Several procedures for preparation exist, predominantly using cell separators such as continuous flow plasmapheresis. This technique is preferred by several authors even though it is complicated to be used on a daily basis. The main advertised method of P-PRP is known under the commercial name PRGF26 [Plasma Rich in Growth Factors or EndoRet, Biotechnology Institute BTI]. P-PRP has been used in various clinical conditions, predominantly in sports medicine⁵⁹. The literature on this technique, however, is somewhat biased as the promoting company has published most of the articles.

Another technique of P-PRP that was extensively sponsored for skin ulcers and is known under the commercial name: Vivostat PRF (Platelet-Rich Fibrin, Vivostat A/S, Alleroed, Denmark). The name is misleading as this technique is not a PRF as promoted by the company, instead a P-PRP product.

Leukocyte and Platelet Rich Plasma (L-PRP)

Leukocyte-and Platelet-Rich Plasma (L-PRP) products are formulations with leukocytes and platelets in low-density fibrin network. All the products of this family can be applied as liquid solutions or in an activated gel form⁵⁹. This is the platelet concentrate family with the largest number of commercial systems with interesting results in different medical fields⁶⁶. L-PRP has shown to improve acute nerve, muscle and cartilage injury caused by trauma by improving chemotaxis, angiogenesis and production of extracellular matrix⁶⁷. Several automated protocols have been advanced in recent years. These protocols require the use of specific kits that

permit minimum handling of the blood samples and maximum standardization of the preparations. For example, Harvest Smart-PreP (Harvest Technologies, Plymouth, MA, USA) and Biomet GPS III (Biomet Inc., Warsaw, IN, USA) are two such commercial systems. Regarding the efficacy, most published investigations are case reports and further controlled studies are needed⁵⁹.

Pure Platelet Rich Fibrin (P-PRF)

These are platelet rich preparations without leukocytes and with a high-density fibrin network. They only exist in a sturdily activated gel form, and cannot be injected or used like traditional fibrin glues. Nevertheless, due to their strong fibrin matrix, they can be utilized like a solid material for other applications. There is only one known commercial product in this family, called Fibrinet PRFM (Platelet-Rich Fibrin Matrix, Cascade Medical, Wayne, NJ, USA). The principal inconvenient of this technique remains its high cost and relative difficulty in contrast to the other types of P-PRF available.

Leukocyte and Platelet Rich Fibrin (L-PRF)

L-PRF preparations have a high concentration of leukocytes and platelets within a high-density fibrin network. These ones cannot be injected because L-PRF are composed of a membrane-like structure. L-PRF was developed by Choukroun and colleagues⁵⁹ and the technique consists of one-step centrifugation and no anticoagulants or blood activators are required, which is the key difference with other families⁵⁹. The commercial Intra-spin L-PRF protocol has FDA approval (Intra-Lock Inc., Boca Raton, FL, USA). Differently to the other families, the production of this platelet concentrate chair-side in clinical setting is very simple.

This protocol remains the main platelet concentrate technique used in oral and maxillofacial surgery, because the L-PRF membranes and clots are very easy to combine with current surgical techniques. Some interesting results have been obtained with L-PRF applications in dentistry, sports medicine, orthopedics, however, vigorous randomized clinical trials are largely missing⁶⁸.

4.3 Differences Between P-PRP and L-PRF

Unlike P-PRP, L-PRF is acquired by single centrifugation of venous blood by use of a specially programmed centrifuge. L-PRF includes autologous growth factors originated from platelets and a fibrin matrix. One of the most significant differences between L-PRF and most P-PRP preparations is that L-PRF manufacture does not require other reagents, i.e. no need of anticoagulant during blood harvesting or calcium chloride and thrombin for platelet activation and fibrin polymerization. In contrast, L-PRF itself gradually polymerizes during centrifugation similarly to natural polymerization *in vivo*. The polymerization is essential for correct three-dimensional (3D) organization of a fibrin network. L-PRF fibrin structure is advantageous for cytokine enmeshment and cellular migration and slowly releases platelet growth factors for at least 7–10 days^{69,70}. The behavior of L-PRF membrane and P-PRP gel has been compared. These two products were placed in culture medium for 7 days and the growth factor release was compared⁵⁸. L-PRF remained solid and intact for 7 days and continuously released growth factors (PDGF, VEGF, TGF- β 1), while P-PRP gels released most of the growth factors during the first hours and completely dissolved in 3 days⁷¹.

The benefits of leukocytes of the L-PRF membrane remain debatable. Some authors proposed that they could have a negative effect due to the potential stimulation of the inflammatory process⁵⁸. However, other authors claim that they play a key role in the healing and inflammation process⁵⁸. For instance, Shär et al. (2015) described that VEGF is mainly produced by leukocytes and, therefore, without them less growth factors would be present⁷². Lundquist et al. (2013) investigated the effect of L-PRF on fibroblasts. The results showed a strong mitogenic effect of the human fibroblasts confirming the biological activity of

L-PRF. In addition, the investigators reported release of high levels of growth factors involved in angiogenesis (VEGF) as well as in re-epithelization (HGF), and cytokines (IL-8, IL-16)⁷³.

Chapter 5: L-PRF

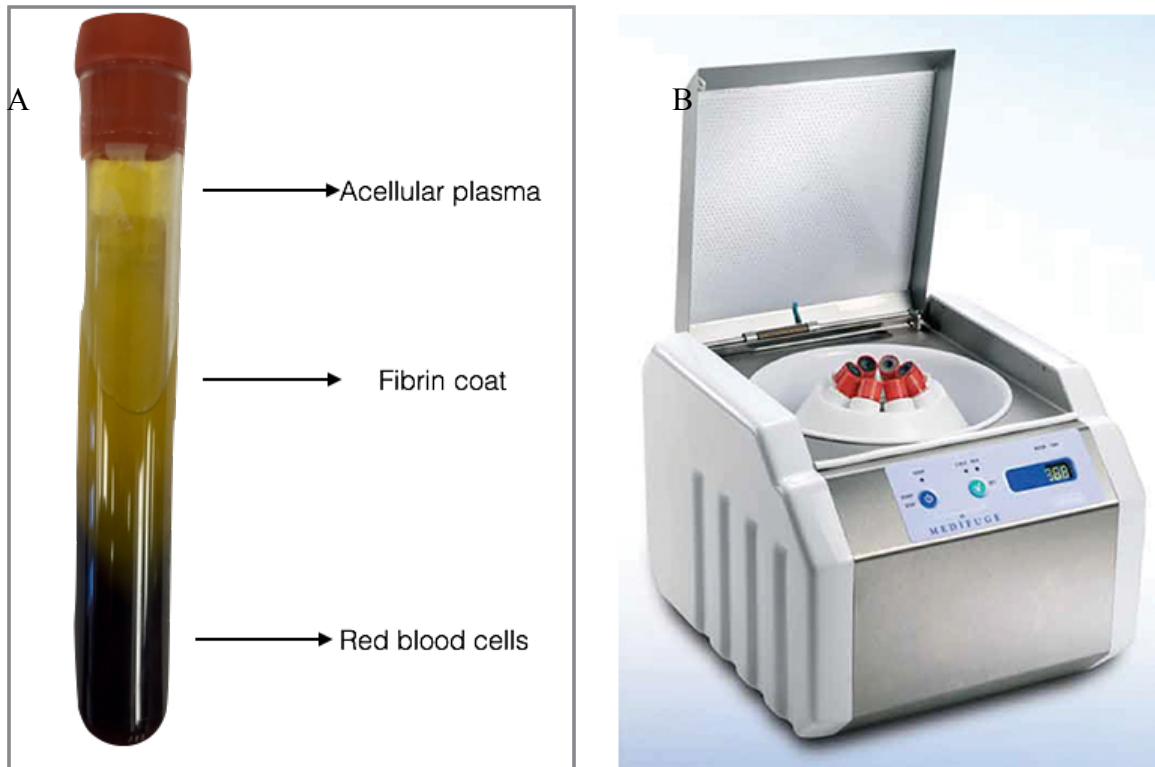
Choukroun developed L-PRF protocol in France for specific use in oral and maxillofacial surgery⁷⁴. Because L-PRF was the protocol used in this thesis work, it will be described in detail below.

5.1 Preparation and Handling

The L-PRF protocol is very simple, however, the membranes need to be prepared just prior to application^{75,76}. To make the L-PRF membranes, a specific centrifuge is needed in addition to glass collection test tubes and blood collection armamentarium. There is some controversy about the speed of the centrifugation and the timing. In 2014, a study was conducted to compare the effect of the centrifugal force using protocols to produce standard platelet-rich fibrin (S-PRF) (2700 rpm, 12 minutes) and advanced platelet-rich fibrin (APRF) (1500 rpm, 14 minutes). Immunohistochemistry for monocytes, T and B -lymphocytes, neutrophilic granulocytes, CD34-positive stem cells, and platelets was performed on clots produced from four different human donors. Platelets were detected throughout the clot in both groups, although in the A-PRF group, more platelets were found in the distal part, away from the buffy coat (BC). Stem cells, T- and B-lymphocytes and monocytes were detected around the BC in both groups.

Decreasing the rpm while increasing the centrifugation time in the A-PRF group gave a greater presence of neutrophilic granulocytes in the distal part of the clot. In the S-PRF group, neutrophils were found mostly at the red blood cell (RBC)-BC interface. The clinical relevance of these differences remains unclear⁷⁷.

Figure 5.1: L-PRF Preparation and Handling. (A) The L-PRF clot was produced in glass-coated plastic tubes. It contains the following layers: Upper plasma, middle contains the fibrin coat, lower is composed by RBCs, (B) Medifuge- Silfradent centrifuge.



