A Highly Perfused Tissue-Engineered Construct for Gingival Tissue Regeneration using Degradable/Polar/Hydrophobic/Ionic Polyurethanes and A Dynamic Co-culture System

by

Jane Wing Chi Cheung

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

© Copyright by Jane Wing Chi Cheung 2015
A Highly Perfused Tissue-Engineered Construct for Gingival Tissue Regeneration using Degradable/Polar/Hydrophobic/Ionic Polyurethanes and A Dynamic Co-culture System

Jane Wing Chi Cheung
Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto
2015

Abstract

Gingival tissue engineering represents a promising method for regenerating the gingival lamina propria. In this thesis, a degradable polyurethane (degradable/polar/hydrophobic/ionc polyurethane (D-PHI)) scaffold was shown to support the proliferation and collagen production by human gingival fibroblasts (HGFs). As the gingival lamina propria is highly vascular, a perfusion bioreactor was designed to facilitate medium perfusion through HGF-seeded scaffolds. In the perfused (dynamic) culture, HGFs showed enhanced proliferation, metabolic activity and collagen production when compared with static cultures. These data suggest that the metabolic, phenotypic and differentiation state of HGFs were altered in dynamic cultures. Hence, the influence of the D-PHI material and perfusion on myofibroblast differentiation was explored. This study demonstrated that D-PHI does not enable myofibroblast differentiation over the 28-day culture. Moreover, it was shown that the perfused D-PHI scaffold modulated the fibroblast phenotype through complex mechanisms involving transforming growth factor (TGF)-β1, basic fibroblast growth factor (FGF-2), β1-integrin, and focal adhesion kinase, all of which were upregulated at the early time point (1 day) of the dynamic culture. These data provided new insights into the signalling pathways that are associated with the regulation of myofibroblast
differentiation in a perfused system with elastomeric polymers such as D-PHI. Furthermore, using the bioreactor system developed in this thesis, a dynamic co-culture system with human umbilical vein endothelial cells (HUVECs) and HGFs was established. With the perfusion of medium, the production of vascular endothelial growth factor, an angiogenic factor, was induced in the HGFs. This study also investigated different co-culturing conditions (e.g. cell density, type of culture medium, cell seeding ratio) that can optimize cell growth, HUVEC cluster formation, angiogenic factor production, inhibition of myofibroblast differentiation, and collagen expression. It was found that at 80,000 cells/scaffold (12.95 mg, 80% porosity), a greater proportion of HGFs in the co-culture (i.e. 1:2 (HUVEC:HGF)) cultured in a 50/50 mixture of the HUVEC medium and HGF medium (by volume) can potentially induce HUVEC cluster formation, augment cell proliferation, and increase angiogenic factor production. The knowledge from this thesis could enable rational developments of the co-culture systems for building a functional, highly vascularized tissue-engineered construct for gingival tissue regeneration.
Acknowledgments

I would like to take this opportunity to offer my outmost gratitude and appreciation to my co-supervisors and mentors, Dr. Paul Santerre and Dr. Chris McCulloch. The successful completion of this thesis would not have been possible without their infinite generosity, wisdom, invaluable support and inspiring guidance. They have enriched my growth as a student and a researcher throughout my Ph.D. studies, and I am forever grateful for been given the opportunity to learn from them.

I would also like to express my sincere thanks to my committee member Dr. Lidan You for her time and scientific guidance. Her insightful comments and positive criticism has been instrumental in the improvement of this Ph.D. project.

Many thanks to Dr. Kuihua Cai for kindly teaching me cell work, Dr. Meilin Yang for his invaluable knowledge and support in chemical synthesis, Dr. Jian Wang for teaching me scanning electron microscopy, Miss Feryal Sarraf for her infinite support in histology and immunohistochemistry, and Mr. Milos Legner for his expertise in fluorescence microscopy. Thank you for their patience and kindly granting me their time to answer my many questions.

My daily laboratory experience was incredibly fun and memorable. It has been a real pleasure to work with all my friends and colleagues in the Santerre lab from the past and present, namely Dr. Joanne McBane, Dr. Patrick Blit, Dr. Marisa Lopez-Donaire, Yasaman Delaviz, Maneesha Rajora, Meghan Wright, Kate Brockman, Yi Guo, Emily Rose, Hans Shih, Arezou Ossareh, Devika Jain, Mitch Nascimento, Zac Grodzinski and Ken Shiguetomi. Thank you for offering both your scientific support and friendship. I would like to specially thank Kyle Battiston for sharing numerous professional and scientific exchanges as well as making my days in the lab (and in the conferences) amusing and enjoyable, and Dr. Soroor Sharifpoor for her valuable mentorship throughout my Ph.D. studies.

I am also eternally grateful to my family and friends for their infinite love, support and encouragement. I am forever indebted to my parents for their guidance, endless support (including the car rides during late hours and weekends, and being here with me in the few difficult moments over the past 5 years), and for always believing in me.
# Table of Contents

Abstract ................................................................................................................................. ii  
Acknowledgments ................................................................................................................ iv  
Table of Contents .................................................................................................................. v  
List of Figures ....................................................................................................................... xi  
List of Abbreviations ........................................................................................................... xxi  
List of Equations ................................................................................................................... xxvi

## Chapter 1: Introduction

1.1 Introduction ..................................................................................................................... 1  
1.2 Hypothesis ....................................................................................................................... 4  
1.3 Objectives ......................................................................................................................... 4  
  1.3.1 Objective I .................................................................................................................... 4  
  1.3.2 Objective II .................................................................................................................. 6  
  1.3.3 Objective III ............................................................................................................... 7  
  1.3.4 Objective IV ............................................................................................................... 8  
1.4 Additional contributions ................................................................................................ 10  
1.5 References ...................................................................................................................... 11

## Chapter 2: Literature review

2.1 The periodontium and the lamina propria of the gingiva .............................................. 21  
  2.1.1 Fibroblasts and myofibroblasts ................................................................................ 24  
  2.1.2 Vascular endothelial cells (ECs) .............................................................................. 32  
2.2 Gingival atrophy ............................................................................................................. 34  
  2.2.1 Current treatment for gingival atrophy ................................................................. 35  
2.3 Gingival tissue engineering ........................................................................................... 36  
  2.3.1 Scaffolds for tissue engineering .......................................................................... 37  
  2.3.2 Cell types for gingival tissue engineering ............................................................ 41  
2.4 Medium perfusion ......................................................................................................... 43  
  2.4.1 Effect of shear stress on ECs ............................................................................... 44  
  2.4.2 Perfusion bioreactors ......................................................................................... 45
Chapter 3: Perfused culture of gingival fibroblasts in a degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold leads to enhanced proliferation and metabolic activity

3.1 Foreword
3.2 Introduction
3.3 Materials and methods
  3.3.1 2D D-PHI flat film fabrication
  3.3.2 3D D-PHI scaffold fabrication
  3.3.3 Culture of HGF cell line and seeding
  3.3.4 Perfusion bioreactor
  3.3.5 DNA mass quantification
  3.3.6 Water-soluble tetrazolium (WST)-1 assay
  3.3.7 Histology
  3.3.8 Scanning electron microscopy (SEM)
  3.3.9 Total protein measurement and Western blotting
  3.3.10 Statistical analysis
3.4 Results
  3.4.1 Viability of HGF on 2D D-PHI flat films
  3.4.2 HGF proliferation and metabolic activity in perfused 3D D-PHI scaffolds
  3.4.3 Scanning electron micrographs of HGF on perfused D-PHI scaffolds
  3.4.4 Type I collagen (Col I) production
3.5 Discussion
  3.5.1 Dynamic culture enhanced HGF proliferation, metabolic activity, and infiltration in the scaffold
  3.5.2 Dynamic culture promoted Col I production in HGF
Chapter 4: Establishing a gingival fibroblast phenotype in a perfused degradable polyurethane scaffold: mediation by TGF-β1, FGF-2, β1-integrin, and focal adhesion kinase

4.1 Foreword ................................................................. 110
4.2 Introduction ............................................................. 111
4.3 Materials and methods ................................................ 114
  4.3.1 D-PHI film and scaffold fabrication ....................... 114
  4.3.2 Culture of HGF cell line and setup of perfusion (dynamic) culture ........................................ 115
  4.3.3 Immunohistochemistry and immunofluorescence ............................................................. 116
  4.3.4 Western blotting .................................................... 117
  4.3.5 TGF-β1 Enzyme linked immunosorbent assay (ELISA) ................................................ 118
  4.3.6 FGF-2 ELISA ........................................................ 118
  4.3.7 Inhibition of the Smad pathway ................................ 119
  4.3.8 Inhibition of β1-integrin ......................................... 119
  4.3.9 Statistical analysis .................................................. 119
4.4 Results ........................................................................ 120
  4.4.1 D-PHI inhibits myofibroblast differentiation ................ 120
  4.4.2 Dynamic culture modulates α-SMA and Col I production over time ......................... 121
  4.4.3 Both TGF-β1 and FGF-2 were increased in the dynamic culture ........................................ 124
  4.4.4 Level of total β1-integrin was increased and FAK was activated in the dynamic culture ................................. 127
4.5 Discussion ................................................................... 129
  4.5.1 Perfused D-PHI scaffolds inhibit myofibroblast differentiation ................................. 129
  4.5.2 High levels of TGF-β1 and FGF-2 in perfused culture regulates α-SMA production ................................................................................................................. 131
  4.5.3 β1-integrin and FAK may mediate α-SMA and Col I expression in the dynamic culture ................................................................. 133
4.6 Conclusion ................................................................. 134
4.7 Acknowledgments ....................................................... 135
Chapter 5: Pro-angiogenic character of endothelial cells and gingival fibroblasts co-cultures in perfused degradable polyurethane (D-PHI) scaffolds

5.1 Foreword

5.2 Introduction

5.3 Materials and methods

5.3.1 D-PHI scaffold fabrication

5.3.2 Culture of HGF and HUVEC cell lines and perfused (dynamic) co-culture

5.3.3 VEGF enzyme-linked immunosorbent assay (ELISA) for HGF monoculture study

5.3.4 Cell seeding density

5.3.5 DNA quantification

5.3.6 Cell culture medium

5.3.7 Water soluble tetrazolium assay

5.3.8 Immunofluorescence

5.3.9 HUVEC and HGF counting in immunofluorescence images

5.3.10 Quantification of α-SMA-positive cells (myofibroblasts) and type I collagen (Col I) coverage in immunofluorescence images

5.3.11 ELISAs for co-culture studies

5.3.12 Cell seeding ratio

5.3.13 Statistical analysis

5.4 Results

5.4.1 Enhanced VEGF production by HGFs in perfused D-PHI scaffolds

5.4.2 Effect of cell seeding density

5.4.3 Effect of culture medium type

5.4.4 Effect of cell seeding ratio

5.5 Discussion

5.6 Conclusion

5.7 Acknowledgments

5.8 References
Chapter 6: Conclusion

6.1 Summary

6.2 Scientific contributions

6.3 Recommendations and future perspectives

6.4 References

Appendix A: Characterization of collagen expression from human gingival fibroblasts on a degradable/polar/hydrophobic/ionic polyurethane

A.1 Introduction

A.2 Materials and methods
   A.2.1 HGF cell culture
   A.2.2 PLGA films
   A.2.3 D-PHI films preparation
   A.2.4 Collagen assay
   A.2.5 DNA quantification
   A.2.6 Statistical analysis

A.3 Results
   A.3.1 Collagen assay
   A.3.2 HGF cell growth
   A.3.3 Cell seeding efficiency
   A.3.4 Normalized collagen content

A.4 Discussion

A.5 Conclusion

A.6 References

Appendix B: Assessment of human umbilical vein endothelial cell growth and migration on degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold

B.1 Introduction

B.2 Materials and methods
   B.2.1 D-PHI scaffolds fabrication
   B.2.2 Culture of HUVECs
   B.2.3 Cell seeding
List of Figures

Chapter 2: Literature review

Figure 2.1. (a) The anatomical structure of the periodontium – (i and ii) gingiva, (iii) cementum, (iv) mandibular bone, (v) periodontal ligament, (vi) dentine, (vii) alveolar mucosa, and (viii) alveolar process. Adapted from Schroeder (Schroeder, 1986). (b) The histological image (left) and schematic drawing (right) of oral gingival tissues. OGE: oral gingival epithelium, OSE: oral sulcular epithelium, JE: junctional epithelium, GM: gingival margin, GS: gingival sulcus, E: enamel, AP: alveolar process. Adapted from Schroeder (Schroeder, 1986) ........................................... 22


Figure 2.3. The development of myofibroblasts. Upon injury, inflammatory signals and profibrotic growth factors such as TGF-β1 stimulate resident fibroblasts to undergo transition into myofibroblasts. Myofibroblasts are characterized by α-SMA, ED-A Fn, stress fibre formation, and supermature focal adhesion. Under normal conditions, myofibroblasts undergo apoptosis after wound healing is completed. Adapted from Hinz (Hinz, 2007) ......................... 26

Figure 2.4. Various signaling molecules and pathways that are involved in myofibroblast differentiation. TGF-β1 can be delivered to the fibroblasts from other cells or from the release from the latent TGF-β1 complex upon mechanical stimulation (Blumbach et al., 2010; Wipff and Hinz, 2009). TGF-β1 can induce α-SMA production via the Smad signalling pathway or other pathways such as PI3K (Blumbach et al., 2010; Hinz, 2007). Myofibroblast differentiation can also be TGF-β-independent and can be stimulated by other proteins such as endothelin-1 (Leask, 2011). FGF-2, which can be released by other cells or from the ECM by mechanical stress, has been shown to have antagonistic effect on TGF-β1-induced myofibroblast differentiation by either inhibiting the Smad pathway or α-SMA expression at the transcription level (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). Mechanical stress such as flow-induced...
shear stress can also promote myofibroblast differentiation via integrins, the phosphorylation of focal adhesion kinase in the supermature focal adhesion, Rho kinase, actin polymerization, mitogen activated protein kinase (MAPK) such as p38, as well as by the conformational changes in the ECM proteins (Hinz, 2006; Small, 2012; Wang et al., 2000; Wang et al., 2002; Wipff and Hinz, 2009; Zhao et al., 2007). Actin polymerization can induce an increased level of myocardin related transcription factors (MRTFs), which then can bind to the serum response factor (SRF) for the activation of α-SMA expression (Small, 2012; Zhao et al., 2007). In addition to MRTFs, SRF has been reported to be associated with various mitogen activated protein kinases such as p38 and ERK in regulating α-SMA expression (Kretzschmar et al., 1999; Wang et al., 2000; Wang et al., 2002).