Another study made a comparison between the different centrifugation forces used in the most popular machines available in the market, namely the original L-PRF centrifuge (Intra-Spin, Intra-Lock) and three other laboratory including Advanced platelet rich fibrin (APRF) (1500 rpm, 14 minutes), LW-UPD8 (LW Scientific) and Salvin 1310 (Salvin Dental). The author evaluated the level of mechanical vibrations during centrifugation and the impact of the centrifuge characteristics on the cell and fibrin architecture of a L-PRF membrane. The second objective of this article was to assess how changing some parameters of the L-PRF protocol may influence its biological behaviour⁷⁸. The original L-PRF centrifuge (Intra-Spin) was the steadiest machine in all configurations and persisted below the threshold of resonance, revealed a strongly

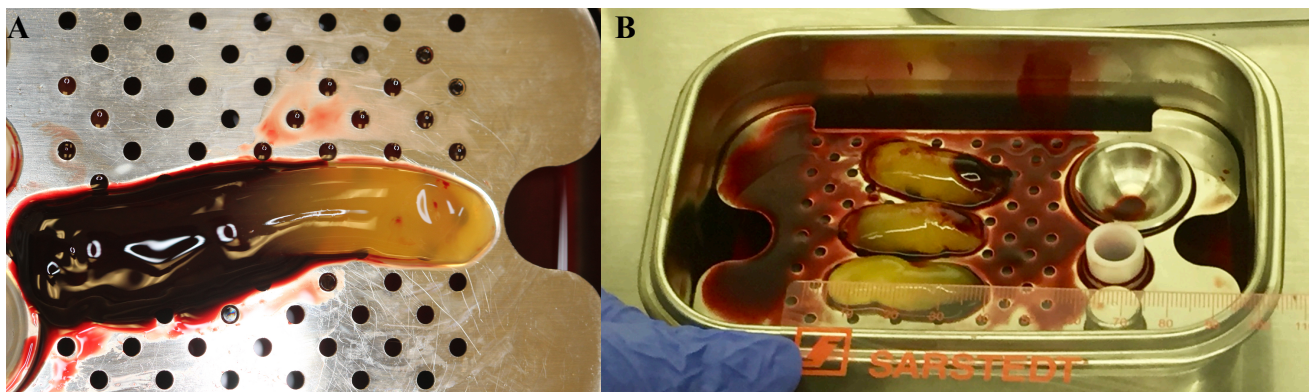
polymerized thick fibrin matrix and the cell population seemed alive with a normal shape. The A-PRF, Salvin and LW PRF membranes presented a weaker polymerized fibrin gel and most of the visible cell bodies appeared damaged. Furthermore, a slow release of TGF- β 2, PDGF, VEGF factors from original L-PRF membranes was significantly stronger (more than twice stronger, $p < 0.001$) at all experimental times than the release from A-PRF membranes⁷⁸.

L-PRF Membrane

The main advantages in L-PRF preparation are the non-existence of anticoagulant suggests the activation in a few minutes of most platelets of the blood sample in contact with the tube walls and the initiation of the coagulation cascades. After centrifugation, the upper part of the tube will contain acellular plasma, the middle part has the fibrin membrane and cells and the lower part contains the red blood clot. The main disadvantage of this technique is that it requires quick handling (transferring the blood tube to the centrifuge). Due to the lack of anticoagulant, the blood samples start to coagulate almost immediately upon contact with the tube glass. If the transferring of the blood tube to the centrifuge is overly long, fibrin will polymerize in a diffuse way in the tube, producing membranes with inconsistent quality⁷⁹. The membrane and blood clot are extracted from the tube followed by removal of the blood clot (Figure 3.2). Discussion has been ongoing on which was an adequate way to conserve the L-PRF membrane when prepared, because storing them under compression for too long would increase an early release of growth factors and the shrinkage of the fibrin network and could also dehydrate and damage the leukocyte content. This issue is concerning when considering that many clinicians using L-PRF do not comprehend that L-PRF is a living clinical component. One option to conserve the L-PRF clot is to store it in its centrifugation tube. If the serum has not been removed from the clot, the

growth factor content remains stable⁸⁰. It is a good way to gain 5-15 minutes, however, it is not a long-term solution, because with extended time the L-PRF membranes blend with the red blood clot base, leading to an unusable material. A second option is to keep the clots in a sterile metal cup and to press them into membranes with a sterile metal spoon and keep them in a wet serum environment. The disadvantage of this technique is that is not easy to handle when numerous L-PRF membranes are produced.

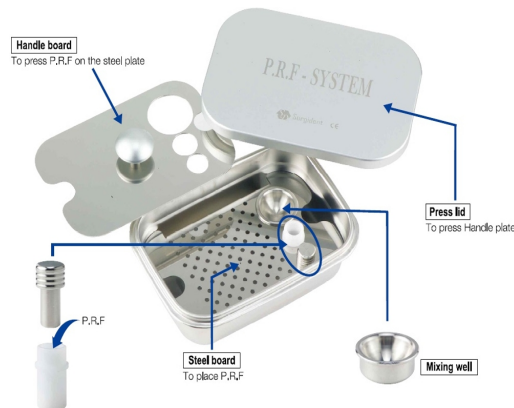
Figure 5.2: L-PRF membranes. (A) L-PRF and blood clot after removal from tube. (B) L-PRF membranes after removal of the clot.



In 2007, a new instrument for the preparation and standardization of L-PRF membranes were developed, namely the L-PRF Box (Process, Nice, France). This metallic box (Figure 3.3) was designed to produce membranes of constant thickness that remain hydrated for several hours and to retrieve the serum exudate expressed from the fibrin clots which is rich in the proteins vitronectin and fibronectin⁷⁶. The L-PRF membranes are then positioned on the grid in the L-PRF Box® (Process Ltd., Nice, France), and covered with the compressor lid. This result is an inexpensive autologous fibrin membrane in approximately one minute. The L-PRF Box also

contains compression wells to produce L-PRF products for filling cavities (such as extraction sockets)⁶⁹.

Figure 5.3: L-PRF Box and its Components.



5.2 Structure and Content

L-PRF is a firm fibrin-based dense biomaterial with a three-dimensional architecture containing 95% of the platelets and 50% of the leukocytes from the initial blood harvest⁸¹.

Dohan et al. 2010, evaluated the cell composition and three-dimensional organization of the L-PRF membranes and determined the influence of different collection tubes (glass-coated plastic tubes or dry glass) and compression procedures (forcible or soft) on the final L-PRF-membrane architecture. Roughly 97% of the platelets and 50% of the leukocytes were maintained in the L-PRF membranes showing a specific three-dimensional distribution and mature fibrin network. There was no significant difference in the L-PRF architecture between groups using the different collection tubes and compression techniques⁸¹.

5.2.1 Cytokines Present in L-PRF Preparations

L-PRF membranes contain several cytokines and growth factors that will be released when the membranes gradually dissolve in vivo. Fibroblasts and other cells in the gingival wounds will be, therefore, exposed to these mediators that may significantly regulate their functions. Some of these factors are released from platelets during activation while others come from leucocytes present in the L-PRF. Among pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α are present in the L-PRF products. IL-1 β is likely derived from neutrophils present in the L-PRF⁸². It is a key mediator of inflammation control. Its role in normal wound repair is, however, somewhat unclear. IL-1 receptor knockout mice have normal skin wound closure but less fibrosis^{83,84}. Interestingly, oral wound healing seems to require IL-1 signaling for protection from bacterial insults⁸⁴. Interleukin 6 (IL-6) is another leucocyte-derived pro-inflammatory cytokine present in L-PRF. Its functions related to IL-1 β and TNF- α circuit that could stimulate and constitutes a major amplification pathway for signals transmitted to immune cells.

Furthermore, IL-6 will support the reaction chains leading to inflammation, destruction and remodeling. Interestingly, IL-6 promotes proliferation of fibroblasts²⁰. In addition, IL-6 appears to have positive effects on wound healing as it promotes keratinocyte migration, macrophage infiltration and angiogenesis⁸⁵.

TNF- α is yet another cytokine released from leucocytes in the L-PRF. TNF- α activates monocytes and stimulates the remodeling capacities of fibroblasts⁸⁶. Moreover, it promotes phagocytosis and neutrophil cytotoxicity and modulates the expression of key pro-inflammatory mediators such as IL-1 β and IL-6⁸⁷. Furthermore, promotes or stimulate proliferation of fibroblasts and suppresses myofibroblasts differentiation²⁰. (Table 3.2.1) TNF- α seems to

promote early wound healing as mice treated with anti-TNF- α antibody have delayed wound closure, and inflammatory cell and fibroblast density⁸⁸.

IL-4 is also released from the L-PRF. It serves as pleiotropic cytokine that stimulates ECM synthesis in fibroblast and inhibits stimulation of MMP-1 and MMP-3 by IL-1 β . In fact, it counteracts many transduction pathways from IL-1 β mediated signals⁸⁹. In healing wounds, topical application of IL-4 accelerates wound healing⁹⁰ (Table 3.2.1)

L-PRF membranes also contain several growth factors and cytokines that are believed to be beneficial for wound healing. Many of these factors are released from platelets during their activation. TGF- β 1 is released and activated during platelet activation. It serves as a crucial cytokine for surveillance of inflammation as it keeps inflammation under control⁹¹. TGF- β 1 stimulates chemotaxis of fibroblasts and increases their integrin and matrix production⁹². It also reduces matrix breakdown by reducing collagenase activity and increasing TIMP expression⁹¹.

As mentioned above, TGF- β 1 is a key cytokine required for myofibroblast differentiation⁹³. Excessive or prolonged TGF- β 1 expression is associated with fibrotic scars⁹³. In addition to TGF- β 1, L-PRF membranes release PDGF, FGF and IGF-1 that also promote fibroblast migration, proliferation and ECM synthesis²⁰. All these growth factors released from platelets have been also tested for periodontal wound healing and PDGF has shown sufficient evidence of efficacy for commercialization²⁰.

L-PRF also contains factors such as VEGF and FGF that promote angiogenesis, a crucial process for wound repair⁹⁴. FGF also promotes migration and proliferation of fibroblasts and their ECM and collagenase synthesis^{20,95}. (Table 3.2.1)

L-PRF releasate seems to stimulate osteoblast proliferation, matrix production and differentiation⁹⁶. Less is known about effects of L-PRF on fibroblasts, particularly on gingival fibroblasts. A few studies suggest that L-PRF has positive effect on fibroblast proliferation. In this thesis, we will explore in more detail how L-PRF modulates the expression of wound healing related genes in gingival fibroblasts.

5.2.2 Current Clinical Use

Oral Applications

a. Direct sinus floor elevation procedures

The use of L-PRF has been widely described in the literature, for instance an animal study compared the potentials of L-PRF-mixed with Bio-Oss® and Tisseel®-mixed Bio-Oss® to improve bone regeneration in a canine sinus model⁹⁷. The mean osseointegration rate was $43.5 \pm 12.4\%$ and new bone formation rate $41.8 \pm 5.9\%$ in the L-PRF/Bio-Oss® composite sites. In the Tisseel®/Bio-Oss® composite sites they were $30.7 \pm 7.9\%$ and $31.3 \pm 6.4\%$. There were statistically significant differences between the groups. The findings from this study suggest that when L-PRF is used as an adjunct to Bio-Oss® particles for bone augmentation in the maxillary sinus, bone formation in the graft sites is significantly greater than when Tisseel® is used⁹⁸.

Another study evaluated the effects of L-PRF on post-operative complications after direct sinus elevation procedure. They concluded that L-PRF and allogeneous bone graft in combination with L-PRF membrane does not significantly improve postoperative complications following direct sinus lifting⁹⁹. However, using L-PRF may promote better soft tissue healing and less post-operative pain after extractions¹⁰⁰. A recent systematic review found that the benefits of using L-

PRF in sinus lifts is controversial. Some studies have shown no difference in amount of vital bone or residual bone, whereas, other studies have shown faster healing. However, due to the lack of standardization, further RCTs with longer follow ups are needed¹⁰¹.

b. Protection and stabilization of graft materials during ridge augmentation procedures and socket preservation.

L-PRF could aid as a resorbable membrane for guided bone regeneration (GBR), blocking the premature migration of epithelial cells into bone defect and providing a space that allows the migration of mesenchymal stem cells and facilitate the underlying blood clot to mature into lamellar mineralized bone. However, L-PRF membranes dissolve rather rapidly. In a recent study a comparison of treatment outcome after extraction of impacted third molars with and without the application of L-PRF was performed. It was concluded that L-PRF reduces the severity of immediate postoperative sequelae, decreases preoperative pocket depth and had more bone density after 3 months but this aspect was not statistically different⁹². In a recent systematic review, it was concluded that using L-PRF as an adjunct for ridge preservation warrants further randomized clinical trials¹⁰¹.

d. For root coverage with single and multiple teeth recession.

The effect of L-PRF and coronally advanced flap (CAF) has been compared with CAF only. In this meta-analysis, no significant differences were found in term of recession depth reduction or keratinized tissue width gain after 6 months. However less side effects such as pain, swelling and hypersensitivity were associated with L-PRF group⁶⁸.

e. Regenerative procedures in treatment of 3-walled osseous defect and furcations.

In a recent meta-analysis, it was concluded that L-PRF vs open flap debridement (OFD) showed a statistical significant difference in favor of OFD+L-PRF in terms of pocket depth reduction, clinical attachment gains and amount of bone fill⁶⁸.

f. To enhance palatal wound healing after free gingival graft.

It has been proposed that L-PRF enhances wound healing due to its stimulatory effect on angiogenesis and epithelialization. Therefore, this material has been used to enhance palatal donor side wound healing after harvesting free gingival grafts. In a recent study, 18 patients were treated with free gingival grafts (FGGs). L-PRF was applied on the donor site of 10 patients and in the other 18 patients only periodontal pack was applied. Sites where L-PRF was used showed faster wound closure and lesser morbidity than the control group¹⁰².

Chapter 6: Aim of the Study

L-PRF has shown some clinical promise in case studies for instance improving palatal wound healing, improving periodontal regeneration and facilitating faster socket healing¹⁻³ and has been, therefore, adopted to clinical use because of it is easy to produce in one step centrifugation process. Little is known about the effects of the combined growth factors and cells present in L-PRF membranes on gingival fibroblasts. In many surgical procedures, L-PRF membranes will be placed underneath surgical flaps facing gingival fibroblasts. The aim of this study was, therefore, to investigate the effects of soluble biological factors released from L-PRF on expression of wound healing related genes in gingival fibroblast.

Hypothesis:

1. Soluble factors released from L-PRF regulates the expression of wound healing related genes in gingival fibroblast
2. The effect of soluble factors from L-PRF on fibroblast gene expression are time- and dose-dependent
3. Soluble factors from L-PRF regulate collagen gel contraction in vitro

Chapter 7: Material and Methods

Cell Culture:

Normal human gingival fibroblasts (HGFs)¹⁴. They were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 23 mM sodium bicarbonate, 20 mM HEPES, 1% antibiotics (50 µg/ml streptomycin sulfate, 100 U/ml penicillin; Gibco) and 10% heat-inactivated fetal bovine serum (FBS; Gibco).

Human Blood Samples:

The Clinical Research Ethics Board at the University of British Columbia reviewed and approved all procedures involving human tissue donors [certificate number H1501881]. Informed written consent was obtained from the patients providing the blood samples per the Helsinki Declaration (1975). Peripheral venous blood (4 vials 9ml each) was collected from 6 healthy volunteers (appendix b) by venipuncture with using a 21 x three quarter gauge butterfly vacuette needle.

L-PRF:

To prepare L-PRF, 9 mL of blood was collected in 10-mL polyethylene tubes (Vacuette test tubes), coated with silica micro particles and immediately centrifuged at variable rpm from 2400-3000 rpm for 12 minutes at room temperature using a table centrifuge specifically designed for this application (Silfradent Medifuge MF200) (Figure 7.1). This centrifuge device uses a program with the following characteristics: 2,700 rpm 2 min, 2,400 rpm 4 min, 2,700 rpm 4 min,

and 3,000 rpm 3 min. After centrifugation, the L-PRF membrane and blood clot was removed from the tube. The red blood component (RBC) was discarded with scissors leaving approximately 1-2 mm of the RBC remaining.

Figure 7.1: L-PRF Membrane Preparation: (A): L-PRF in the tube. (B): L-PRF clot. (C): Removal of RBC. (D): L-PRF membrane



Treatment of Fibroblasts with L-PRF

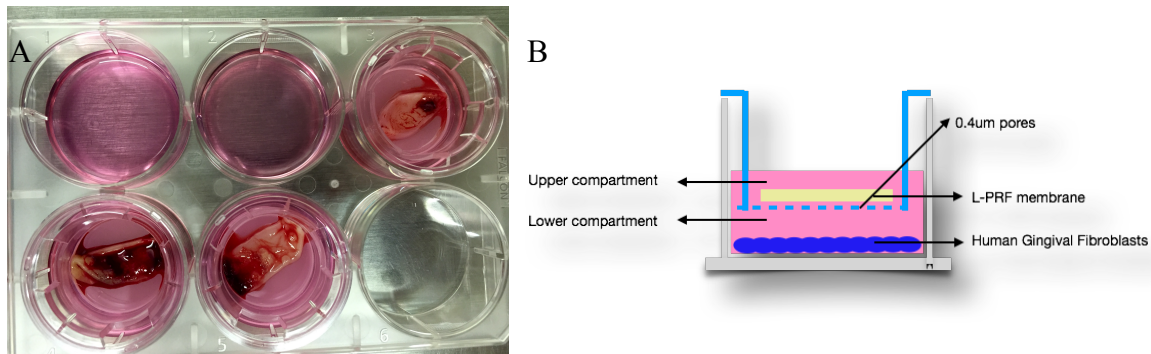
HGFs were seeded in 6-well plates in their normal growth medium (2×10^5 cells per well) for 48 h. The cell layer was then rinsed with phosphate buffered saline (PBS) twice. Transwell inserts (Costar; $0.4 \mu\text{m}$ pore size, Pittston, PA, USA) were then placed into the culture wells.

Freshly isolated L-PRF membranes were placed into the inserts and filled with FBS-free culture medium. The pore size ($0.4 \mu\text{m}$) allows soluble agents from the L-PRF to pass but prevents cellular migration. Control cultures contained the transwells without the membranes.

Cultures were continued for 48 h followed by RNA isolation for real-time quantitative polymerase chain reaction (RT-qPCR; protocol details described below). To study the effect of L-PRF on protein expression in HGF, the L-PRF membranes were removed after 48 h, followed with rinsing the cell layer with PBS twice. The cultures were kept for another 24 h in DMEM

medium without serum. Medium and cell layer proteins were then subjected to Western blotting (protocol details described below).

Figure 7.2: L-PRF in Tranwells: (A) *L-PRF membranes were placed on the transwell insert, that separated the membranes from fibroblasts;*(B) *Schematic figure of L-PRF and HGFs inside the transwell.*



RT-qPCR:

RT-qPCR was performed as previously described¹⁰³. Total RNA was extracted from cells grown in 6-well plates using NucleoSpin RNA II kit according to the manufacturer's protocol (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and assessed for purity by RNA/DNA Calculator (GeneQuant Pro, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). RNA integrity was further assessed by electrophoresis using a denaturing agarose gel containing formaldehyde, followed by staining of RNA with 0.5 µg/ml of ethidium bromide in 0.1 M ammonium acetate for 30 min. Gels were assessed for integrity of 18S and 28S rRNAs bands (1.9 kb and 5 kb, respectively). Samples with A260/280 ratio approximately 2.0, and approximately 2.1 ratio of 28S/18S rRNA, were used for the study. 1.0 µg of total RNA was reverse-transcribed using high-capacity cDNA reverse transcription kits (Applied Biosystems, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The cDNA was synthesized using Mastercycler gradient 5331

Reverse-Transcriptase PCR Instrument (Eppendorf AG, Hamburg, Germany) with the program: 1 cycle at 25°C for 10 min, 1 cycle at 37°C for 120 min and 85°C for 5 min to heat-inactivate the reverse transcriptase. The primers used for real-time PCR are listed in (Appendix A). All primers were designed on the boundaries of exons and analyzed by BLASTn software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for their specificity. The primers were designed to yield a target sequence that was 60–150 base pairs long with a GC content between 40–70%. The efficiency of the target amplification was optimized (which includes annealing temperature, primer and sample concentration) up to 95% for each primer set using a 10-fold dilution series of cDNA while standard curves were made. For the reaction, cDNA from each sample was diluted to a concentration (1 ng/ml) such that the Ct values were well within the range of their standard curves, and 5 µl of diluted cDNA was mixed with 10 µl of 2 X iQ SYBR Green I Supermix (Bio-Rad), and 5 p-moles of primers, for a final volume of 20 µl. Real-time PCR amplification was performed on the CFX96 System (Bio-Rad) using the following program: 1 cycle at 95°C for 30s, 40 cycles at 95°C for 10s, 60°C for 24s, and reaction completion with reading plate and a melt curve analysis from 65°C to 95°C, 5s for each 0.5°C. Amplification reactions were conducted using asparagine-linked glycosylation 9 (*ALG9*), glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and beta-2-microglobulin (*B2M*) as reference genes. For every experiment, both of two reference genes with the M-value below 0.5 were used. Non-transcribed RNA samples were used as a negative control. The PCR reactions were performed in triplicate for each sample. The data was analyzed and is presented based on the comparative Ct method (CFX Manager Software Version 2.1, Bio-Rad).