Figure 2.5. The morphology of ECs in static condition (top) and under flow (bottom). Under static condition, ECs are cobble-stone like. When exposed to shear stress, they are typically spindle-shaped under lamina flow, and they align with the long axis parallel to the direction of blood flow. Adapted from Ando and Yamamoto (Ando and Yamamoto, 2009).

Figure 2.6. (a) Loss of gingival connective tissues as a result of gingival atrophy associated with tooth mal-positioning, which exposes the root of the tooth to the surrounding oral environment. (b) A soft connective tissue graft was used to cover the area where gingival recession occurred. Adapted from Camargo et al. (Camargo et al., 2001).

Figure 2.7. A schematic outlining gingival tissue engineering. Cells are harvested and isolated from the host’s non-diseased gingival tissues, which are then expanded in cell population. The cells are seeded onto a natural or synthetic scaffold for further culturing and maturation. The resulting tissue-engineered construct can be transplanted back into the affected area to replace damaged tissues. Adapted from Chen et al. (Chen et al., 2010).

Figure 2.8. The design of parallel circuit perfusion bioreactors generally consists of a peristaltic pump, gas exchange, parallel perfusion chambers that contains one or more tissue-engineered constructs, and medium reservoirs.

Figure 2.9. Interaction between VEGF, TGF-β1, and FGF-2 in the process of angiogenesis.
Chapter 3: Perfused culture of gingival fibroblasts in a degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold leads to enhanced proliferation and metabolic activity

Figure 3.1. (a) Schematic of the setup for the bioreactor chamber in one of the pump’s channels. (b) Parts of the bioreactor’s chamber: (A) the platform, (B) the gasket, (C) the top, (D) wing nuts, and (E) hex bolts. The locations of the three samples in the bioreactor are indicated by arrow heads. (c) A fully assembled bioreactor chamber. ................................................................. 87

Figure 3.2. HGF cell population and metabolic activity on 2D D-PHI flat films over a 14-days culture period were assessed by (a) DNA mass quantification and (b) WST-1 assay respectively \((n = 9, \pm\) standard error (SE)). Initial cell seeding density was 20000 cells/well. *Significantly higher value compared to day 1 \((p<0.05)\). †Significantly higher value compared to day 7 \((p<0.05)\). ...................................................................................................................................... 90

Figure 3.3. (a) HGF proliferation on D-PHI scaffolds in static (no perfusion) vs. dynamic (with perfusion) culture was measured by DNA contents. (b) The metabolic activity of the cells was assessed by (b) a WST-1 assay and (c) total protein production. \((n = 9, \pm SE, value was significantly different from each other, †: p<0.05, *: p<0.01, §: p≤0.001.).) ................................................................. 92

Figure 3.4. Histology images of HGFs in 3D D-PHI scaffolds in static vs. dynamic culture at days 1, 7, 14, and 28. The control scaffold (no cells) is shown at the bottom. Cells were stained with hematoxylin and eosin (H&E) and the extent of their distribution indicated by the arrows. Scale bar = 500 µm. ..................................................................................................................... 94

Figure 3.5. Scanning electron micrographs of spindle-shaped HGFs (arrows) stretched out with their processes in the static culture (b – d) while they formed clusters in the dynamic culture (e – h) on D-PHI scaffolds. (a) No cells were seeded in the control scaffold. (e) A 4000-times magnification of the boxed area in (f), where strands of extracellular matrix (arrow heads) were observed. ...................................................................................................................................... 95

Figure 3.6. Col I production in HGFs in D-PHI scaffolds over 28 days of culture: (a) Western blot image, \(\text{Vim} = \text{vimentin}\), (b) Col I content normalized to value at day 0 \((n = 6, \pm SE)\), (c) Col I content normalized to vimentin content at corresponding time point, then normalized to value
(Col I/Vim) at day 0 (n = 6, ± SE). This shows the amount of Col I produced relative to cell content at specific time points. * Significantly different from each other (p<0.05); † significantly different from other time points in that culture condition (p<0.05).

Chapter 4: Establishing a gingival fibroblast phenotype in a perfused degradable polyurethane scaffold: mediation by TGF-β1, FGF-2, β1-integrin, and focal adhesion kinase

Figure 4.1. Immunofluorescence images of HGFs cultured on (a – c) D-PHI films and (d – f) TCPS after 24 hours of static culture. HGFs were stained for (a, d) Col I (red) and (b, e) α-SMA (green), with the nuclei stained with Hoechst (c, f, blue). Scale bar = 60 µm.

Figure 4.2. α-SMA production in perfused (dynamic) vs. non-perfused (static) D-PHI scaffold over 28 days of culture. n = 9, ± standard error (SE). * Significantly different from each other (p<0.05).

Figure 4.3. Immunohistochemistry images of HGFs cultured in (a, c) non-perfused (static) and (b, d) perfused (dynamic) D-PHI scaffolds at day 28 of culture. HGFs were stained for (a – b) Col I and (c – d) α-SMA. The area of low Col I content in (a) is indicated below the dotted line. Immunofluorescence images of HGFs cultured in (e) non-perfused and (f) perfused D-PHI scaffolds. HGFs were stained for FSP (green, indicated with white arrow heads) and Col I (red), with the nuclei and scaffolds stained with Hoechst (blue). (a – d) Scale bar = 500 µm, (e – f) Scale bar = 60 µm.

Figure 4.4. The production of TGF-β1 from HGFs cultured in static and dynamic cultures. n = 9, ± SE. * Significantly different from each other (p<0.01).

Figure 4.5. The production of FGF-2 from HGFs cultured in static and dynamic cultures. n = 6, ± SE. † Significantly different from each other (p<0.05).

Figure 4.6. The total β1-integrin production and FAK phosphorylation (pFAK) was measured 2h after the initiation of the dynamic culture. For static culture, the samples remained in the 96-well plate and were taken out at the same time as those in the dynamic culture. For β1-integrin,
the results were normalized to Vim; for pFAK, the results were normalized to total FAK. \( n = 9, \pm \text{SE.} \) *Significantly different from static culture (p<0.05).”

Figure 4.7. \( \alpha \)-SMA content at days 1 and 14 with or without antibody-mediated inhibition of \( \beta 1 \)-integrin in static and dynamic cultures. Data were normalized to day 0 values. ‘+’: with antibody; ‘-’: without antibody. Significantly different from each other (*: p<0.05; †: p<0.001). \( n = 6, \pm \text{SE.} \)

Supplementary Figure 4.1. A schematic diagram outlining the proposed mechanisms that are involved in the modulation of type I collagen (Col I) and \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) expression (myofibroblast differentiation). [Flow-induced shear stress can act as one of the mechanical stimuli]. Transforming growth factor (TGF)-\( \beta 1 \) is believed to be a regulator of collagen production and it is a potent inducer of \( \alpha \)-SMA expression via the Smad signaling pathway (Galie et al., 2012; Hinz, 2007; Hinz, 2010; Horan et al., 2008; Tomasek et al., 2002), however this process can be inhibited by basic fibroblast growth factor (FGF-2), which has been shown to inhibit Smad nuclear translocation, as well as downregulating \( \alpha \)-SMA expression at the transcription level (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). [Flow] has been reported to stimulate focal adhesion kinase (FAK) signaling via \( \beta 1 \)-integrin (Galie et al., 2012; Ng et al., 2005). Collagen production has also been shown to be mediated by \( \beta 1 \)-integrin (Langholz et al., 1995; Liu et al., 2009; Liu et al., 2010) and FAK (Dalla Costa et al., 2010; Surazynski et al., 2005). Both integrins and FAK are associated with myofibroblast differentiation (i.e. \( \alpha \)-SMA production) (Hinz, 2010).

Supplementary Figure 4.2. Control images of D-PH I scaffolds with HGFs in static culture stained without the primary antibodies for (a) immunohistochemistry and (b) immunofluorescence. Non-specific binding of the DAB peroxidase substrate (in (a)) and the secondary antibodies (in (b)) was not observed. The cell nuclei and scaffolds were stained with (a) hematoxylin and (b) Hoechst (blue). (a) Scale bar = 500 \( \mu \text{m} \), (b) Scale bar = 60 \( \mu \text{m} \)........

Supplementary Figure 4.3. \( \alpha \)-SMA content (normalized to Vim) of HGFs cultured in perfused (D0 Dyn) or non-perfused (D0 Stat) D-PH I scaffolds, inhibited with 3\( \mu \text{M} \) of SIS3. HGFs cultured in untreated medium (SIS3-) was used as negative control. Data was obtained at day 0 of culture (D0, i.e. 24 hours after seeding) after treatment with or without SIS3. \( n = 9, \pm \text{SE.} \).
Chapter 5: Pro-angiogenic character of endothelial cells and gingival fibroblasts co-cultures in perfused degradable polyurethane (D-PHI) scaffolds

Figure 5.1. VEGF production from HGF monoculture in static or dynamic condition. n = 9, ± standard error (SE). Significantly different from each other (*p<0.05, ǂp<0.01). .......................... 153

Figure 5.2. DNA content of two different cell seeding densities at various time points. Total number of cells are expressed in cells/ scaffold. Cells were cultured under perfusion at a HUVEC:HGF ratio of 1:1, in 50/50 mix medium. n = 9, ±SE. Significantly greater than co-culture with 40,000 cells (*p<0.05, ǂp<0.01). ............................................................................ 154

Figure 5.3. (a) DNA content and (b) metabolic activity (as measured by WST-1 assay) of HUVEC-HGF co-cultures in 50/50 mix medium or DMEM only at various time points. Cells were cultured under perfusion at a HUVEC:HGF ratio of 1:1 with a seeding density of 80,000 cells/ scaffold. n = 9, ±SE. Significantly different from each other (*p<0.05, ǂp<0.01). ................................................................. 155

Figure 5.4. Immunofluorescence images of HUVEC-HGF co-cultures at day 28 of culture in (a) – (d), (i) – (j) 50/50 mix medium, or (e) – (h), (k) – (m) DMEM only. Cells were cultured under perfusion at a HUVEC:HGF ratio of 1:1 with a seeding density of 80,000 cells/ 12.95 mg scaffold. Cells were stained for von Willebrand factor (vWF, (a, e)), fibroblast surface protein (FSP, (b, f)), cell nuclei (Hoechst 33342; (c, g)), caspase 3 (d, h), type I collagen (Col I, (i, k)), and α-smooth muscle actin (α-SMA, (j, m)). Hoechst 33342 also stained the D-PHI scaffolds (negative control with no primary antibodies (n)), hence a higher magnification (15x) was used to distinguish between scaffold sections and cell nuclei. HGFs were treated with staurosporine to induce apoptosis (i.e. caspase 3 activation), which was used as a positive control (o). The top of the scaffold is indicated by a dotted line. Scale = 60 µm. (p) The area of Col I (per cell) in the scaffold sections in 50/50 mix medium vs. DMEM at days 0 and 28 of culture. (q) The number of myofibroblasts (i.e. α-SMA+ cells) in 50/50 mix medium vs. DMEM at day 28 of culture, normalized to day 0 values (denoted as 1 (dotted line)). For (p) and (q), n = 9, ±SE. Significantly different from each other (*p<0.05). ................................................................................................. 157

Figure 5.5. (a) VEGF, (b) FGF-2, (c) TGF-β1 release from the HUVEC-HGF perfused co-culture (HUVEC:HGF = 1:1, at seeding density of 80,000 cells/ scaffold) in either 50/50 mix medium or DMEM. n = 9, ±SE. Significantly different from each other (*p<0.05). ......................... 159
Figure 5.6. (a) DNA content and (b) metabolic activity of HUVEC-HGF perfused co-cultures in 50/50 mix medium at various time points. HUVECs and HGFs were seeded at ratios 2:1, 1:1, or 1:2 (HUVEC:HGF), with a seeding density of 80,000 cells/ scaffold. n = 8, ±SE. Significantly different from each other (*p<0.05, †p<0.01). ................................................................. 161

Figure 5.7. Immunofluorescence images of HUVEC-HGF co-cultures at day 28 of culture at seeding ratios (a, d, g, j, n) 2:1, (b, e, h, k, o) 1:1, or (c, f, i, m, p) 1:2 (HUVEC:HGF). Cells were cultured under perfusion in 50/50 mix medium with a seeding density of 80,000 cells/ scaffold. Cells were stained for vWF (a) – (c), FSP (d) – (f), Col I (j) – (m), and α-SMA (n) – (p). Cell nuclei were stained with Hoechst 33342, which also non-specifically stained the D-PHI scaffolds (g) – (i), hence a higher magnification (15x) was used to distinguish between cell nuclei and scaffold sections. HUVEC clustering/ structural formations were indicated by white circles. The top of the scaffold is denoted by a dotted line. Scale = 60 µm. (q) The area covered by Col I in the scaffold sections at all three ratios for days 0 and 28 of culture, normalized to the amount of cells. (r) The number of myofibroblasts at all three ratios at day 28 of culture, normalized to day 0 values (denoted as 1 (dotted line)). For (q) and (r), n = 9, ±SE.......... 163

Figure 5.8. The proportion of HUVECs to HGFs in the perfused co-culture at (a) day 0 and (b) day 28 with seeding ratios 2:1, 1:1 or 1:2 (HUVEC:HGF). Cells were cultured in 50/50 mix medium with a seeding density of 80,000 cells/ scaffold. n = 9, ±SE. Significantly different from each other (*p<0.05, †p<0.01). ........................................................................................................... 164

Figure 5.9. (a) VEGF, (b) FGF-2, (c) TGF-β1 release from the HUVEC-HGF perfused co-culture (in 50/50 mix medium, at seeding density of 80,000 cells/ scaffold) at seeding ratios 2:1, 1:1, or 1:2 (HUVEC:HGF). n = 9, ±SE. Significantly different from each other (*p<0.05, †p<0.01)........................................................................................................... 165

Supplementary Figure 5.1. The monoculture of HGFs on D-PHI (40,000 cells/ sample) cultured in F-12 K medium only. HGFs were stained for FSP (green). The morphologies of HGFs were abnormal (i.e. not spindle-shaped). Scale = 60 µm. ................................................................. 149
Supplementary Figure 5.2. The proportion of HUVECs to HGFs in the perfused co-culture over 28 days in either 50/50 mix medium or DMEM. Cells were cultured at a HUVEC:HGF ratio of 1:1 with a seeding density of 80,000 cells/scaffold. n = 9, ±SE. ......................................................... 158

Appendix A: Characterization of collagen expression from human gingival fibroblasts on a degradable/polar/hydrophobic/ionic polyurethane

Figure A.1. Collagen expression over 14 days by HGFs seeded at various densities onto TCPS. Values are reported as mean ± standard error (SE). * indicates significantly different from day 1 (p<0.05). ± indicates significantly different from day 7 (p<0.05). n = 3. ......................................................... 203

Figure A.2. Collagen expression over 14 days by HGFs seeded at a cell seeding density of 20000 cells/well onto three different substrates. Values are reported as mean ± SE. * indicates significantly different from day 1 (p<0.05). ± indicates significantly different from day 7 (p<0.05). n = 3. ................................................................. 203

Figure A.3. DNA content of HGFs seeded at a cell density of 40000 cells/well onto TCPS, over 14 days. Values are reported as mean ± SE. * indicates significantly different from day 1 (p<0.05). n = 3. ................................................................. 204

Figure A.4. DNA content of HGFs seeded at a cell density of 20000 cells/well onto three different substrates, over 14 days. Values are reported as mean ± SE. * indicates significantly different from day 1 (p<0.05). n = 12 for TCPS, n = 9 for D-PHI and PLGA. ......................... 205

Figure A.5. Cell seeding efficiency for HGFs seeded onto three different substrates. Values are reported as mean ± SE. n = 12 for TCPS, n = 9 for D-PHI and PLGA. ................................. 205

Figure A.6. Collagen production normalized to DNA content, for HGFs cultured on three different substrates, over 14 days. Values are reported as mean ± SE. * indicates significantly different from day 1 (p<0.05). Θ indicates significantly different from TCPS day 14 (p<0.05). n = 12 for TCPS, n = 9 for D-PHI and PLGA. ................................. 206
Appendix B: Assessment of human umbilical vein endothelial cell growth and migration on degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold

Figure B.1. a) Each scaffold was placed into a 10mL sterile disposable syringe. b) The plunger was then re-inserted to remove most of the air from the syringe. c) The cell suspension was drawn into the syringe by pulling the plunger backward to ensure that the scaffold was in the cell suspension. d) The syringe was closed with a luer lock cap. e) The plunger was pulled back for 2mL for 5 seconds in order to assure that a vacuum was created inside the syringe. This procedure was repeated 3 times (the scaffold is floating in the media). ............................................ 211

Figure B.2. Average DNA content of HUVECs, seeded with the static seeding technique, at days 0, 1 and 7 of culture for 40,000 and 80,000 cells/scaffold. n = 9, ±SE. ........................................... 213

Figure B.3. Average DNA content of HUVECs, seeded with the vacuum seeding technique, at days 0, 1 and 7 of culture for 40,000 and 80,000 cells/scaffold. n = 9, ±SE. ........................................ 214

Figure B.4. DNA content of HUVECs cultured in D-PHI scaffolds in static or dynamic cultures over 7 days. All samples (40,000 cell/scaffold) were cultured statically at day 0, followed by separation into static or dynamic cultures at day 1. n = 9, ±SE. ¶Significantly different from each other (p<0.001). ........................................... 215

Appendix C: Supplementary data

Figure C.1. (a) Amount of vimentin (Vim) produced by HGFs in static vs. dynamic monocultures throughout 28-day period. (b) Direct relationship between the amount of Vim and DNA content in HGFs in static and dynamic monocultures. n = 9, ±SE. *Significantly different from each other (p<0.05). ................................................................. 217

Figure C.2. (Left) Immunofluorescence image of HGFs in a D-PHI scaffold section (5x magnification). Cell nuclei were stained with Hoechst, which also stained the scaffold. (Right) A magnified view (15x) of the framed area in the image on the left. Oval-shaped nuclei can be distinguished from the scaffold (arrows). Scale bar = 60 µm. .................................................. 217
Figure C.3. Immunofluorescence images of HUVEC-HGF co-culture in a D-PHI scaffold section (15x magnification). (Left) Cell nuclei were stained with Hoechst, which also stained the scaffold. (Right) Cells were stained for von Willebrand factor (vWF, red) and cell nuclei were stained with Hoechst (blue). The nuclei can be seen localized with the vWF stain (arrows), which can be distinguished from the scaffold. These images were used for the ease of counting. Scale bar = 60 µm.

Figure C.4. Immunofluorescence images of HUVEC-HGF co-culture (in 50/50 mix medium, at 1:2 (HUVEC: HGF)) in a D-PHI scaffold section (15x magnification). (Left) Cell nuclei were stained with Hoechst, which also stained the scaffold. (Right) HUVECs were stained for vWF (red) and cell nuclei were stained with Hoechst (blue). It was observed that HUVECs (arrows) formed large clusters at a ratio of 1:2 (HUVEC:HGF). Scale bar = 60 µm.

Figure C.5. Amount of ED-A Fn produced by HGFs in static vs. dynamic monocultures throughout 28-day period. n = 9, ±SE. *Significantly different from each other (p<0.05).

Figure C.6. HGFs were cultured on TCPS for 24 hrs, after which 3µM SIS3 (a Smad inhibitor) was added to selected wells to test the functionality of SIS3. After 1 hr of incubation, α-SMA production was significantly reduced in the treated vs. non-treated condition, proving that SIS3 was effective in blocking the Smad pathway. The results also indicate that Smad was activated when HGFs were cultured on TCPS. n = 9, ±SE. *Significantly different from each other (p<0.05).

Appendix D: Mechanical drawings of the perfusion bioreactor

Figure D.1. Plan views of the top and the platform of the bioreactor chamber.

Figure D.2. Side views of cross-sections A and B indicated in Fig. D.1 at the platform and at the top of the bioreactor’s chamber.

Figure D.3. Side view of the bioreactor’s chamber with the parts mounted together. Cross-sections A and B correspond to those indicated in Fig. D.1.
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ABP</td>
<td>Alveolar bone proper</td>
</tr>
<tr>
<td>AME</td>
<td>Alveolar mucosa epithelium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Alveolar process</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>BMP-#</td>
<td>Bone morphogenic protein-#</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
</tr>
<tr>
<td>Col I</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>D</td>
<td>Dentine</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s phosphate buffer saline</td>
</tr>
<tr>
<td>D-PHI</td>
<td>Degradable/polar/hydrophobic/ionic polyurethane</td>
</tr>
<tr>
<td>DVO</td>
<td>Divinyl oligomer</td>
</tr>
<tr>
<td>E</td>
<td>Enamel</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>EpiC</td>
<td>Epithelial cell</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF-1</td>
<td>Acidic fibroblast growth factor</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR-#</td>
<td>Fibroblast growth factor receptor-#</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FSP</td>
<td>Fibroblast surface protein</td>
</tr>
<tr>
<td>GM</td>
<td>Gingival margin</td>
</tr>
<tr>
<td>GS</td>
<td>Gingival sulcus</td>
</tr>
<tr>
<td>GTR</td>
<td>Guided tissue engineering</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HDI</td>
<td>Hexamethylene-1,6-diisocyanate</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon-α</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-#</td>
<td>Interleukin-#</td>
</tr>
<tr>
<td>JE</td>
<td>Junctional epithelium</td>
</tr>
<tr>
<td>LDI</td>
<td>Lysine diisocyanate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDI</td>
<td>Methylene di(4-phenyl isocyanate)</td>
</tr>
<tr>
<td>MRTF</td>
<td>Myocardin-related transcription factor</td>
</tr>
<tr>
<td>OGE</td>
<td>Oral gingival epithelium</td>
</tr>
<tr>
<td>OSE</td>
<td>Oral sulcular epithelium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCN</td>
<td>Polycarbonate diol</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>pFAK</td>
<td>Phosphorylated focal adhesion kinase</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SIS3</td>
<td>(2E)-1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl)-propenone hydrochloride</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TDI</td>
<td>2,4-toluene diisocyanate</td>
</tr>
<tr>
<td>T&lt;sub&gt;g&lt;/sub&gt;</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V-CAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial-cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGF-R#</td>
<td>Vascular endothelial growth factor receptor #</td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WST-1</td>
<td>Water soluble tetrazolium-1</td>
</tr>
</tbody>
</table>
List of Equations

Appendix E: Calculations of Reynolds number and shear stress in the perfusion bioreactor

Equation E.1. Speed of fluid flow.................................................................223
Equation E.2. Reynolds number (Re) calculation........................................223
Equation E.3. Average speed of fluid flow in a 3D porous scaffold....................224
Equation E.4. Estimation of Re for flow in a 3D porous scaffold.........................225
Equation E.5. Wall shear stress in a parallel plate model................................225
1.1 Introduction

Periodontal diseases are high prevalence infections that affect over 47.2% of Americans 30 years and older (Eke et al., 2012; Hau et al., 2014). They are commonly caused by bacterial biofilm but can also be related to atrophic processes, which are related to loss of metabolic perfusion to the gingival lamina propria in conditions such as tooth mal-positioning (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Tonetti et al., 2014; Wennström, 1996). In First World countries, gingival atrophy affects more than 20% of adults (Albandar and Kingman, 1999; Kassab and Cohen, 2003; Sarfati et al., 2010; Susin et al., 2004). Current treatments for bacteria-induced periodontal diseases include scaling and root planing (Álvarez et al., 2011; Page and Schroeder, 1976; Shue et al., 2012) and medications such as antibiotics (Álvarez et al., 2011; Giuliani and Rinaldi, 2011; Hau et al., 2014; Page and Schroeder, 1976). The lost tissues due to bacterial biofilm-induced degradation or atrophy can be treated with tissue grafts (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Harris, 1992; Langer and Langer, 1985; Paolantonio et al., 2002; Pini-Prato et al., 2010; Schroeder, 1986; Thombre et al., 2013; Tonetti et al., 2014; Wennström, 1996; Zabalegui et al., 1999). Tissue grafts are often limited by tissue abundance and donor site morbidity (Camargo et al., 2001; Chambrone et al., 2010; Moharamzadeh et al., 2007; Tonetti et al., 2014). To regenerate the lost gingival connective tissues which include the lamina propria, gingival tissue engineering has emerged as a promising treatment.