L-PRF Releasate

In a set of experiments, agents released from the L-PRF membranes into the serum-free culture medium without the presence of fibroblasts were produced (called L-PRF releasate). For these experiments, L-PRF membranes were prepared as described above. Each L-PRF membrane was placed in 35 mm cell culture dish with 2 ml serum-free DMEM for 48 h. The medium was collected followed by centrifugation at 108 g for 10 min. The supernatant was collected as the L-PRF releasate.

Collagen I Gel Contraction Assay

To test the effect of L-PRF on human gingival fibroblasts ECM remodeling, collagen I gel contraction assay was used. To create gingival fibroblast inoculated collagen gels, 600 μ l of collagen I solution (PureCol type I bovine collagen solution; Advanced BioMatrix, San Diego, CA, USA) was mixed with 20% (V/V) 5X DMEM (Sigma) containing 2.5% 100 mM NaHCO_3 and 1% FBS (Gibco), and adjusted to neutral pH. HGFs (2×10^5 cells per 0.33 cm^2 dish) homogeneously mixed with 0.5 ml of neutralized collagen mixture and incubated for 2 h during which time the solution elated. Then sterile needle was then used to detach the solid gels from the walls of the wells. FBS-free medium (1%) was added into each well with or without L-PRF releasate (5 mg protein/ml). The photos were taken for recording the gel size on day 0, 3 and 7.

The sizes of gels were then quantified based on the areas measured by ImageJ software.

Statistical analysis

Experiments were repeated separately at least three times. The mean of the six donors was calculated and Student's t-test for paired comparisons was performed to compare the control

versus test. Statistical analysis for RT-qPCR data (performed in triplicates) was done using log₂-transformed data¹⁰⁴. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was performed. Statistical significance was set at $p < 0.05$ and required a minimum of 1.5-fold change. The data in the figures is presented either as box blots (Figure 8.1.) when data is obtained from six donors (mean of three replicate measurements per each donor were used), or as the mean \pm SEM when single donor data is presented with replicate measurements.

Chapter 8: Results

L-PRF Modulates Gene Expression in Human Gingival Fibroblasts

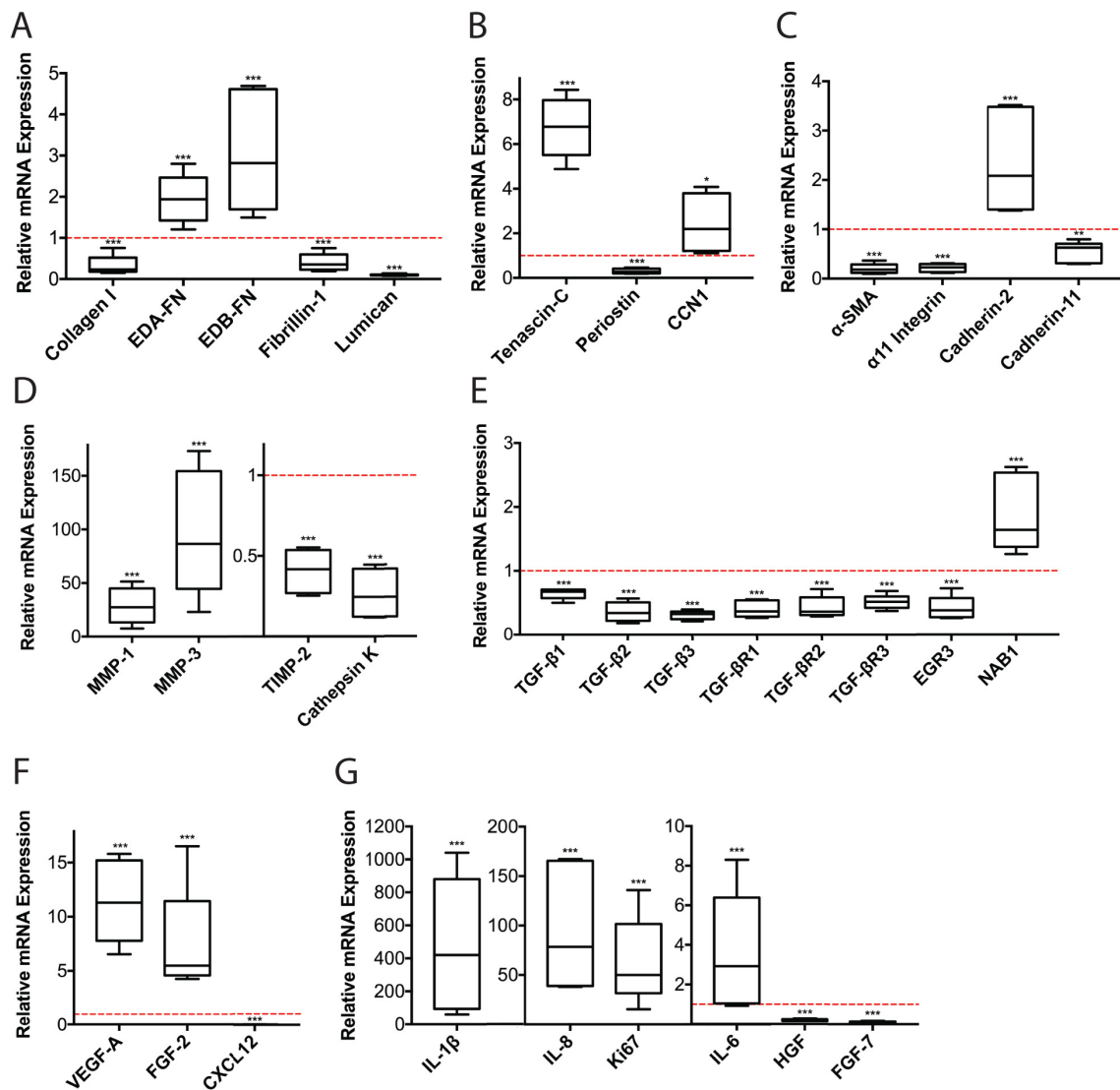
To investigate how L-PRF regulates the expression of wound healing related genes in HGFs (appendix A), and whether there are variations in regulating function of L-PRF between different blood donors, L-PRFs were made from six healthy donors. Among 86 genes encoding extracellular matrix (ECM) proteins, matrix metalloproteinases, myofibroblast- and angiogenesis-associated proteins, transforming growth factors, cytokines, connexin, basement membrane and neural crest differentiation-associated proteins, 35 genes (40.6%) showed significantly ($p < 0.05$) altered expression in all samples from six blood donors (Table 8.1).

Sixteen genes (18%) showed no change in expression or inconsistent results between six donors. Several genes tested ($N=35$; 41%) showed low or non-detectable expression in both treated (in all individuals tested) and control samples.

Table 8.1. L-PRF Effect in Gene Response

CONSISTENT	35	Collagen I, EDA-FN, EDB-Fn, Fibrillin-1, Tenascin-C, Periostin, CCN-1, α -sma, α -11 integrin, cadherin-2, cadherin011, mm-1, mmp-3, TIMP-2, cathepsin-k, Lumican, , VEGF- α , FGF-2, CXCL12, TGF- β 1, TGF- β 2, TGF- β 3, TGF- β R1, TGF- β R2, TGF- β R3, EGR3, NAB1, IL-1 β , IL-8, Ki67, IL-6, HGF, FGF-7
NO CHANGE/ INCONSISTENT	16	Emilin-2, BGN, DCN, P311, EGR1, NMMIIA, β -Actin, r-Actin, MMP2, TIMP-1, -3, -4, Cx45, Collagen IV, Slug, FBN
LOW EXPRESSION/ UNDETECTABLE	35	Collagen III, Elastin, Emilin-1, Emilin-3, THBS1, THBS2, SPARC1, TNX, Osteopontin, CCN2, CCN3, FMOD, ASPN, SMAD7, Cthrc-1, EGR2, NMMIIB, MMP-7, -9, -10, -11, -12, -13, -14, IL-1 α , HB-EGF, GM-CSF, TNF- α , Collagen VII, Laminin-1 α 1, Laminin-5 β 3, FOXD3, Twist1, SNAIL, Nestin2
TOTAL	86	

Figure 8.1: The Effect L-PRF on Human Gingival Fibroblast Gene Expression. Expression of significantly regulated human gingival fibroblast genes (35 of 86 genes tested) was grouped by function and presented for both control- and L-PRF-treated samples. The control (HGF + FBS-free DMEM medium) was set as 1; (A) extracellular matrix related genes; (B) matricellular protein genes; (C) myofibroblast- and cell contractility-associated genes; (D) matrix-metalloproteinase-1 and -3, TIMP-2 and cathepsin k; (E) TGF- β signaling-related genes; (F) angiogenesis-related genes; (G) Interleukines, Ki67, HGF and FGF-7 genes. Results show box blots displaying the distribution of data from six donors (Minimum, maximum, median, first quartile, third quartile; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).



Among the assessed genes, we first tested mRNA expression of the major fibroblast matrix proteins because of their key functions in wound healing (total 24 genes tested). Seven of these genes (29.2%) were specifically regulated by L-PRF. Collagen type I mRNA was significantly down-regulated by L-PRF, whereas the extra domain-A fibronectin (EDA-FN) and the extra domain-B fibronectin (EDB-FN) genes were up-regulated by 2- to 3.5-fold by L-PRF.

Furthermore, fibrillin-1 was significantly down-regulated by L-PRF (Fig. 8.1A). Among matricellular proteins, tenascin-C was up-regulated significantly by L-PRF, whereas periostin was significantly down-regulated by L-PRF (Fig. 8.1B). Connective tissue growth factor 1 (CCN1) was modestly up-regulated by L-PRF (Fig. 8.1B).