Natural or synthetic scaffolds have been used in gingival tissue engineering for cell culture and tissue growth. Collagen-based scaffolds are the most commonly used natural scaffolds in tissue engineering (Mathes et al., 2010; Moharamzadeh et al., 2008; Rouabhaia and Allaire, 2010); however their mechanical stability decreases quickly upon biodegradation if the collagen has not been cross-linked (Friess, 1998; Hillmann et al., 1999; Ma et al., 2003; Moharamzadeh et al., 2008) and the material may be immunogenic due to the source (Moharamzadeh et al., 2007; Moharamzadeh et al., 2008). Synthetic scaffolds are more mechanically stable and the
synthetic polymers are more reproducible. It is also easier to control the scaffold’s properties (e.g. elastic modulus, degradation rate, microstructure, etc.) (Benatti et al., 2007; Dhandayuthapani et al., 2011). Among the synthetic scaffolds used for tissue engineering to date, polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), and polyglycolic acid (PGA) have been explored the most extensively (Aframian et al., 2002; Blackwood et al., 2008; Buurma et al., 1999; Bäumchen et al., 2009; Dhandayuthapani et al., 2011). The degradation by-products of PLA, PGA, PLGA are, however, acidic and often promote an inflammatory response (Yang et al., 2001; Yang et al., 2005). After subcutaneous implantation in rats for 60 days, PLGA scaffolds also showed that they excluded cell and tissue infiltration. In addition, the scaffolds lost their structural integrity after this period (McBane et al., 2011b).

Many degradable aliphatic polyurethanes (PUs) make up a promising class of materials being considered for tissue engineering applications because of their high mechanical compliance, their ability to undergo biodegradation by hydrolysis, oxidation, or enzymes, and the potential for formulating the material in a manner that they can generate non-cytotoxic degradation byproducts (Saxena, 2008; Yang et al., 2009). Previous studies have shown that a degradable/polar/hydrophobic/ionic PU (D-PHI) hydrogel is non-cytotoxic and shows good wettability (Sharifpoor et al., 2009), as well as reducing the levels of inflammatory cytokines in monocytes (McBane et al., 2011a). D-PHI porous scaffolds, under cyclic mechanical strain, promoted growth and a more contractile phenotype for human coronary artery smooth muscle cells (Sharifpoor et al., 2011). From in vivo studies, D-PHI scaffolds displayed good biocompatibility (McBane et al., 2011b) and enhanced endothelial cell (EC) micro-vessel formation (McDonald et al., 2011). The degradation byproducts of D-PHI include lysine, linear water-soluble hydrocarbon chains and carbon dioxide, which are all non-cytotoxic and can be cleared easily by the human body (Sharifpoor et al., 2009). D-PHI scaffold has a degradation rate of 21 wt% over 100 days in vivo during which its porosity had increased from 82% to 87%, and its physical form and structure was retained (McBane et al., 2011b). In this thesis, the biocompatibility of the D-PHI material with human gingival fibroblasts (HGFs) and ECs was assessed to verify that this material is suitable for gingival tissue engineering.

HGFs have been commonly used to test the biocompatibility of dental implants (Jin et al., 2012; Ma et al., 2011) and soft tissue constructs (Chung et al., 2009; Moscato et al., 2008). Previous studies also show that HGFs can be used to populate in vitro models of gingival
connective tissues (Blackwood et al., 2008; Hillmann et al., 1999; Mathes et al., 2010; Mohammadi et al., 2007; Moharamzadeh et al., 2007; Moharamzadeh et al., 2008; Moscato et al., 2008; Mussig et al., 2008; Sachar et al., 2012; Yamada et al., 2006; Yamada et al., 2006). HGFs mainly produce type I collagen.

In cell culture, perfusion systems have been shown to improve metabolic exchange and enhance cell proliferation (Du et al., 2008; Navarro et al., 2001; Timmins et al., 2007; Zhao et al., 2009). Thus a perfusion bioreactor system was designed and developed in this thesis to facilitate the perfusion of medium through the HGF-seeded D-PHI scaffolds and the cell behaviour (i.e. cell growth and phenotype) was examined.

In wound healing, fibroblasts can differentiate into myofibroblasts to deposit and organize extracellular matrix, as well as to facilitate contraction of the granulation tissues for establishing a framework enabling tissue repair and reconstruction (Arora et al., 1999; Blumbach et al., 2010; Gabbiani, 2003; Hinz, 2006; Hinz, 2007; Hinz, 2010; Mayrand et al., 2012; Micallef et al., 2012; Moulin et al., 1999; Moulin et al., 2010; Tomasek et al., 2002; Wipff and Hinz, 2009). The appearance and contractility of myofibroblasts during wound healing are advantageous for tissue remodeling in the short term but become detrimental when there is a prolonged presence of myofibroblasts, which is often associated with fibrotic diseases (Blumbach et al., 2010; Galie et al., 2012; Hinz, 2006; Hinz, 2007; Horan et al., 2008; Micallef et al., 2012; Tomasek et al., 2002; Wipff and Hinz, 2009). This thesis investigated how the D-PHI material and the perfusion system established a transient myofibroblast phenotype in early culture time and the subsequent phenotypic change back to fibroblasts. The change in the production of myofibroblast markers was examined and various signalling pathways such as the TGF-\(\beta\)-Smad 2/3 (Blumbach et al., 2010; Hinz, 2007) and \(\beta\)1-integrin-FAK pathways (Chan et al., 2009; Tomasek et al., 2002) were explored. The antagonistic effect of FGF-2 on TGF-\(\beta\)-induced myofibroblast differentiation (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999) was also investigated.

In healthy gingival tissues, the lamina propria is highly vascular and has been modelled by a coculture system of HGFs and ECs (Moharamzadeh et al., 2007; Saxena, 2008; Taba Jr. et al., 2005). As ECs require both flow-induced shear stress (Ando and Yamamoto, 2009; Davies, 1995; Shi and Tarbell, 2011) and co-culture with other cell types for survival and micro-vessel formation (Baiguera and Ribatti, 2013; Davis et al., 2002; Guillemette et al., 2010; Newman et al.,
a perfused co-culture system was established in this thesis to promote HGF and EC proliferation, collagen production by HGFs, and EC tubulogenesis in D-PHI scaffolds. The aim here was to populate and establish vascular network in the *in vitro* model of gingival lamina propria for tissue regeneration.

Although previous studies showed some success in EC-fibroblast co-cultures in promoting blood vessel formation, the culture conditions such as the choice of culture medium (Choong et al., 2006; Salem et al., 2002) and cell seeding ratios (Kunz-Schughart et al., 2006; Ma et al., 2011) were not well defined. The current thesis investigated some of the favourable co-culture conditions (i.e. cell seeding density, type of culture medium, and cell seeding ratio) with the perfused D-PHI scaffold for optimizing cell proliferation, EC clustering, angiogenic factor production, and collagen production, such that a highly perfused tissue-engineered construct could potentially be developed for gingival tissue regeneration.

1.2 Hypothesis

**Central Hypothesis:** *In vitro* dynamic co-culture of HGFs with ECs on D-PHI scaffolds will produce a highly perfused tissue-engineered construct which physically (i.e. tissue-like structure) and biologically (i.e. expression of type I collagen (Col I), as well as displaying the potential for capillary formation) models phenotypic characteristics of the natural gingival lamina propria.

1.3 Objectives

The central objective for this thesis was to build a tissue-engineered construct with high collagen content and early signs of HUVEC cluster formation using HGFs, ECs, D-PHI scaffolds and a perfusion system for the regeneration of gingival lamina propria.

1.3.1 Objective I

To synthesize D-PHI scaffold (80% porosity, 2 mm thickness) and assess its biocompatibility with HGFs based on cell viability, proliferation and collagen production.

*Approach:*
• Synthesized D-PHI porous scaffolds from lysine-based polycarbonate divinyl oligomer (DVO) and acrylate monomers, with sodium bicarbonate and polyethylene glycol as porogens (Sharifpoor et al., 2009).
• Investigated HGF cell viability and proliferation (i) on two-dimensional D-PHI films over a static culture period of 14 days and (ii) in three-dimensional D-PHI porous scaffolds over a static culture period of 28 days by measuring DNA content and metabolic activity (water soluble tetrazolium-1 (WST-1) assay).
• Examined HGF cell morphology in the D-PHI scaffolds throughout the 28-day static culture by scanning electron microscopy (SEM).
• Observed the spatial localization of HGFs in the D-PHI scaffolds throughout the 28-day static culture period by histological staining (hematoxylin and eosin (H&E) staining).
• Measured Col I production from HGFs cultured statically in D-PHI scaffolds by Western blotting and normalized the Col I content to vimentin (Vim) (measured by Western blotting).

Related Paper and Abstracts:


1.3.2 Objective II

To design and develop a perfusion bioreactor system for facilitating medium perfusion (dynamic culture) through HGF-seeded D-PHI scaffolds such that cell proliferation and collagen production are enhanced.

**Approach:**

- Designed, built and troubleshooted a perfusion bioreactor system which consisted of a parallel circuit of perfusion chambers (each perfusion chamber contains three channels (one scaffold per channel)), a peristaltic pump, and medium reservoirs.
- Investigated HGF cell viability and proliferation in D-PHI porous scaffolds over a dynamic culture period of 28 days (500 µL/min) by measuring DNA content and metabolic activity (WST-1 assay).
- Examined HGF cell morphology in the perfused D-PHI scaffolds throughout the 28-day culture by SEM.
- Observed the spatial localization of HGFs in the perfused D-PHI scaffolds throughout the 28-day culture period by histological staining (H&E staining).
- Measured Col I production from HGFs cultured in perfused D-PHI scaffolds by Western blotting and normalized the Col I content to Vim (measured by Western blotting).
- Compared results from the dynamic culture with those from the static culture to show the effect of medium perfusion on cell proliferation and collagen production.

**Related Paper and Abstracts:**


Cheung JWC, Santerre JP. Characterization of human gingival fibroblasts on a degradable polar hydrophobic ionic polyurethane (D-PHI), 35th Society for Biomaterials Meeting, Orlando, FL, USA, April 13 – 16, 2011, #3.


1.3.3 Objective III

To investigate the interactions of HGFs with D-PHI scaffolds under medium perfusion by characterizing the cell phenotypic change and examining signalling pathways that are activated during dynamic culture.

Approach:

- Measured the production of α-smooth muscle actin (α-SMA, one of the distinctive myofibroblast markers) and Col I by HGFs in the perfused vs. non-perfused D-PHI scaffolds by Western blotting and normalized the contents to Vim (measured by Western blotting).
- Observed the α-SMA and Col I production and localization of cells spatially in the perfused vs. non-perfused D-PHI scaffolds by immunohistochemical and immunofluorescence staining, and confirmed that HGFs deposited Col I onto the D-PHI porous scaffolds using immunofluorescence for staining of the cells’ cytoplasm and Col I.
- Measured the release of TGF-β1 and basic fibroblast growth factor (FGF-2) in the culture medium from static and dynamic cultures by enzyme-linked immunosorbent assay (ELISA).
- Investigated the signaling pathways that affect the production of α-SMA and Col I by (i) inhibition of the Smad pathway, which can be activated by TGF-β1, (ii) measuring the total β1-integrin production and FAK phosphorylation (i.e. focal adhesion-related pathways), and (iii) by the inhibition of β1-integrin. Compared the results from the dynamic culture with those from the static culture.
Related Paper and Abstracts:


Cheung JWC, Santerre JP. Characterization of human gingival fibroblasts on a degradable polar hydrophobic ionic polyurethane (D-PHI), 35th Society for Biomaterials Meeting, Orlando, FL, USA, April 13 – 16, 2011, #3.


Cheung JWC, McCulloch CAG, Santerre JP. Dynamic culture of gingival fibroblasts in a degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold leads to enhanced proliferation and collagen production, 9th World Biomaterials Congress, Chengdu, China, June 1 – 5, 2012, #S073.

1.3.4 Objective IV

To establish a perfused co-culture system with HGFs and ECs by incorporating ECs and HGFs simultaneously in D-PHI scaffolds using the perfusion bioreactor system that was built in Objective II, and to select optimal culture conditions (cell seeding density, type of culture medium, and cell seeding ratio) based on cell proliferation, EC cluster formation, angiogenic factor production, and Col I production.

Approach:

- Measured the production of the angiogenic factor vascular endothelial growth factor (VEGF) in HGF monoculture in perfused vs. non-perfused D-PHI scaffolds by ELISA to show the angiogenic potential of HGFs cultured under medium perfusion in D-PHI scaffolds.
• Investigated the effect of medium perfusion and cell seeding density (40,000 cells vs. 80,000 cells per 12.95 mg D-PHI scaffold) on the co-culture of ECs and HGFs by measuring the DNA content over 28 days of culture.

• Examined the effect of culture medium (50/50 mix by volume of EC culture medium and HGF culture medium vs. HGF culture medium only) on the EC-HGF dynamic co-culture, based on cell growth, metabolic activity, EC cluster formation, α-SMA and Col I production, the proportion of ECs to HGFs over time, and angiogenic factor (VEGF, TGF-β1, FGF-2) production in the 28-day dynamic culture by measuring the DNA content, WST-1 assay, immunofluorescence staining (for EC cluster formation and production of α-SMA and Col I), cell counting in the immunofluorescence images, and ELISAs respectively.

• Examined the effect of cell seeding ratios (2:1, 1:1, 1:2 (EC:HGF)) on the EC-HGF dynamic co-culture based on the aforementioned parameters measured or observed using the above analysis methods.

Related Paper and Abstracts:


Cheung JWC, McCulloch CAG, Santerre JP. Co-culture of human gingival fibroblasts and vascular endothelial cells in a perfused degradable/polar/hydrophobic/ionic polyurethane (D-PHI), 36th Society for Biomaterials Meeting, Boston, MA, USA, April 10 – 13, 2013, #816.

Cheung JWC, McCulloch CAG, Santerre JP. The effect of culture medium on the co-culture of human gingival fibroblasts and vascular endothelial cells in a perfused polyurethane (D-PHI) scaffold, 30th Canadian Biomaterials Society Meeting, Ottawa, ON, Canada, May 29 – June 1, 2013, Podium #24.

Cheung JWC, McCulloch CAG, Santerre JP. Optimization of endothelial cell (HUVEC)-gingival fibroblasts co-cultures in perfused degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffolds, 37th Society for Biomaterials Meeting, Denver, CO, USA, April 16 – 19, 2014, #275.

1.4 Additional contributions

I also contributed to a literature review of co-culture systems used in tissue engineering and the role of biomaterials in these systems. I have made equal contributions with Kyle G. Battiston in writing this review. The abstract of this published review paper is in Appendix F. (Battiston KG, Cheung JWC, Jain D, Santerre JP. Biomaterials in co-culture systems: Towards optimizing tissue integration and cell signaling within scaffolds. Biomaterials 2014; 35: 4465-4476. (Journal impact factor: 8.312)). In addition, I have provided important contributions to a publication that highlights the research work in the Santerre group at the University of Toronto. My contributions consisted of providing the results from the investigation of HGF behaviour on two-dimensional surfaces (see Appendix A), and from the study of HUVEC monoculture on perfused D-PHI scaffolds (see Appendix B). The abstract of this published paper is in Appendix G. (Zhang X, Wright MEE, Cheung JWC, Battiston KG, Sharifpoor S, Labow RS, Santerre JP. New degradable polyurethanes for use in tissue engineering: Inspired by mechanisms of biodegradation and wound healing. Journal of Biomaterials and Tissue Engineering 2014; 4: 1-14. (Journal impact factor: 1.167)).
1.5 References

Aframian, D. J., Redman, R. S., Yamano, S., Nikolovski, J., Cukierman, E., Yamada, K. M.,
Kriete, M. F., Swaim, W. D., Mooney, D. J. and Baum, B. J. (2002). Tissue compatibility of two
biodegradable tubular scaffolds implanted adjacent to skin or buccal mucosa in mice. *Tissue
Eng.* 8, 649-659.

Akasaka, Y., Ono, I., Tominaga, A., Ishikawa, Y., Ito, K., Suzuki, T., Imaizumi, R., Ishiguro, S.,
Jimbow, K. and Ishii, T. (2007). Basic fibroblast growth factor in an artificial dermis promotes
apoptosis and inhibits expression of a-smooth muscle actin, leading to reduction of wound

calculus in adults 30 years of age and older in the united states, 1988-1994. *J. Periodontol.* 70,
30-43.


regulates transforming growth factor-β induction of a-smooth muscle actin in fibroblasts. *Am. J.
Pathol.* 154, 871-882.

*Angiogenesis* 16, 1-14.

polyglycolide scaffold structure on the proliferation of gingival fibroblasts in vitro: A pilot study.


Chapter 2
Literature review

2.1 The periodontium and the lamina propria of the gingiva

The periodontium consists of hard and soft connective tissues and is responsible for supporting the teeth by attaching them to the jaw bones. The hard connective tissues include the cementum and the alveolar bone (alveolar process); and the soft connective tissues are composed of the lamina propria of the gingiva and the periodontal ligament (Schroeder, 1986). The soft connective tissues are covered by the gingival epithelium. The periodontal ligament and the lamina propria connect to the dentine of the tooth via cementum, and to the jaw bone via the alveolar process (Fig. 2.1a) (Schroeder, 1986). The periodontal ligament is bounded centrally by the cementum, peripherally by the alveolar bone, and merges cervically into the lamina propria.

Gingiva is a strip of mucosa that surrounds the neck of the tooth (Schroeder, 1986). It is the most peripheral part of the periodontium and covers the alveolar process externally and the cementum cervically (Fig. 2.1a and b). Gingival tissues consist of two distinct layers: (i) a stratified squamous epithelium and (ii) the lamina propria, which is a fibrous, subepithelial connective tissue layer (Fig. 2.1b) (Benatti et al., 2007; Izumi et al., 2003; Moharamzadeh et al., 2007; Schroeder, 1986; Taba Jr. et al., 2005). The epithelium is attached to the lamina propria via hemidesmosomes and a basement membrane, which consists of type IV collagen, laminin, and fibronectin (Moharamzadeh et al., 2007). Collagen represents 60% of the extracellular matrix (ECM) in the healthy gingiva, and approximately 27.3% consists of acid mucopolysaccharides (e.g. hyaluronic acid), glycoproteins, and proteoglycans (e.g. chondroitin sulphate) (Schroeder, 1986). In an adult human, the width of the gingival tissue ranges from 1 – 10 mm and the height ranges from 5 – 7 mm (including free and attached gingiva) (Schroeder, 1986). The variation in the dimensions depends on the age and the location of the gingival tissue relative to the tooth.
Figure 2.1. (a) The anatomical structure of the periodontium – (i and ii) gingiva, (iii) cementum, (iv) mandibular bone, (v) periodontal ligament, (vi) dentine, (vii) alveolar mucosa, and (viii) alveolar process. Adapted from Schroeder (Schroeder, 1986). (b) The histological image (left) and schematic drawing (right) of oral gingival tissues. OGE: oral gingival epithelium, OSE: oral sulcular epithelium, JE: junctional epithelium, GM: gingival margin, GS: gingival sulcus, E: enamel, AP: alveolar process. Adapted from Schroeder (Schroeder, 1986).

The multi-layered epithelium mainly consists of epithelial cells (epiC) and it is responsible for protecting the underlying connective tissues (Schroeder, 1986). The gingival epithelium is capable of continuous renewal (Ellegaard et al., 1976; Mackenzie et al., 1991; Mackenzie and Tonetti, 1995; McKeown et al., 2003; Schroeder, 1986).

The lamina propria provides additional support for the teeth and it protects the underlying alveolar bone. It has an abundant fibrous network that consists of ~91% type I collagen fibres, ~8% of type III collagen fibres in the deeper layer, and less than 1% of type V collagen
Reticulin fibres are also found in the lamina propria near the basement membrane. Fibroblasts are the main cell type residing in the lamina propria, along with endothelial cells, pericytes, nerve cells, and very occasionally, small amount of monocytes, macrophages, mast cells, and lymphocytes (Moharamzadeh et al., 2007; Schroeder, 1986; Taba Jr. et al., 2005). Recent studies showed that the lamina propria also contains undifferentiated ectomesenchymal cells that serve as a replacement source for the fibroblasts (Taba Jr. et al., 2005). The lamina propria is highly vascular. It contains extensive capillary loops whose blood supply is from the arteriols from the interdental septum, the periodontal ligament and the oral mucosa (Melcher and Bowen, 1969; Moharamzadeh et al., 2007; Saxena, 2008; Schroeder, 1986; Taba Jr. et al., 2005). In addition, it contains lymphatic vessels, nerves, nerve endings, and ducts of salivary glands (Moharamzadeh et al., 2007; Taba Jr. et al., 2005).

The collagen fibres in the lamina propria are arranged into densely woven meshes and the cells are oriented parallel to the fibre bundles (Schroeder, 1986). The fibre bundles constitute the supra-alveolar fibre apparatus and most fibre bundles have preferential orientation, thus they are categorized based on their orientation and architectural arrangement (Fig. 2.2) (Schroeder, 1986). For example, the circular and semicircular fibres encircle the tooth partially and entirely in the gingiva; and they cross interdentally to the fibre groups of the adjacent tooth. Thus these fibres are denser, more compact and larger. On the other hand, the dentogingival fibres have three subgroups and fibres from one of the subgroups radiate coronally under the junctional epithelium. All of the fibre groups are intermingled and distributed differently to account for the stresses from all tooth movement (Schroeder, 1986). The turnover of soluble collagen in gingiva is faster than that in the alveolar bone and skin (Schroeder, 1986).
2.1.1 Fibroblasts and myofibroblasts

Fibroblasts are spindle-shaped cells that are responsible for the growth and maintenance of connective tissues, ECM production, wound healing, inflammation, and fibrosis (Giannopoulou and Cimasoni, 1996; Taba Jr. et al., 2005). There are approximately $2 \times 10^8$ fibroblasts per cubic centimeter in the lamina propria of the gingiva (Schroeder, 1986). They are typically characterized by the expression of collagen and vimentin (Giannopoulou and Cimasoni, 1996; Hinz, 2007; Smith and Martínez, 2006; Wipff and Hinz, 2009). Vimentin (Vim) is an intermediate filament protein that is typically expressed in mesenchymal cells such as fibroblasts (Lodish et al., 2003). Since fibroblasts are responsible for the production and remodelling of the connective tissue, they secret an abundant amount of types I, III, and IV collagen, fibronectin and heparin sulfate proteoglycans (Hillmann et al., 1999; Hughes, 2008).

In the event of wound healing following an injury, fibroblasts can differentiate into myofibroblasts to deposit and organize ECM, as well as to facilitate contraction of the
granulation tissues for establishing a framework enabling tissue repair and reconstruction (Arora et al., 1999; Blumbach et al., 2010; Gabbiani, 2003; Hinz, 2006; Hinz, 2007; Hinz, 2010a; Mayrand et al., 2012; Micallef et al., 2012; Moulin et al., 1999; Moulin et al., 2010; Tomasek et al., 2002; Wipff and Hinz, 2009). The appearance and contractility of myofibroblasts during wound healing are advantageous for tissue remodeling in the short term but become detrimental when there is a prolonged presence of myofibroblasts, which is often associated with scarring (fibrosis) (Blumbach et al., 2010; Galie et al., 2012; Hinz, 2006; Hinz, 2007; Horan et al., 2008; Micallef et al., 2012; Tomasek et al., 2002; Wipff and Hinz, 2009). Myofibroblasts are characterized by the expression of α-smooth muscle actin (α-SMA) and its assembly into stress fibres (Blumbach et al., 2010; Chan et al., 2009; Galie et al., 2012; Hinz, 2006; Hinz, 2007; Hinz, 2010b; Micallef et al., 2012; Wipff and Hinz, 2009), extensive cell-to-matrix adhesion sites (Hinz, 2007), increase in intercellular adherens (e.g. N-cadherin, OB-cadherin) (Hinz, 2007; Pittet et al., 2008), synthesis and remodelling of collagen (types I, III, IV, V) (Blumbach et al., 2010; Chan et al., 2009; Hinz, 2006; Hinz, 2007), and the expression of the fibronectin (Fn) splice variant ED-A Fn (Hinz, 2006; Hinz, 2007). The α-SMA-positive stress fibres and ED-A Fn endow the myofibroblasts with contractility (Blumbach et al., 2010; Hinz et al., 2001; Micallef et al., 2012; Wipff and Hinz, 2009).

Fibroblast-myofibroblast transition depends on fibroblasts’ interaction with the surrounding ECM, where mechanical stress in the matrix is transduced to the cells across adhesion sites, and on the presence of growth factors such as transforming growth factor (TGF)-β1 (Blumbach et al., 2010; Galie et al., 2012; Hinz, 2006; Hinz, 2010b; Horan et al., 2008; Tomasek et al., 2002; Wipff and Hinz, 2009). After the normal repair of the tissue, myofibroblasts typically undergo apoptosis and α-SMA expression is significantly decreased (Fig. 2.3) (Gabbiani, 2003; Hinz, 2006; Hinz, 2007; Micallef et al., 2012; Moulin et al., 1999; Tomasek et al., 2002).
Figure 2.3. The development of myofibroblasts. Upon injury, inflammatory signals and profibrotic growth factors such as TGF-β1 stimulate resident fibroblasts to undergo transition into myofibroblasts. Myofibroblasts are characterized by α-SMA, ED-A Fn, stress fibre formation, and supermature focal adhesion. Under normal conditions, myofibroblasts undergo apoptosis after wound healing is completed. Adapted from Hinz (Hinz, 2007).