To investigate the effect of L-PRF on HGF differentiation to myofibroblasts, we analyzed the expression of eight myofibroblast- and cell contractility-associated genes. Half of these showed (50%) significant changes by L-PRF treatment. Expression of alpha-smooth muscle actin (α -SMA), α 11 integrin and cadherin-11 showed significant down-regulation by L-PRF treatment while the expression of cadherin-2 showed up-regulation by about 2-fold by L-PRF (Fig. 8.1C). Matrix remodeling is an important part in wound healing, during which MMPs play a crucial role in ECM degradation¹⁸. MMPs are also known to regulate inflammation. Therefore, we investigated the effect of L-PRF on the expression of 14 matrix metalloproteinase (MMP) related genes in HGFs. Both MMP-1 and MMP-3 showed significant up-regulation by L-PRF treatment (Fig. 1D). Other MMPs showed no change, or the expression was below the detection level in HGFs by RT-qPCR (data not shown). In tissue inhibitors of metalloproteinase (TIMP) genes, only TIMP-2 was down-regulated significantly by L-PRF (Fig. 8.1D).

We tested cathepsin K that plays an important role in intracellular ECM degradation. Cathepsin K, was significantly down-regulated by L-PRF (Fig. 8.1E).

During wound healing, blood vessel formation and regression is important¹⁰⁵. Therefore, the effect of L-PRF treatment on gene expression of vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor-2 (FGF-2), and C-X-C motif chemokine ligand 12 (CXCL12) was investigated. All of these genes showed significant changes in HGFs by L-PRF (Fig. 6.1F). VEGF-A and FGF-2 were up-regulated by L-PRF significantly while CXCL12 was significantly down-regulated by L-PRF (Fig. 8.1F).

Transforming growth factor- β (TGF- β) signaling is a key regulator of inflammation and a key regulator to number of key factors in wound healing^{106,107}. Total 14 TGF- β signaling related genes were tested, and 9 of these showed significant differences between control and L-PRF treatment (Fig. 1H). Expression of Nuclear polyadenylated RNA-binding protein (NAB1) was significantly up-regulated by L-PRF in HGFs, however, all the other genes showed significant down-regulation by L-PRF, including TGF- β 1-3, TGF- β receptor (TGF- β R) 1-3, early growth response protein 3 (EGR3) and NAB2 (Fig. 8.1H).

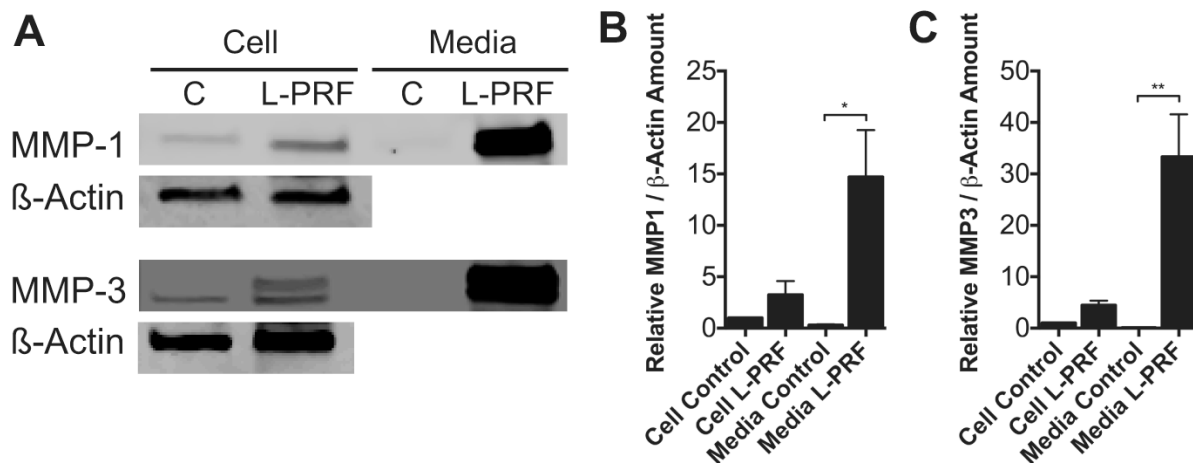
Cytokines and chemokines play important roles during wound healing¹⁰⁸. In this group, three cytokines were significantly up-regulated by L-PRF, including IL-1 β , IL-6 and IL-8 (Fig. 8.1I). For chemokines, both HGF and FGF-7 were significantly down-regulated by L-PRF (Fig. 8.1I). For the other genes, Ki67 which is considered as cell proliferation marker¹⁰⁹ was significantly up-regulated by L-PRF (Fig. 8.1I). However, there was no regulation of basement membrane related genes by L-PRF in HGFs.

L-PRF Stimulates MMP-1 and -3 Protein Production in Human Gingival Fibroblasts

MMP-1 and -3 were among the genes with multi-fold regulation with L-PRF treatment.

To verify whether these gene expression changes were translated to the target proteins, we used Western blotting to analyze the protein level of MMP-1 and -3 in HGFs with or without L-PRF treatment. The treatment with L-PRF significantly increased the accumulation of MMP-1 and MMP-3 in the cell culture media (Fig. 8.2A). The protein levels of MMP-1 and MMP-3 in cell layer were also increased, but did not reach statistical significance (Fig. 8.2B, C).

Figure 8.2 The Effect of L-PRF on The Expression of MMP-1 and -3 Protein Levels in Human Gingival Fibroblasts (HGFs). (A) HGFs were treated with FBS-DMEM (control) or L-PRF membrane for 48 hours, rinsed and cultured for another 24 h without the L-PRF membrane. Protein abundance of MMP-1 and MMP-3 in the HGF conditioned medium or in the cell layer was measured by Western blotting. β -actin was used as a loading control. (B) MMP-1 protein levels relative to the loading control were quantified. (C) MMP-3 protein levels relative to the loading control were quantified. Results show mean \pm SEM from triplicate experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

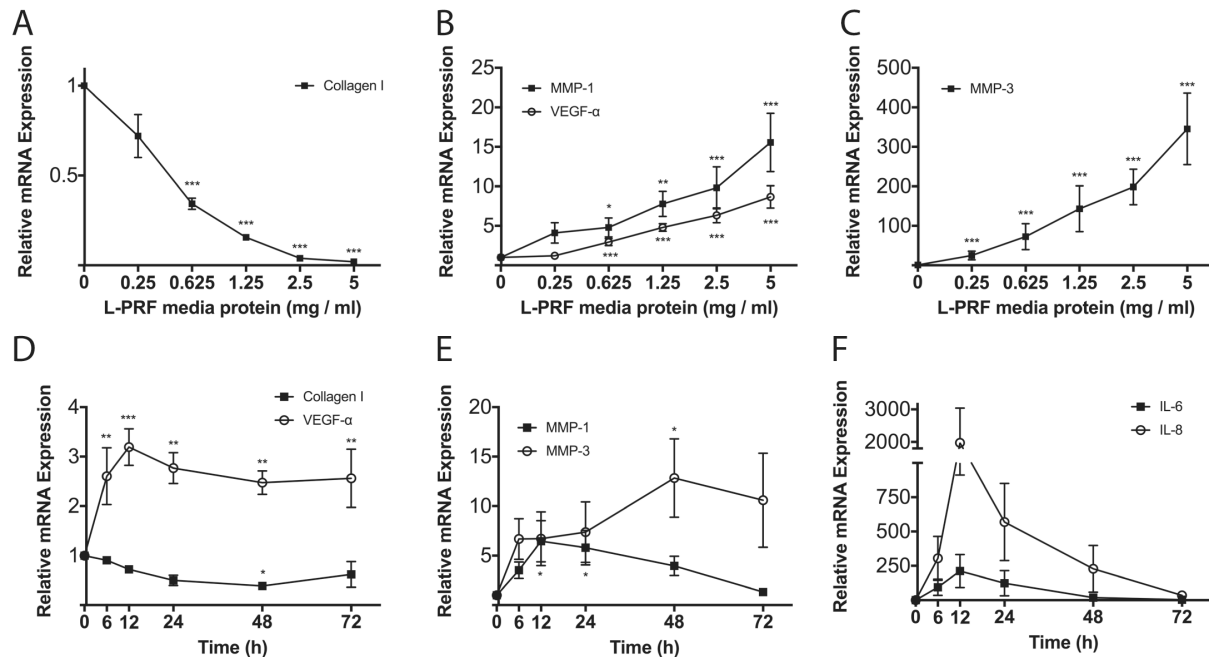


L-PRF Releasate Regulates Gingival Fibroblasts Dose-/Time-Dependently

Having demonstrated that L-PRF regulates expression at multiple genes and proteins in HGFs, we assessed whether these differences were also induced by the L-PRF releasate. About 10 mg proteins were released from a single L-PRF membrane in 48h into medium (data not shown). Then we used different concentrations (dilutions) of the L-PRF releasate to treat the HGFs for 48 h, following by RNA isolation and RT-qPCR. Collagen type I, MMP-1, MMP-3 and VEGF- α , were tested again as the target genes. Collagen type I showed dose-dependent down-regulation by treatments with the L-PRF releasate while the expression of MMP-1, MMP-3 and VEGF- α all showed dose-dependent up-regulation by L-PRF releasate treatments, indicating that regulation of gene expression by L-PRF membranes is mediated by soluble factors in one-way communication without feedback regulation by fibroblasts (Fig. 8.3A-C).

Using the releasate, we then tested regulation of selected genes in a time course experiment to find out the dynamics of the regulation of different genes. Expression of collagen type I was decreased slowly by L-PRF releasate and reached the lowest expression at 48 h (Fig. 3D). However, the expression of VEGF- α started to increase significantly already at 6 h by the L-PRF and reached the peak of expression at 12 h and then leveled until the end of the experiment at 72 h (Fig. 8.3D). Similarly, up-regulation of MMP-3 expression by L-PRF started from early time point and reached the peak at 48 h (Fig. 8.3E). Up-regulation of MMP-1 expression by L-PRF peaked at 12 h and then declined although remained higher than the control to the end of the experiment (Fig. 8.3E). Up-regulation of IL-6 and IL-8 gene expression peaked at 12h and then declined to the basal level by 48-72 h (Fig. 8.3F). Clearly, the dynamics of regulation seems to be different for type I collagen compared to the others, perhaps requiring the expression of secondary mediators.