2.1.1.1 Mediation of myofibroblast differentiation – growth factors

TGF-β1 is an important growth factor that regulates cell growth, differentiation, motility, vasculogenesis, and ECM production (Horan et al., 2008; Mallet et al., 2006; Washio et al., 2011). There are three isoforms of TGF-β and TGF-β1 is a major inducer of myofibroblast differentiation (Fig. 2.4), which is tightly regulated by cooperative and antagonistic growth factors (Blumbach et al., 2010; Galie et al., 2012; Hinz et al., 2001; Hinz, 2006; Hinz, 2007; Horan et al., 2008; Wipff and Hinz, 2009). TGF-β can be produced by fibroblasts, myofibroblasts, vascular cells and keratinocytes (Blumbach et al., 2010). Recent studies have reported that TGF-β1 is synthesized with the latency-associated protein and latent TGF-β binding protein as a large latent complex in the ECM (Blumbach et al., 2010; Hinz, 2007; Wipff and Hinz, 2009). The release and activation of TGF-β from these complexes require dissociation by either proteases, or the induction of the process by mechanical forces (Blumbach et al., 2010; Wipff and Hinz,
Tight regulation of this process is essential due to the potent effect of this growth factor (Hinz, 2007). In response to interstitial flow, the production of TGF-β1 has been shown to mediate myofibroblast differentiation (Galie et al., 2012; Ng et al., 2005). TGF-β regulates α-SMA expression commonly via the Smad signalling pathway (Smad 2/3) (Fig. 2.4) (Blumbach et al., 2010; Hinz, 2007). There are also Smad-independent pathways that induce α-SMA expression such as the phosphatidylinositol 3-kinase (PI3K) and its downstream effector p21-activated kinase-2 (Hinz, 2007). TGF-β1 can regulate α-SMA transcription via the TGF-β1 control element. TGF-β1 control element in the α-SMA promoter is a non-Smad-binding element and it regulates α-SMA expression independent of Smad signalling (Tomasek et al., 2005). Myofibroblast differentiation can also be TGF-β-independent. Cytokines such as endothelin-1 (Leask, 2011), increase in intracellular stress (Hinz, 2007), and the production of Fizz1 by alveolar epiC (Hinz, 2007) have been shown to promote myofibroblast differentiation.

There are various antagonists to TGF-β and its induction in α-SMA expression such as interleukin (IL)-1 produced by keratinocytes (Hinz, 2007), interferon (IFN)-γ produced by T-cells (Hinz, 2007), and basic fibroblast growth factor (FGF-2) commonly produced by fibroblasts (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999) (Fig. 2.4). FGF-2 can inhibit TGF-β/Smad signaling by activating the ERK1/2 signaling pathway (Kretzschmar et al., 1999), thereby suppressing α-SMA expression (Ishiguro et al., 2009). FGF-2 can also inhibit α-SMA expression at the transcription level (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). Under perfusion, FGF-2 release can be augmented, which can inhibit the formation of myofibroblast in vitro (Sterpetti et al., 1994).
Figure 2.4. Various signaling molecules and pathways that are involved in myofibroblast differentiation. TGF-β1 can be delivered to the fibroblasts from other cells or from the release from the latent TGF-β1 complex upon mechanical stimulation (Blumbach et al., 2010; Wipff and Hinz, 2009). TGF-β1 can induce α-SMA production via the Smad signalling pathway or other pathways such as PI3K (Blumbach et al., 2010; Hinz, 2007). Myofibroblast differentiation can also be TGF-β-independent and can be stimulated by other proteins such as endothelin-1 (Leask, 2011). FGF-2, which can be released by other cells or from the ECM by mechanical stress, has been shown to have antagonistic effect on TGF-β1-induced myofibroblast differentiation by either inhibiting the Smad pathway or α-SMA expression at the transcription level (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). Mechanical stress such as flow-induced shear stress can also promote myofibroblast differentiation via integrins, the phosphorylation of focal adhesion kinase in the supermature focal adhesion, Rho kinase, actin polymerization, mitogen activated protein kinase (MAPK) such as p38, as well as by the conformational changes in the ECM proteins (Hinz, 2006; Small, 2012; Wang et al., 2000; Wang et al., 2002; Wipff and Hinz, 2009; Zhao et al., 2007). Actin polymerization can induce an increased level of myocardin related transcription factors (MRTFs), which then can bind to the serum response factor (SRF) for the activation of α-SMA expression (Small, 2012; Zhao et al., 2007). In addition to MRTFs, SRF has been reported to be associated with various mitogen activated protein kinases such as p38 and ERK in regulating α-SMA expression (Kretzschmar et al., 1999; Wang et al., 2000; Wang et al., 2002).
2.1.1.2 Mediation of myofibroblast differentiation – mechanical stress

Mechanical forces can be transmitted across focal adhesion (FA) to fibroblasts, thus affecting their response (Blumbach et al., 2010; Chan et al., 2009; Wang et al., 2000; Wang et al., 2002). FAs are junctions for cell adhesion, migration and spreading. They contain a complex organization of cytoskeletal and signalling proteins (Chan et al., 2009). In addition to TGF-β, mechanical stress from, for example, increased ECM rigidity and slow interstitial flow that accompanies inflammation and tissue regeneration has been shown to stimulate myofibroblast differentiation (Chan et al., 2009; Hinz, 2007; Ng et al., 2005; Shi and Tarbell, 2011; Wang et al., 2000; Wang et al., 2002; Wipff and Hinz, 2009). Mechanical stress-induced myofibroblast differentiation can be mediated by mechanosensing ion channels, integrins, and mechanically-induced conformational change in the ECM proteins that exposes cryptic signaling domains (e.g. TGF-β1 activation upon release from large latent complex) (Hinz, 2007; Wipff and Hinz, 2009) (Fig. 2.4). Upon myofibroblast differentiation, specialized FAs called supermature FAs are developed and they are characterized by large size (~8 – 30 µm), high levels of phosphorylated focal adhesion kinase (FAK), vinculin, paxillin, tensin, and integrins αvβ3 and α5β1 (Hinz, 2006; Hinz, 2007; Wipff and Hinz, 2009).

In previous studies, myofibroblast differentiation was not observed in soft tissues (elastic modulus of 100-20,000 Pa) but in matrix or substrate that has an elastic modulus of above 20 kPa (e.g. 50 kPa for granulation tissues) (Hinz, 2007; Wipff and Hinz, 2009). Cyclic, uniaxial strain has been shown to increase type I collagen (Col I) and α-SMA expression in cardiac fibroblasts cultured on two-dimensional (2D) elastic substrate (Galie et al., 2011; Galie et al., 2012). The regulation of α-SMA expression by mechanical stimuli can be mediated by integrin-mediated pathways such as p38 (Sato et al., 2002; Wang et al., 2000; Wang et al., 2002; Wang et al., 2006), FAK (Dalla Costa et al., 2010; Suer et al., 2009; Wang et al., 2006), and c-Jun NH(2)-terminal kinase (MacKenna et al., 1998; Wang et al., 2000; Wang et al., 2006).

Many researchers have attempted to inhibit myofibroblast differentiation by decreasing substrate stiffness, limiting FA sizes using micropatterned structures, or designing novel biomaterials (Acharya et al., 2008; Arora et al., 1999; Dreier et al., 2013; Galie et al., 2012; Goffin et al., 2006; Hinz, 2006; Hinz, 2007; Liu et al., 2010; Tomasek et al., 2002; Wipff and Hinz, 2009). With regards to substrate compliance, Dreier et al. found that a more compliant 2D, collagen-coated
polyacrylamide substrate (4 kPa) can inhibit TGF-β induced myofibroblast differentiation (Dreier et al., 2013). Fibroblasts cultured on soft polydimethylsiloxane (PDMS) with a modulus of 9.6 kPa was α-SMA-negative, while those cultured on a stiffer PDMS surface (780 kPa) differentiated into myofibroblasts with strong α-SMA expression and stress fibre formation (Goffin et al., 2006). For the design of biomaterials, Acharya et al. conjugated silk fibroin with lactose to fabricate scaffolds that inhibit myofibroblast differentiation (Acharya et al., 2008). Using micropatterned silicone substrate, FA size was limited and by varying the substrate stiffness from 23 kPa to 12 kPa, α-SMA expression was inhibited (Goffin et al., 2006).

Interstitial flow is increased during inflammation and tissue regeneration as a result of increased microvascular permeability and increased lymphatic drainage (Ng et al., 2005). Short-term interstitial flow culture (10 µL/min) of up to 4 days with cardiac fibroblasts demonstrates myofibroblast differentiation with increased α-SMA and type III collagen contents (Galie et al., 2012). Flow-induced shear stress has also been shown to stimulate intracellular signaling via integrin α1β1 which influences the proliferation and orientation of dermal fibroblasts on collagen matrices (Galie et al., 2012; Ng et al., 2005); and the downstream signaling involves FAK-ERK signaling. It was reported that at 8 dynes/cm², fibroblasts differentiated into myofibroblasts after 24 h; while at shear stresses between 9 and 13 dynes/cm², fibroblast proliferation was promoted (Sterpetti et al., 1994) and myofibroblast differentiation was suppressed.

Fibroblasts often adhere to the ECM and perceive mechanical signals via integrins (Chan et al., 2009; Hinz, 2010b; Tomasek et al., 2002; Wipff and Hinz, 2009). Specific integrins have been shown to mediate the activation of TGF-β1 (integrin-mediated pulling) from the latent complex on stiff substrates (Wipff and Hinz, 2009). Thus by interfering with these specific integrins, the profibrotic effect of TGF-β1 can be diminished without impairing other beneficial effects of TGF-β1 (Wipff and Hinz, 2009). Integrins α1β1 and α2β1 are responsible for collagen binding (Heino, 2000; Langholz et al., 1995; Warstat et al., 2010) and knocking out β1-integrin results in decreased collagen expression and delayed wound healing (Liu et al., 2009; Liu et al., 2010), which suggests the important role of β1-integrin in the regulation of collagen synthesis. β1-integrin mediates fibroblast-ECM crosstalk, which is followed by activation of various kinases such as integrin-linked kinase (Blumbach et al., 2010), FAK (Chan et al., 2009), and Rho kinases (Small, 2012; Zhao et al., 2007). Mechanical stress can cause clustering of integrins and
development of FAs, which are linked to the actin filaments intracellularly (Wang et al., 2002; Wipff and Hinz, 2009; Zhao et al., 2007).

Mechanical stress can increase the phosphorylation of FAK (e.g. tensile stress of 0.65 pN/µm² (Chan et al., 2009)) and thus mediating myofibroblast differentiation (Hinz, 2006) (Fig. 2.4). FAK is widely known to be co-localized with integrins at the cell adhesion sites in fibroblasts (Schlaepfer et al., 1999) and it has been shown to be closely related to β1-integrin via linkage proteins such as talin (Schlaepfer et al., 1999; Sieg et al., 2000). The N terminus of FAK has been demonstrated to contain sequences that bind to peptides derived from β1-integrins (Schlaepfer et al., 1999). Hence FAK is important for integrin-based signaling (Chan et al., 2009; Tomasek et al., 2002). Upon clustering of integrins, FAKs are recruited to FAs and subsequently autophosphorylate at tyrosin residue Tyr397 (Chan et al., 2009; Schlaepfer et al., 1999), which then interact with other FA proteins such as Src and paxillin. Tyr397 phosphorylation is required for full FAK activation and downstream signaling (Chan et al., 2009). Some of the downstream signalling proteins include extracellular signal-regulated kinase (ERK) (Hayashida et al., 2007) and p38 (Sato et al., 2002). There are also non-integrin stimuli that may upregulate FAK phosphorylation such as growth factors (e.g. vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF)) and other actin-related proteins (e.g. Rho) (Schlaepfer et al., 1999; Sieg et al., 2000).

Aside from FAK, mechanical stimuli can mediate α-SMA production via actin polymerization. Mechanical stress and growth factors have been shown to regulate actin polymerization (Fig. 2.4) (Small, 2012). For example, the Rho-signalling pathway has been reported to be involved in actin polymerization, which requires β1-integrin and intact actin filaments (Small, 2012; Zhao et al., 2007). Actin polymerization can induce the nuclear translocation of myocardin-related transcription factors (MRTFs), which can bind to the serum response factor (SRF) for the activation of α-SMA expression (Fig. 2.4) (Small, 2012; Zhao et al., 2007). SRF is a transcription factor that is involved in the regulation of α-SMA expression by binding to the CArG elements in the α-SMA promoter (Tomasek et al., 2005; Wang et al., 2002; Zhao et al., 2007). CArG elements such as CArG B can respond to mechanical stimulation and regulate the activity of the promoter and α-SMA expression (Tomasek et al., 2005; Wang et al., 2002; Zhao et al., 2007). CArG elements have the general sequence motif CC(A/T)6GG (Wang et al., 2002) and are believed to contain the core sequence of serum response element that is required for
transcriptional responses following serum, growth factor or mechanical stimulation (Small, 2012; Wang et al., 2002).

In addition to MRTFs, SRF has been reported to be associated with various mitogen activated protein kinases such as p38 and ERK in regulating α-SMA expression (Fig. 2.4) (Kretzschmar et al., 1999; Wang et al., 2000; Wang et al., 2002).

2.1.1.3 Gingival fibroblasts

Gingival fibroblasts are the main type of cells located in the gingival lamina propria (Schroeder, 1986). They are mostly responsible for the production and remodelling of collagen in the lamina propria (Moharamzadeh et al., 2007; Mussig et al., 2008). Gingival fibroblasts are the most common cell type used for assessing the biocompatibility of dental implants (Jin et al., 2012; Ma et al., 2011) and for populating in vitro models of gingival connective tissues (Blackwood et al., 2008; Hillmann et al., 1999; Mathes et al., 2010; Mohammadi et al., 2007; Moharamzadeh et al., 2007; Moharamzadeh et al., 2008; Moscato et al., 2008; Mussig et al., 2008; Sachar et al., 2012; Yamada et al., 2006).

2.1.2 Vascular endothelial cells (ECs)

ECs are cells that line the blood vessel walls in a monolayer (Baiguera and Ribatti, 2013). They are essential in the homeostasis of the circulatory system (Ando and Yamamoto, 2009). By responding to shear stress from blood flow, they regulate vascular remodelling and lumen diameter, facilitate antithrombotic activity, control infiltration of leukocytes into the blood stream, as well as secrete various growth factors such as PDGF (Ando and Yamamoto, 2009; Davies, 1995).

ECs are characterized by their expression of proteins such as vascular cell adhesion molecule (V-CAM)-1 (Ando and Yamamoto, 2009), cluster of differentiation 31 (CD31) or platelet EC adhesion molecule (PECAM)-1 (Ando and Yamamoto, 2009; Hofmann et al., 2008), von Willebrand factor (vWF) or factor VIII (Baiguera and Ribatti, 2013; Hofmann et al., 2008), and vascular endothelial cadherin (VE-cadherin) (Ando and Yamamoto, 2009; Dejana and Giampietro, 2012; Hofmann et al., 2008). CD31 is localized to the cell membrane of ECs (Ando and Yamamoto, 2009) and vWF is a glycoprotein involved in coagulation (Lenting et al., 2012).
The morphology of ECs is malleable and is dependent on the flow-induced shear stress (Davies, 1995). They are typically spindle-shaped under laminar flow, and they align with the long axis parallel to the direction of blood flow (Fig. 2.5) (Ando and Yamamoto, 2009; Davies, 1995). Without the influence of flow, ECs are cobble-stone like (Ando and Yamamoto, 2009); and under turbulent or stagnant flow, ECs adopt a rounder shape and they lack uniform orientation (Ando and Yamamoto, 2009; Davies, 1995).

Typically ECs require shear stress for angiogenesis (formation of new blood vessels) and vascular remodelling (Ando and Yamamoto, 2009). The effect of shear stress on ECs will be described in section 2.4.1.

There are two types of adhesive structures for adhesion between ECs – the tight junction and the adherens junction known as VE-cadherin (Dejana and Giampietro, 2012). When ECs are in confluent conditions, they do not divide and VE-cadherin interacts with signaling partners to induce contact inhibition of growth in ECs (Dejana and Giampietro, 2012). When these junctions are partially or completely dismantled, ECs begin to migrate and proliferate.

**Figure 2.5.** The morphology of ECs in static condition (top) and under flow (bottom). Under static condition, ECs are cobble-stone like. When exposed to shear stress, they are typically spindle-shaped under lamina flow, and they align with the long axis parallel to the direction of blood flow. Adapted from Ando and Yamamoto (Ando and Yamamoto, 2009).
In the highly vascular gingival lamina propria, ECs form capillaries and maintain homeostasis (Schroeder, 1986). As the flow in capillaries is laminar, ECs in the gingival lamina propria are exposed to slow laminar flow (Mathes et al., 2010) with low shear stress (4 dyn/cm²) (Shaik et al., 2009). In the progression of periodontal diseases, ECs are reported to produce various cytokines and adhesion molecules that can mediate leukocyte migration and infiltration into the diseased area (An et al., 2014).

2.2 Gingival atrophy

Periodontal diseases include atrophic processes in the gingiva (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Wennström, 1996). Gingival atrophy is highly prevalent (Albandar and Kingman, 1999; Kassab and Cohen, 2003; Sarfati et al., 2010; Susin et al., 2004) and is defined as the oral exposure of the root surface due to the recession of the gingiva (i.e. irreversible displacement of the gingival margin apical to the cemento-enamel junction, Fig. 2.6a) (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Tonetti et al., 2014; Wennström, 1996). The exposed root surface may be associated with root sensitivity (Chambrone et al., 2010; Kassab and Cohen, 2003; Tonetti et al., 2014), non-carious cervical lesions (Tonetti et al., 2014), root caries (Kassab and Cohen, 2003; Tonetti et al., 2014), etc. Gingival atrophy may also lead to tooth loss, and it has a negative impact on the quality of life with regards to impaired aesthetics of the smile (Chambrone et al., 2010; Tonetti et al., 2014). Gingival atrophy can be caused by multiple factors such as tooth mal-alignment, alveolar bone dehiscences, chronic trauma, aging, smoking, etc. (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Wennström, 1996).
Figure 2.6. (a) Loss of gingival connective tissues as a result of gingival atrophy associated with tooth mal-positioning, which exposes the root of the tooth to the surrounding oral environment. (b) A soft connective tissue graft was used to cover the area where gingival recession occurred. Adapted from Camargo et al. (Camargo et al., 2001).

2.2.1 Current treatment for gingival atrophy

Current treatment for gingival atrophy often involves soft connective tissue grafts (Fig. 2.6b) (Chambrone et al., 2010; Graziani et al., 2014; Harris, 1992; Tonetti et al., 2014; Tözüm et al., 2005), free gingival grafts (Camargo et al., 2001; Chambrone et al., 2010), and matrices derived from natural materials such as porcine collagen (Aroca et al., 2013; Cardaropoli et al., 2012; Graziani et al., 2014; Thombre et al., 2013).

Soft connective tissue grafts are commonly derived from the palate (Aroca et al., 2013; Chambrone et al., 2010; Paolantonio et al., 2002; Rastogi et al., 2009; Schroeder, 1986; Wennström, 1996; Zabalegui et al., 1999). They have been shown to reduce gingival recession and have the potential to re-establish gingival tissue mass for complete root coverage (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Harris, 1992; Langer and Langer, 1985; Pini-Prato et al., 2010; Thombre et al., 2013; Tonetti et al., 2014; Wennström, 1996; Zabalegui et al., 1999). However, tissue grafts have been reported with limitations such as difficulty in retaining the original characteristics of donor tissue, pain and discomfort, donor site morbidity (secondary defect), tissue insufficiency to cover the entire recession area and high cost (Camargo et al., 2001; Chambrone et al., 2010; Karring et al., 1975; Moharamzadeh et al., 2007; Paolantonio et al., 2002; Rastogi et al., 2009; Tonetti et al., 2014). For example, in a clinical
study with monkeys, connective tissue grafts from the gingiva are reported to induce formation of a keratinized gingival epithelium after 4 weeks of implantation; whereas grafts from the alveolar mucosa failed to induce keratinization (Karring et al., 1975), which emphasized on the specificity of the source of the tissue graft. Moreover, the study of Rastogi et al. demonstrated that tissue grafts from the oral mucosa can potentially cause secondary defects which cannot be closed (Rastogi et al., 2009); these opened defects are prone to bacterial challenge in the moist oral cavity. Clinicians have used porcine collagen matrices and acellular dermal matrices as alternatives to these grafts but the clinical outcome (e.g. complete root coverage) was not significantly superior when compared with tissue grafts (Aroca et al., 2013; Cardaropoli et al., 2012; Thombre et al., 2013). Pertaining to the disadvantages of current treatments, biomedical devices such as tissue-engineered constructs are currently being explored.

2.3 Gingival tissue engineering

For gingival tissue engineering, the goal is to regenerate the highly vascular lamina propria (Melcher and Bowen, 1969; Moharamzadeh et al., 2007; Saxena, 2008; Schroeder, 1986; Taba Jr. et al., 2005). The four main components for vascular tissue engineering are scaffolds, cells, cell-cell signals, and micro-vessel blood supply (Chen et al., 2010; Moharamzadeh et al., 2007; Taba Jr. et al., 2005) (Fig. 2.7). The choice of scaffolds and cell types will be outlined in sections 2.3.1 and 2.3.2 respectively, and the establishment of capillary networks in the scaffolds (angiogenesis) will be discussed in section 2.5.

![Figure 2.7](image)

**Figure 2.7.** A schematic outlining gingival tissue engineering. Cells are harvested and isolated from the host’s non-diseased gingival tissues, which are then expanded in cell population. The
cells are seeded onto a natural or synthetic scaffold for further culturing and maturation. The resulting tissue-engineered construct can be transplanted back into the affected area to replace damaged tissues. Adapted from Chen et al. (Chen et al., 2010).

2.3.1 Scaffolds for tissue engineering

In the context of gingival tissue regeneration, scaffolds need to provide the structure for cell growth and tissue formation (Chen et al., 2010; Dhandayuthapani et al., 2011; Taba Jr. et al., 2005), elicit minimal inflammatory response, be non-cytotoxic, be porous, and have matching mechanical properties with the natural lamina propria (Dhandayuthapani et al., 2011; Moharamzadeh et al., 2007). Cells have been shown to behave differently on 2D substrates and three-dimensional (3D) scaffolds (Benatti et al., 2007; Bott et al., 2010; Hillmann et al., 1999; Moharamzadeh et al., 2007; Teng et al., 2000). For example, gingival fibroblasts cultured on 2D substrate assembled into a monolayer and generated a lower amount of types III and V collagen when compared with cells cultured on a 3D polyglycolic acid (PGA) scaffold (Hillmann et al., 1999), indicating that 3D cell organization is important for tissue formation. As such, 3D scaffolds are preferred over 2D substrates for tissue engineering. The interconnectivity of pores in porous scaffolds is also essential in tissue engineering for encouraging uniform cell seeding (Benatti et al., 2007) and migration (Murphy et al., 2002). For biodegradable scaffolds, the scaffold structure must remain stable for tissue growth and the rate of degradation should be well-suited for the specific tissue reconstruction (Shue et al., 2012; Taba Jr. et al., 2005).

2.3.1.1 Natural vs. synthetic scaffolds

For natural scaffolds, collagen sponges, gels or matrices are often chosen as scaffold materials for gingival tissue regeneration (Mathes et al., 2010; Mohammadi et al., 2007; Moharamzadeh et al., 2008; Navarro et al., 2001). Human gingival fibroblasts (HGFs) cultured on collagen gels have induced increased amount of gingival tissues after implantation (Mathes et al., 2010; Moharamzadeh et al., 2008; Rouabhia and Allaire, 2010). Collagen-based scaffolds, however, often curl up a few days after cell seeding due to cell contraction and their mechanical stability decreases quickly with biodegradation if the collagen is not cross-linked (Friess, 1998; Hillmann et al., 1999; Ma et al., 2003; Moharamzadeh et al., 2008). Other natural scaffolds including gelatinous (Moharamzadeh et al., 2007; Moharamzadeh et al., 2008; Moscato et al.,
2008; Sachar et al., 2012) and fibrin-based scaffolds (Moharamzadeh et al., 2007; Ye et al., 2000) have been used. For example, the work by Sachar et al. showed that HGFs, which had been cultured in nanofibrous gelatin scaffolds, demonstrated enhanced proliferation near the core of the scaffold after 14 days of culture (Sachar et al., 2012). However, the degradation profile of this material remained a challenge as gelatinous scaffolds generally have a fast degradation rate (e.g. >60 wt% after 4 weeks in vivo (Wang et al., 2008)), which can result in the collapse of the supporting structure before complete tissue reconstruction actually takes place (i.e. months). Similarly, fibrin gels exhibit a fast degradation rate (Ye et al., 2000) and the availability of fibrin in large quantity is limited (Moharamzadeh et al., 2007; Taba Jr. et al., 2005).

Processed acellular cadaver skin scaffolds such as de-epidermized dermis and AlloDerm™ (Izumi et al., 1999; Moharamzadeh et al., 2007) were used in gingival tissue engineering. These scaffolds are often derived from non-host biopsies, and the tissues are denuded of cells using reagents such as sodium dodecyl sulfate (SDS), which leaves the ECM available for host cell attachment as well as providing structural support for host tissue regeneration. Acellular dermis and de-epidermalized dermis were shown to promote epiC proliferation but had limited fibroblast infiltration (Maia. et al., 2011; Moharamzadeh et al., 2007). Recently 3D scaffolds developed from decellularized human palatal gingiva shows the promotion of blastema cell migration and activity (Naderi et al., 2013), however decellularized matrices are often limited by availability and lack of reproducibility. They are also not cost-effective and can have problems with immunogenicity (Moharamzadeh et al., 2007; Moharamzadeh et al., 2008).

Scaffolds made of synthetic polymers are preferred over natural ones because of the reproducibility of synthetic polymers and their versatility in terms of controlling their properties (e.g. elastic modulus, degradation rate, microstructure, etc.) (Benatti et al., 2007; Dhandayuthapani et al., 2011). Among the synthetic scaffolds used for tissue engineering to date, PLA, PLGA, and PGA have been explored the most extensively (Aframian et al., 2002; Blackwood et al., 2008; Buurma et al., 1999; Bäumchen et al., 2009; Dhandayuthapani et al., 2011). These three polymers have been reported as biocompatible material and are FDA-approved (Moharamzadeh et al., 2007; Yang et al., 2001). PLGA (70/30) has been widely used in tissue engineering for constructing vascular grafts (Wen et al., 2007). For gingival tissue engineering, Bäumchen et al. cultured primary HGFs on PGA scaffolds (Bäumchen et al., 2009) while work by Buurma et al. cultured human pulp and gingival fibroblasts onto non-woven PGA
scaffolds (Buurma et al., 1999). Both studies showed that the PGA scaffolds supported the proliferation of HGFs. The degradation by-products of PLA, PGA, PLGA are, however, acidic and often promote an inflammatory response (Yang et al., 2001; Yang et al., 2005). In vitro degradation of PLGA films showed increased concentrations of acidic degradation by-products in the sub-surface regions. These films were implanted into rat skeletal muscles and resulted in the recruitment of neutrophils and monocytes after one week (Pamula and Menaszek, 2008). In vivo study of 3D PLGA scaffolds also showed that it excluded cells and tissue infiltration over 60 days and the scaffolds lost their structural integrity after this period (McBane et al., 2011c).