Figure 8.3. Dose and Time Dependency of L-PRF-mediated Regulation of Human Gingival Fibroblasts (HGF) Genes. (A) Effects of different concentrations of the L-PRF releasate on the regulation of type I collagen gene expression; (B) Effects of different concentrations of the L-PRF releasate on the regulation of MMP-1 and VEGF α gene expression (C) Effects of different concentrations of the L-PRF releasate on the regulation of MMP-3 gene expression (D-F) Dynamics (time-course) of the L-PRF releasate-mediated gene regulation in HGFs (Type I collagen, VEGF α , IL-6 and IL-8). Results show the mean \pm SEM from triplicate experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

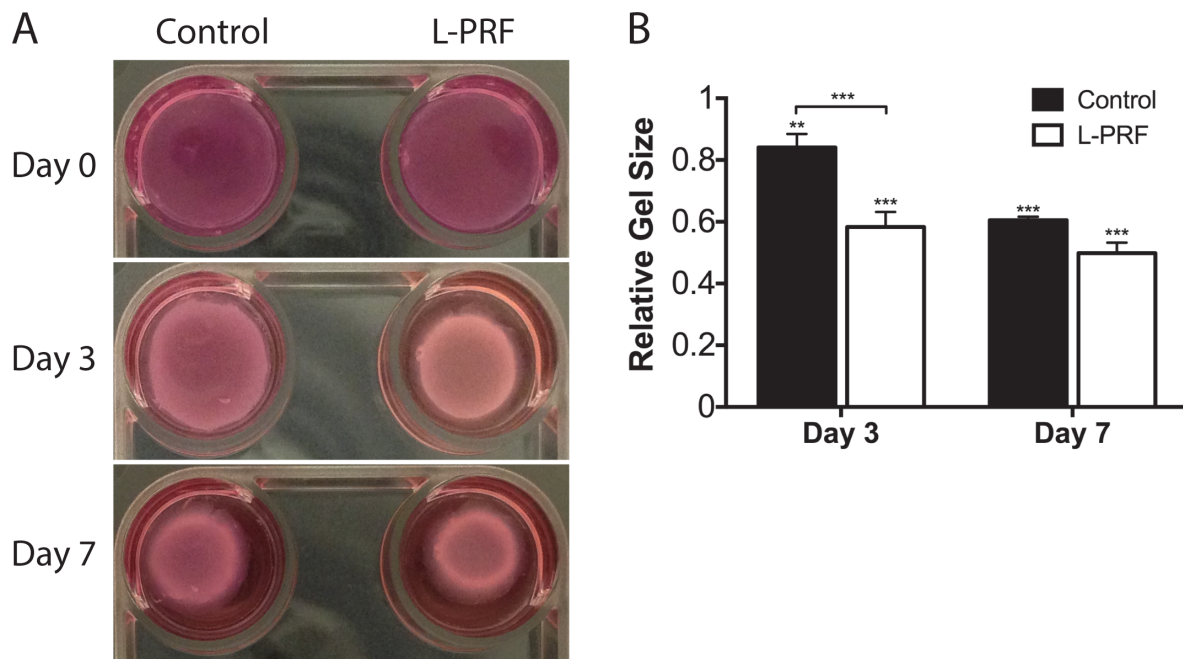


Effect of L-PRF Releasate on Collagen Gel Contraction

Next, to find out how soluble factors from L-PRF regulate fibroblast functions in 3-dimensional environment in the presence of 1% serum, we conducted an assay that measure fibroblast collagen gel contraction with or without L-PRF releasate treatment. The cells were seeded with type I collagen to create the gel structure, and regular cell culture medium without FBS was added to incubate the cells. Comparing to initial gel size on day 0, both the control and

L-PRF releasate treatments showed promotion of collagen gel contraction on both day 3 and 7 time points (Fig. 8.4B). However, on day 3, L-PRF releasate treatment showed significantly more contraction comparing to control.

Figure 8.4. Effect of L-PRF on Type I Collagen. (A) Cell media were treated with control medium (1%FBS+DMEM) or 50% L-PRF in the same medium. By day 3, L-PRF releasate treatment showed significantly enhanced initial gel contraction compared to control. (B) Collagen I contraction relative to the initial gel size was quantified. Results show mean \pm SEM from triplicate experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).



Chapter 9: Discussion

Platelet-rich concentrates have been progressively used to enhanced wound healing by providing additional growth factors at the injury site. These concentrates contain a mixture of anabolic factors derived from platelets and catabolic factors derived from leukocytes. Their concentrations, however, fluctuate among many different preparation systems. Several studies have shown L-PRF growth factor concentration, release kinetics⁷⁷.

Randomized clinical trials using significant population size to demonstrate efficacy for the L-RF in soft and hard tissue grafting are still missing.

Nevertheless, L-PRF has shown some clinical promise in case studies and has been, therefore, adopted to clinical use because of it is easy to produce in one step centrifugation process. Little is known about the effects of the combined growth factors and cells present in L-PRF membranes on gingival fibroblasts. In the present study, we demonstrate that L-PRF has a significant effect on human gingival fibroblast gene expression and may promote events in early wound healing.

In our study, seven of the major fibroblast matrix proteins were specifically regulated by L-PRF. Collagen type I mRNA was significantly down-regulated by L-PRF, whereas the extra domain-A fibronectin (EDA-FN) and the extra domain-B fibronectin (EDB-FN) genes were up-regulated by 2- to 3.5-fold by L-PRF (Fig. 1A). EDA and EDB are fibronectins involved in cell adhesion to the ECM, fibroblast migration and possibly regulation of growth factor responses and granulation tissue angiogenesis^{110,111}. Tenascin-C was up-regulated significantly by L-PRF, whereas the expression of periostin and lumican was significantly down-regulated by L-PRF (Fig. 1B). Connective tissue growth factor 1 (CCN1) was modestly but significantly up-regulated by L-PRF treatment (Fig. 1B). Tenascin-C is an anti-adhesive protein that modulates cell

adhesion to the ECM, apparently allowing development of dynamic cell adhesions (like activation of $\beta 1$ integrins) necessary for migration¹¹². Connective tissue growth factor 1 (CCN1) plays a role in fibroblasts response to growth factors, such as TGF- β 1, or to cellular stress and hypoxia during granulation tissue formation^{113,114}.

These results suggest that L-PRF seen to positively regulate the expression of matrix molecules that are associated with early wound matrix (FN, TN). However, L-PRF appeared at the same time down regulate the expression of genes that are highly expressed at later stages at wound healing (granulation tissue formation) such as type I collagen, lumican and periostin^{20,115}.

Consistent with promotion of early wound healing, L-PRF treatment significantly down-regulated the expression of genes related to myofibroblast differentiation.

For instance, alpha-smooth muscle actin (α -SMA) expression is related with wound contraction, $\alpha 11$ Integrin is a fibroblast receptor to interact directly with collagen and cadherin-2 and 11 are important during myogenic differentiation. They promote myoblast differentiation, and increase wound contraction to promote wound closure¹¹⁶. The later may promote fibrosis by facilitating the differentiation of resident tissue fibroblasts into myofibroblasts¹¹⁷.

Promotion of genes involved in expression of early wound matrix, inhibition of myofibroblast differentiation, intracellular matrix degradation and stimulation of expression of certain MMPs, all support the notion that L-PRF treatment would indeed positively regulate early wound healing in fibroblasts. Among 14 matrix metalloproteinase (MMP) in HGFs tested, MMP-1 and MMP-3 showed significant up-regulation by L-PRF. MMPs are involved in matrix degradation or modulation of inflammation via activation/deactivation of inflammatory mediators¹¹⁸. However, upregulation of MMPs in early phases has been described in scar-less

wound healing. For instance, fetal skin wound healing that does not produce scars has increased levels of MMPs relative to TIMPs compared to scar-forming adult skin¹¹⁹. Unpublished data from our laboratory also showed significant and early up-regulation of MMP-1 and -3 in scarless gingival wound healing (data not shown). Collectively these observations suggest that certain MMPs may play a significant role in regulation of functions other than matrix degradation, likely activating and deactivating cytokines and chemokines¹²⁰. Obviously, MMP activity in later stages of wound healing is associated with matrix degradation/re-modeling. Interestingly, expression of cathepsin K was significantly down-regulated by L-PRF treatment. Cathepsin K plays an important role in extracellular matrix turn-over but also associates with fibrotic wounds.

Again, prevention of excessive matrix degradation in early wound could be positive for optimal healing. Recently, targeting cathepsin K to improve diabetic wound healing has been proposed¹²¹.

Formation of a new blood supply is critical to wound healing, VEGF and FGF-2 are potent angiogenic factors that play a role in the regulation of initiation and formation of new vascular structures. CXCL12 plays a role in the inflammatory response by recruiting lymphocytes and promoting angiogenesis. In our results, VEGF-A and FGF-2 were up-regulated by L-PRF significantly meanwhile CXCL12 was significantly down-regulated by L-PRF. In general, the function of CXCL12 may not relate to early wound healing as previous studies that have shown that there is no expression of CXCL12 at protein level at the first five days after wounding¹²². In addition, suppressing connexin 43 has been related to faster healing but also down-regulation of CXCL12 in early stages gingival wound healing¹²³. In general, these results suggest an overall positive effect in early blood vessel formation by the L-PRF.

TGF- β family members play a complex role in regulating wound inflammation, cell migration, differentiation and matrix production. A total 14 TGF- β signaling related genes were tested, and 9 of these showed significant differences between control and L-PRF treatment.

Expression of nuclear polyadenylated RNA-binding protein (NAB1) showed significant up-regulation by L-PRF, while all the other genes showed significant down-regulation by L-PRF, including TGF- β 1-3, TGF- β receptor (TGF- β R) 1-3, early growth response protein 3 (EGR3) and NAB2. One of many important functions of the TGF- β signaling is to positively regulate myofibroblasts differentiation and matrix production. Consistent with our other results, suppressing of myofibroblast function early in the wounds maybe also beneficial for early healing. Previously, release of TGF-1 β from the L-PRF membranes has been reported⁷². It is possible that soluble TGF- β 1 released from the L-PRF membranes suppressed expression of fibroblast own TGF- β 1 expression using a negative feedback loop.

Cytokines and chemokines play important roles during wound healing¹⁰⁸. Although IL-1 β generally promotes inflammation, its role in normal wound repair is more complex. As discussed above, IL-1 receptor knockout mice have normal skin wound closure but less fibrosis^{83,84}. Normal IL-1 signaling seems to be beneficial for oral wound healing due to protection from bacterial insults⁸⁴. Interestingly, IL-6 is another pro-inflammatory cytokine that may have positive effects in wound healing. It has been reported to promote proliferation of fibroblasts²⁰.

In addition, IL-6 also promotes keratinocyte migration, macrophage infiltration and angiogenesis⁸⁵. IL-8 may also promote wound healing by increasing keratinocyte proliferation and migration while reducing wound contraction¹²⁴. Overall, L-PRF appeared also to increase fibroblast proliferation based on significant upregulation of Ki67 expression confirming findings from a previous study⁹⁴.

The only functional experiment performed for the study was collagen gel contraction assay that showed transient stimulation of gel contraction by L-PRF. This early effect could be regulated by soluble TGF- β 1 released from the L-PRF membrane as it serves as a strong promoter of collagen gel contraction¹²⁵. More functional studies such as cell migration and others are required before the relevance of gene expression changes caused by L-PRF can be confirmed. Thus, the main limitation of our study is that it is an in vitro study focusing on regulation at limited number of gingival fibroblasts genes. In vivo, both soluble factors released from the L-PRF as well as proteins produced under the influence of L-PRF jointly regulate healing outcomes that are further modulated by other factors present at the wound environment.

However, in vitro studies remain an important step toward the understanding of the L-PRF effects in vivo.

In summary, L-PRF appears to promote expression of genes in gingival fibroblasts that are associated with promotion of early wound healing while suppressing those linked to myofibroblast differentiation and function.

Chapter 10: Conclusions

The results demonstrate that the L-PRF membranes have a strong and a specific regulatory effect on gingival fibroblasts that may modulate early phases of wound healing. Further in vitro studies and randomized clinical trials should be conducted with a greater population to confirm its effect in wound healing.

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Appendices

Appendix A

Gene Bank	Gene	Primer sequence	Orientation	Location	Amplicon (bp)
Structural ECM Proteins					
BC036531	Collagen type I (alpha 1)	AACCAAGGCTGCAACC TGGA	Forward	3951-3970	80
		GGCTGAGTAGGGTACA CGCAGG	Reverse	4030-4009	
NM_00090	Collagen type III (alpha 1)	CTCCTGGGATTAATGGT AGT	Forward	1271-1290	70
		CCAGGAGCTCCAGGAA T	Reverse	1340-1324	
NM_00501	Elastin	GGTCCCTGGGGCCAT TC	Forward	174-191	62
		CAGGCCCCAGCGCTGG AT	Reverse	236-219	
NM_000138	Fibrillin-1	AGGAAACGGAGAAGCA CAA	Forward	8577-8595	103
		CTGTCTTCTCAACATCC CAA	Reverse	8679-8660	
NM_007046	Emilin-1	CCTTCGACAGAGTCCT GCTCAA	Forward	3162-3183	65
		CGCTGTGAACACGCCT GTC	Reverse	3226-3208	
NM_032048	Emilin-2	GTGAACATGGCCACTG ACTT	Forward	3513-3532	87
		GCCCTCAGAGTGTAGA TACAG	Reverse	3600-3580	
NM_052846	Emilin-3	TCCCTTAGACGAGATC CTAAGCA	Forward	962-984	72
		TCTAGAAGCTGCACCTT GGTC	Reverse	1033-1013	
NM_212482	EDA-FN (EDA-fibronectin) EDB-FN	CACAGTCAGTGTGGTT GCCT	Forward	5633-5652	68 65
		CTGTGGACTGGGTTCC AATCA CAGTAGTTGCGGCAGG AGAA	Reverse	4168 4188	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
Matricellular Proteins					
NM_003246	Thrombospondin-1 (THBS1)	CATCTTGTTCTGTGACATGTGG	Forward	1513-1534	92
		TTCACAGGGTTTCCCGTTC	Reverse	1604-1586	
NM_003247	Thrombospondin-2 (THBS2)	TTGTGTTCAACCCAGACCA	Forward	2989-3007	107
		CAGGACACACATCATCAATATC	Reverse	3096-3075	
NM_003118	SPARC-1 (Secreted protein acidic and rich in cysteine 1)	AGCAAGAAGCCCTGCC TGA	Forward	164-182	101
		TCCTACTTCCACCTGGACAGGATT	Reverse	264-241	
NM_002160	TNC (Tenascin-C)	CAACCTGATGGGGAGATATGGGGA	Forward	6769-6792	75
		GAGTGTTTCGTGGCCCTTCCAG	Reverse	6846-6826	
NM_019105	TNX (Tenascin-X)	CCTGGAGCCAGACCATAAATACAAG	Forward	6334-6358	57
		CGCTGGCCACCGTGGA A	Reverse	6390-6374	
NM_000582	Osteopontin	CAGTTGCAGCCTTCTCAG	Forward	118-136	82
		CTAGGAGGCAAAGCAAATC	Reverse	199-180	
NM_006475	Periostin	ACACGAGAAGAACGAA TCAT	Forward	199-181	97
		GTAACAATTTCTTCAGAGTTTCTTC	Reverse	199-182	
NM_001554	CCN1	CATGATGATCCAGTCCTGCAA	Forward	199-185	86
		TGTCATTGAACAGCCTGTAGAAG	Reverse	199-186	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
NM_001901	CCN2/CTGF (connective tissue growth factor)	ATGATGTTTCATCAAGA CCTGTGCCTG	Forward	199-183	80
		CTTCCTGTAGTACAGG GATTCAAAGATGTC	Reverse	199-184	
NM_002514	CCN3	CTCAGATCTGGAGCCA TGCGA	Forward	199-187	85
		CGTGCAGATGCCAGTC TGGTTG	Reverse	199-188	
Small Leucine-Rich Proteoglycans					
NM_001711	BGN (Biglycan)	CTCAAGCTCCTCCAGGT GGTC	Forward	1067-1087	93
		CCGAAGCCCATGGGAC AGAAGTC	Reverse	1151-1127	
BT019800	DCN (Decorin)	CTGACACA ACTCTGCT AGAC	Forward	242-261	97
		GACAAGAATCAATGCG TGAAG	Reverse	339-319	
NM_002023	FMOD (Fibromodulin)	CACAATGAGATCCAGG AAG	Forward	761-779	85
		TCCGAAGGTGGTTATA ACTC	Reverse	845-826	
BT006707	LUM (Lumican)	TAGACAACAATAAGAT CAGCAACA	Forward	635-658	85
		TTCGTTGTGAGATAAA CGCAG	Reverse	720-700	
NM_017680	ASPN (Asporin)	CCTCCAGATAATCTTCC TTCATTCTA	Forward	1183-1213	81
		CTTCATCTTTGGCACTG TTGG	Reverse	1244-1264	
TGF-β Signaling					
NM_000660	TGF- β 1 (Transforming Growth Factor- β 1)	CAACGAAATCTATGAC AAGTTCAAGCAG	Forward	1218-1245	76
		CTTCTCGGAGCTCTGAT GTG	Reverse	1294-1275	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
NM_003238	TGF- β 2 (Transforming Growth Factor- β 2)	TGGTGAAAGCAGAGTTCAGAG	Forward	1883-1903	140
		CACAACTTTGCTGTCGATGTAG	Reverse	2022-2001	
NM_003239	TGF- β 3 (Transforming Growth Factor- β 3)	ACACCAATTACTGCTTCGCAA	Forward	1161-1182	81
		GCCTAGATCCTGTCGG AAGTC	Reverse	1242-1220	
NM_001130916	TGF- β R1 (TGF- β Receptor 1)	GTGTATAGCTGAAATTGACTTAA	Forward	286-308	99
		TGATTGCAGCAATATGTTGTA	Reverse	384-364	
NM_003242	TGF- β R2 (TGF- β Receptor 2)	CTGGTGAGACTTTCTTCATGTG	Forward	768-789	127
		CTGATGCCTGTCACTTGAAA	Reverse	894-875	
NM_001195683	TGF- β R3 (TGF- β Receptor 3)	AGGAGGGACTTTAAGACTACTTC	Forward	420~442	136
		TCAGGGCAAAGATGGCAA	Reverse	555~535	
NM_001190823	SMAD7	ATTCCCAACTTCTTCTGGAG	Forward	328-347	96
		TGGACACAGTAGAGCCTC	Reverse	424-407	
NM_138455	Cthrc-1 (Collagen triple helix containing-1)	TGGAAGGACTTTGTGAGAGGA	Forward	689-708	104
		AATTCCATCCAGTAGAAGCATC	Reverse	793-772	
NM_001142483	P311	CCTGGACTGAAGAGAGGG	Forward	321-447	78
		CAGACAAAGAGTTCTGGGTA	Reverse	508-489	
NM_001964	EGR1 (Early Growth Response)	ACGTCTTGGTGCCTTTGTG	Forward	2663-2682	75
		GAGGTGAGCATGTCC	Reverse	2737-	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
NM_001136179	EGR2 (Early Growth Response 2)	AGCTTTGCTCCCGTCTCTG	Forward	469-487	88
		AGCTGGCACCAGGGTACT	Reverse	556-539	
NM_004430	EGR3 (Early Growth Response 3)	CGTTGGACAGCAATCTCTTC	Forward	903-922	74
		AATGGAGCCCATGTCTGTTG	Reverse	976-958	
NM_005966	NAB1 (NGFI-A Binding Protein-1)	CAAAGTCCCACTCATCAGAGA	Forward	1930-1950	114
		TCACAGCTATCTTGAATCTTCAG	Reverse	2043-2020	
NM_005967	NAB2 (NGFI-A Binding Protein-2)	CACATCCCTGCTAAAGCTGAA	Forward	1170-1190	111
		ATGATGCTGTATTTGCGGATCT	Reverse	1280-1259	
Angiogenesis					
NM_001171630	VEGF- α (Vascular Endothelial Growth Factor- α)	AGTGTGTGCCCACTGAGGA	Forward	1316-1334	97
		GTGCTGTAGGAAGCTCATCTC	Reverse	1413-1393	
NM_002006	FGF-2 (Fibroblast Growth Factor-2)	AGTTGGTATGTGGCAC TGAA	Forward	831-850	75
		GTATAGCTTTCTGCCCA GGTC	Reverse	886-906	
NM_199168	CXCL12/SDF-1 α (C-X-C motif chemokine ligand 12 / stromal cell-derived factor 1 alpha)	TACAGATGCCCATGCCGA	Forward	174-191	93
		CTGAAGGGCACAGTTTGGAG	Reverse	266~247	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
Myofibroblast- and Cell Contractility-Associated Genes					
NM_001613	α -SMA (α -Smooth Muscle Actin)	AGCGTGGCTATTCCTTCGT	Forward	637-655	97
		CTCATTTTCAAAGTCCAGAGCTACA	Reverse	733-707	
NM_001004439	α 11 Integrin	GAAGGCACCAACAAGAACGA	Forward	1144-1163	60
		AGGAAAAGCCCGTCTGTGA	Reverse	1204-1186	
NM_001792	Cadherin-2	TGAGGAGTCAGTGAAGGAG	Forward	843-861	91
		CTTCTGCCTTTGTAGGTGG	Reverse	933-915	
NM_001797	Cadherin-11	TTCCTTCGTGTTGTCATTTGTTG	Forward	174-196	89
		CTTCTTCACCCATTGGATACTTG	Reverse	262-240	
NM_002473	NMMIIA (Non-muscle Myosin IIA)	ACCGAGAAGATCAATCCATC	Forward	722-741	81
		AGATACTGGATGACCTTCTTG	Reverse	803-783	
NM_005964	NMMIIB (Non-muscle Myosin IIB)	CCGTTTTACATAATCTGAAGGATC	Forward	395-418	98
		TTGGAAGATTCTTGTAAGGGTT	Reverse	493-472	
NM_001101	β -Actin	AGGCCAGAGCAAGAGAA	Forward	254-270	72
		CAGTTGGTGACGATGCCG	Reverse	320-303	
NM_001614	γ -Actin	GTCGCAATGGAAGAAGAGATCG	Forward	134-155	85
		AGCGTCGTCCCCAGCAAAA	Reverse	218-199	
Intracellular ECM Degradation-Associated Genes					
NM_000396	CTSK (Cathepsin K)	TCGACTATCGAAAGAAAGGATA	Forward	585-606	70
		AAAGCCCAACAGGAA	Reverse	655-637	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
MMPs and their Inhibitors					
NM_002421	MMP-1 (Matrix metalloproteinase 1)	GCTAACAAATACTGGA GGTATGATG	Forward	1250-1275	100
		GTCATGTGCTATCATTT TGGGA	Reverse	1304-1325	
NM_001127891	MMP-2 (Matrix metalloproteinase 2)	AGACATACATCTTTGCT GGAG	Forward	1900-1920	88
		ATCTGCGATGAGCTTG G	Reverse	1988-1972	
NM_001166308	MMP-3 (Matrix metalloproteinase 3)	ATGATGAACAATGGAC AAAGGA	Forward	661-682	91
		GAGTGAAAGAGACCCA GGGA	Reverse	751-732	
NM_214207	MMP-7 (Matrix metalloproteinase 7)	TATGCTGCAACTCATG AAC	Forward	722-740	82
		CGTAGGTTGGATACAT CAC	Reverse	804-785	
NM_004994	MMP-9 (Matrix metalloproteinase 9)	CACTACTGTGCCTTTGA GTCC	Reverse	1260~1243	63
		CGATGGCGTCGAAGAT GTT	Forward	1531~1551	
NM_002425	MMP-10 (Matrix metalloproteinase 10)	TTATACACCAGATTTGC CAAGA	Forward	394-415	56
		TTCAGAGCTTTCTCAAT GG	Reverse	450-432	
NM_005940	MMP-11 (Matrix metalloproteinase 11)	CTATCCTCCAAAGCCAT TGTA	Forward	2258-2179	106
		CAACTGTGTTAATGAC AATCCTC	Reverse	2254-2231	
NM_002426	MMP-12 (Matrix metalloproteinase 12)	GTATGATGAAAGGAGA CAGATGAT	Forward	1303-1326	166
		TACGTTGGAGTAGGAA GTCAT	Reverse	1469-1449	
NM_002427	MMP-13 (Matrix metalloproteinase 13)	CAGGAATTGGTGATAA AGTAGAT	Forward	1302-1324	85
		CTGTATTCAAACGTAT GGGTC	Reverse	1365-1386	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
NM_004995	MMP-14 (Matrix metalloproteinase 14)	TCTCCCAGAGGGTCATT CAT	Forward	1618-1637	70
		TTCCAGTATTTGTTCCC CTGTAG	Reverse	1688-1665	
NM_003254	TIMP-1 (Tissue inhibitor of metalloproteinase 1)	CTGTGTCCCACCCCACC	Forward	267-283	64
		GAAGTTGGCCCTGATG ACGA	Reverse	330-311	
NM_003255	TIMP-2 (Tissue inhibitor of metalloproteinase 2)	ACATTTATGGCAACCCT ATCAA	Forward	481-502	70
		TCAGGCCCTTTGAACAT CTTTA	Reverse	550-529	
NM_000362	TIMP-3 (Tissue inhibitor of metalloproteinase 3)	AGGACGCCTTCTGCAA C	Forward	1281-1297	68
		CTCCTTTACCAGCTTCT TCC	Reverse	1348-1329	
NM_003256	TIMP-4 (Tissue inhibitor of metalloproteinase 4)	ACCTGTCCTTGGTGCA A	Forward	927-944	80
		TGTAGCAGGTGGTGAT TTGG	Reverse	1004-985	
Connexin					
NM_000165	CX43 (connexin 43)	ACTGTGGGTCTCAAAA ATATGCTTA	Forward	1026~1050	95
		AGTAACCAGCTTGTAC CCAG	Reverse	1120~1101	
NM_005497	CX45 (connexin 45)	CTATAACATTGCTGTCA AACCAGA	Forward	1141~1164	120
		TCCTCATGGCTGCCATA C	Reverse	1260~1243	
Cytokines					
NM_000575	IL-1 α (Interleukin-1 alpha)	TGAAGACCAACCAGTG CTG	Forward	1551~1569	135
		CAAGTTTGGATGGGCA ACTG	Reverse	1686~1667	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
NM_000600	IL-6 (Interleukin-6)	CAACCTGAACCTTCCA AAGATG	Forward	380~401	119
		TCT GGC TTG TTC CTC ACT AC	Reverse	539~520	
NM_000584	IL-8 (Interleukin-8)	CAGAGGGTTGTGGAGA AGTT	Forward	407~426	106
		GCTTGAAGTTTCACTGG CA	Reverse	513~493	
NM_001945	HB-EGF (Heparin-binding EGF-like growth factor)	GTACCATAGGAGAGGA GGTTATG	Forward	830~852	132
		TCACCTTGTGTCTTCTC AGAG	Reverse	962~942	
NM_001010932	HGF (Hepatocyte growth factor)	ACCCTAATCAAAATAG ATCCAGC	Forward	310~332	130
		GCTTTATCAAAAACAA AAGCCTTG	Reverse	440~417	
NM_002009	FGF-7/KGF (fibroblast growth factor 7 / Keratinocyte growth factor)	CAAGGAAGGAAAAC TATGC	Forward	895~915	107
		GTGTCCATTTAGCTG ATGC	Reverse	1001~983	
NM_000758	GM-CSF (Granulocyte macrophage colony-stimulating factor)	CAGGCATGGCAGAAGA A	Forward	621~637	131
		CGGTAAAACATCTTGA ATAAATATGG	Reverse	752~727	
NM_000594	TNF- α (Tumor necrosis factor alpha)	TGGGACAGTGACCTGG AC	Forward	1046~1063	138
		TTC TGA GAC AGA GGC AACCT	Reverse	1184~1165	
NM_002417	Ki67	TTCGGAAGCAAATCTG ATTGT TTGTTTCATTGACCTTTG AGGA	Forward Reverse	2145~2165 2255~2235	111

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
Basement membrane					
NM_001845	Collagen type IV	AATCCAGGGCAGCCAG	Forward	1460~1476	85
		ATCCGTCTATATCACAGATGAGG	Reverse	1545~1523	
NM_000094	Collagen type VII	TGGCAGCAGGGGAGAG	Forward	7592-7607	86
		CCCAGGATCACAGCTGAGT	Reverse	7678-7660	
NM_005559	Laminin-1 α 1	TTGACCTTGGCAAAGGCA	Forward	8424~8441	86
		TAACATAGTCTGTCTTGACCGTG	Reverse	8510~8488	
NM_001017402	Laminin-5 β 3	CTCCGAGCTTCATCTACTG	Forward	511~530	92
		GGAGTCACACTTGCAGCA	Reverse	603~586	
Stem Cell Associated Genes					
NM_003068	Slug	GACCCTGGTTGCTTCAAGGA	Forward	762~781	72
		TGTTGCAGTGAGGGCAAGAA	Reverse	833~814	
NM_012183	FOXD3	AGTGAAGCCGCCTTACTCGTACAT	Forward	417~440	275
		AGGAAGCTGCCGTTGTCGAACAT	Reverse	692~670	
NM_000474	Twist1	TGCAGCTATGTGGCTCACGA	Forward	874~983	266
		CCCACGCCCTGTTTCTTTGA	Reverse	1140~1121	
NM_005985	SNAIL	TCGGAAGCCTAACTACAGCGA	Forward	126~146	139
		AGATGAGCATTGGCAGCGAG	Reverse	265~246	
NM_006617	Nestin2	CGGCTGCGGGCTACTGAAAAGT	Forward	890~912	230
		TTGGGGTCCTGAAAGCTGAGGGA	Reverse	1122~1100	

Gene Bank	Gene	Primer Sequence	Orientation	Location	Amplicon (bp)
Housekeeping Genes					
NM_002046	GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	CTTTGTCAAGCTCATTT CCTGGTA	Forward	1020-1043	70
		GGCCATGAGGTCCACC A	Reverse	1089-1073	
NM_004048	B2M (beta-2-microglobulin)	TGTCTTTCAGCAAGGA CTGGTCTTTC	Forward	281-306	92
		ATGGTTCACACGGCAG GCATA	Reverse	351-372	

Appendix B

Donors Information

Donor	Gender	Age	Medical condition
1	Female	27	Healthy NKDA
2	Male	28	Healthy NKDA
3	Female	37	Healthy NKDA
4	Female	38	Healthy NKDA
5	Male	31	Healthy NKDA
6	Male	37	Healthy NKDA