Other biodegradable, synthetic polymers such as polycaprolactone have been used in tissue engineering. Porous polycaprolactone scaffolds can enhance chondrocyte proliferation and cartilaginous ECM production (Meretoja et al., 2012). HGFs transduced with BMP-7 were seeded in porous polycaprolactone scaffolds and after subcutaneous implantation, the HGFs promoted bone regeneration (Williams et al., 2005). Scaffolds made of a combination of natural and synthetic polymers have also been investigated in regenerating the connective tissues (Moscato et al., 2008).

The availability of elastomeric polymers such as degradable polyurethane (PU) has several appealing physical properties for soft connective tissue regeneration where biomechanical forces may play an important role in determining tissue structure (Sharifpoor et al., 2011). The impact of the elastic modulus of the material is an important consideration for tissue engineering designs (Galie et al., 2012; Hinz, 2007; Liu et al., 2010; Tomasek et al., 2002). Many degradable aliphatic PUs make up a promising class of materials for tissue engineering applications because of their high mechanical compliance, their ability to undergo biodegradation by enzymes, and the potential for formulating the material in a manner that they can generate non-cytotoxic products (Gogolewski, 1989; Saxena, 2008; Tang et al., 2001; Tanzi et al., 1997; Xue and Greisler, 2003; Yang et al., 2005; Yang et al., 2009). A detailed description of PUs is illustrated in the next section (section 2.3.1.2).

2.3.1.2 Polyurethane (PU)

PUs are block copolymers that contain hard and soft segments (Ratner et al., 1996). The hard segments, which are composed of diisocyanate and a chain extender, have a glass transition temperature ($T_g$) above the room temperature. 2,4-toluene diisocyanate (TDI) and methylene
di(4-phenyl isocyanate) (MDI) (Ratner et al., 1996) are commonly used diisocyanates in synthesizing PU. However, after the synthesis or degradation of PU with the latter reagents, residual TDI and MDI are reported to be present, and have shown to be toxic and in some studies, reported to yield potential carcinogenic risks (Bolognesi et al., 2001). Hence other types of diisocyanates are used for tissue engineering applications. In a recent study by Hofmann et al., hexamethylene-1,6-diisocyanate (HDI) was used to synthesize PU scaffolds to form a bone substitute and the scaffold supported angiogenesis (Hofmann et al., 2008). The soft segments, which are composed of polyether, polyester or polycarbonate polyols, have T_g’s that are much lower than room temperature (Ratner et al., 1996).

PUs are tough elastomers that have good fatigue properties and blood-compatibility (Ratner et al., 1996). Their physical properties can be easily tailored by modifying the chemical structure (Hofmann et al., 2008; Laschke et al., 2009). Aliphatic PUs allow cells for attachment (Lee, 1996), proliferation (Lee, 1996), and ECM secretion (Grad et al., 2003). Previous studies showed that PU supported cell adhesion and the proliferation of human osteoblasts (Hofmann et al., 2008) and 3T3 mouse fibroblast cell line (Meng et al., 2009). PUs also promote the growth of ECs and the formation of neo-intima; thus PUs are often used for the replacement of vascular structures (Hofmann et al., 2008). In addition, PUs are used in pacemaker lead insulation and artificial heart bladders (De Voogt, 1999; Ratner et al., 1996). A recent study also investigated the use of PUs to form interpenetrating phase composites for the fixation of damaged bone (Yang et al., 2005).

PUs can be degraded by hydrolysis, oxidation or enzymes. Polyester urethane can be degraded by hydrolysis (Lambda et al., 1998; Ratner et al., 1996). Polyether urethane is more resistant to hydrolysis but it is susceptible to oxidation (Lambda et al., 1998; Ratner et al., 1996). PUs can be degraded by enzymes such as papain and esterase (Ratner et al., 1996). The biodegradation of PU by enzymes may be tailored to generate non-cytotoxic products (Yang et al., 2009).

Previous studies have shown that a degradable/polar/hydrophobic/ionic PU (D-PHI) hydrogel is non-cytotoxic and show good wettability (Sharifpoor et al., 2009). The use of the D-PHI scaffolds under cyclic mechanical strain promoted cell growth and a more contractile phenotype for human coronary artery smooth muscle cells (Sharifpoor et al., 2011). When co-cultured with monocytes in D-PHI scaffolds, the migration and distribution of smooth muscle cells in the
scaffold was enhanced and the amount of inflammatory cytokines was reduced (McBane et al., 2011a). D-PHI scaffolds have been shown to induce a low inflammatory, high wound-healing phenotype in monocytes isolated from human whole blood (McBane et al., 2011b) and displayed good biocompatibility in vivo (McBane et al., 2011c). Experiments using a co-culture of monocytes and ECs in vitro in D-PHI scaffolds showed enhanced EC proliferation and a subcutaneous mouse model demonstrated increased tissue in-growth and micro-vessel formation (McDonald et al., 2011).

D-PHI scaffolds are synthesized from polyhexamethylene carbonate diol (PCN) and lysine diisocyanate (LDI). 2-hydroxyethylmethacrylate (HEMA) is incorporated to form a divinyl oligomer (Sharifpoor et al., 2009;Sharifpoor et al., 2011). The biodegradation of D-PHI occurs by hydrolysis of the carbonate and urethane groups (Yang et al., 2005) and dissociation of the ionic containing components. The degradation byproducts of D-PHI include lysine, linear hydrocarbon chains and carbon dioxide, which are all non-cytotoxic and can be cleared easily by the human body (Sharifpoor et al., 2009). The degradation rate of D-PHI in vivo has been reported to be 21 wt% over 100 days in rats and the porosity had increased to 87% in a retained structure (McBane et al., 2011c).

2.3.2 Cell types for gingival tissue engineering

The regeneration of the gingival connective tissues has involved the culturing of fibroblasts in vitro on natural or synthetic scaffolds. Typically, fibroblasts can be harvested from the gingiva (Chung et al., 2009;Jin et al., 2012;Ma et al., 2011;Moscato et al., 2008), the periodontal ligament (Giannopoulou and Cimasoni, 1996;Lekic and Mcculloch, 1996), and the dental pulp (Buurma et al., 1999).

Various commercially available products can now be used to model the oral mucosa. They include EpiOral™ and EpiGingival™ developed by MatTek (Ashland, MA, USA) (Moharamzadeh et al., 2007) and GINTUIT™ from Organogenesis (Iwata et al., 2014). However, these products are monolayers of cells, which are fragile and difficult to handle, and they are costly (Iwata et al., 2014;Moharamzadeh et al., 2007). The drawbacks of these commercial products prompt researchers to explore the use of tissue-engineered constructs for gingival tissue regeneration.
HGFs have been commonly used to test the biocompatibility of dental implants (Jin et al., 2012; Ma et al., 2011), soft tissue constructs (Chung et al., 2009; Moscato et al., 2008), and bone substitutes (Ruediger et al., 2012), due in part to their anatomical location in the gingival tissue. Some studies have been carried out to investigate the use of HGFs for populating in vitro models of gingival lamina propria (Blackwood et al., 2008; Hillmann et al., 1999; Mathes et al., 2010; Mohammadi et al., 2007; Moharamzadeh et al., 2007; Moharamzadeh et al., 2008; Moscato et al., 2008; Mussig et al., 2008; Sachar et al., 2012; Yamada et al., 2006). HGFs have been shown to produce a significantly greater amount of ECM in 3D scaffolds (Hillmann et al., 1999; Moharamzadeh et al., 2007). Under static conditions (i.e. no mechanical stimulation), HGFs are often stretched out extensively and form a cell layer only on the material’s surface with increasing collagen production (Hillmann et al., 1999; Mussig et al., 2008). As such, the infiltration of HGFs into the 3D scaffolds is essential in gingival tissue regeneration and this may require changes in cell seeding or culture methods such as the use of perfusion bioreactors.

In addition to HGFs, stem cells have also been used in gingival tissue engineering and they include periodontal ligament-derived stem cells, bone marrow-derived mesenchymal stem cells, adipose-derived stem cells, and undifferentiated ectomesenchymal cells (Iwata et al., 2014; Lin et al., 2008; Taba Jr. et al., 2005).

After implantation, the fibroblasts that were incorporated in the tissue-engineered construct are expected to remain viable (Barker et al., 2013) and maintain their fibroblastic phenotype (i.e. continue to deposit ECM (Auger et al., 2004; Guerreiro et al., 2012)). The construct’s fibroblast population is anticipated to produce cytokines (e.g. connective tissue growth factor) and deposit ECM (e.g. Col I) that may recruit host ECs and fibroblasts for vascularization and tissue regeneration (Auger et al., 2004; Barker et al., 2013; Cooper et al., 1991; Guerreiro et al., 2012). The construct’s fibroblasts are expected to be slowly replaced by the host fibroblasts as the donor fibroblasts apoptose or are engulfed by host macrophages (Barker et al., 2013). For example, in the study of Barker et al., the donor fibroblasts (pre-seeded, GFP-labelled) in the polycaprolactone/collagen scaffolds remained viable up to 14 days after being implanted in Sprague-Dawley rats (Barker et al., 2013). The donor fibroblasts have been shown to participate in tissue deposition. The fibroblast-seeded scaffold induced rapid vascularization, which was detected at 4 weeks after implant. Since the animals were not immunodeficient, the donor
fibroblasts were phagocytosed by host macrophages at 4 and 8 weeks after implant and they were eventually replaced by host fibroblasts after 12 weeks.

As for the ECs, they are expected to form functional capillary networks in the construct since they are likely to be exposed to more cytokines and ECM from host tissues in vivo (Song et al., 2015). The pre-formed capillary networks in the construct have been shown to recruit host ECs and induce inosculation in vivo, thereby improving vascularization in the scaffold construct (Song et al., 2015; Tremblay et al., 2005; White et al., 2014). For example, in the study of Tremblay et al., a collagen sponge was pre-vascularized with a tri-culture of dermal fibroblasts, keratinocytes and endothelial cells in vitro. The pre-vascularized construct was then implanted in nude mice. After 4 days, inosculation of construct’s capillaries with the host’s vasculature was observed (Tremblay et al., 2005).

With regards to the role of D-PHI scaffolds in supporting the survival, growth and phenotype of fibroblasts and ECs, D-PHI scaffolds have been shown to be non-cytotoxic and having good wettability (Sharifpoor et al., 2009). Methacrylic acid and methyl methacrylate were introduced to D-PHI, which generate anionic function that favours cell attachment. As stated in the previous section, D-PHI demonstrates good biocompatibility in vivo and its degradation supported tissue in-growth (McBane et al., 2011c). Based on these unique and favourable properties of D-PHI, it is anticipated to support the survival and growth of fibroblasts and ECs after implantation. D-PHI has been shown to support capillary formation in a subcutaneous mouse model (McDonald et al., 2011) and it can induce a low inflammatory, high wound-healing phenotype in monocytes (McBane et al., 2011a; McBane et al., 2011b); therefore it is highly anticipated that D-PHI can direct fibroblast’s phenotype toward a pro-wound-healing state after implantation.

2.4 Medium perfusion

For efficient transport of nutrients and wastes in a tissue-engineered construct, the scaffold should be perfused with culture medium and a dynamic flow system is preferred (Du et al., 2008; Navarro et al., 2001; Timmins et al., 2007; Zhao et al., 2009). Medium perfusion has been shown to increase cell proliferation in the tissue-engineered scaffolds (Blackwood et al., 2008; Hillmann et al., 1999; Mohammadi et al., 2007; Moharamzadeh et al., 2007; Moharamzadeh
et al., 2008; Moscato et al., 2008; Mussig et al., 2008; Navarro et al., 2001; Sachar et al., 2012; Yamada et al., 2006). In a study carried out by Navarro et al., with medium perfusion at 1.3 mL/min over a 14-day culture period, the population of human oral keratinocytes cultured in a perfused scaffold made of collagen and chondroitin sulfate showed 88% more growth than that in the non-perfused scaffolds (Navarro et al., 2001). Perfused scaffolds have also been used to increase the density of human mesenchymal stem cells in a 35-day culture (Zhao et al., 2009), which resulted in an increase in the cells’ osteogenic ability, as shown by the greater expression of osteonectin. Moreover, medium perfusion can enhance growth factor production such as TGF-β1 (Galie et al., 2012) and collagen production (Ng et al., 2005), suggesting that medium perfusion is beneficial for cell culture.

Medium perfusion also provides mechanical stimuli for cells (e.g. myofibroblast differentiation under low levels of flow (Ng et al., 2005), and enhanced release of PDGF, FGF-2 (Sterpetti et al., 1994) and nitric oxide (Mcintire et al., 1998) from smooth muscle cells). Flow-induced shear stress can increase the amount of cell-cell contacts in the ECM (Kong and Vazquez, 2009). In particular, flow-induced shear stress can affect EC behaviour and this will be outlined in the next section (section 2.4.1).

Perfusion bioreactors can be used to allow medium flow through the tissue-engineered constructs, thereby providing mechanical stimulus, improving metabolic exchange as well as promoting cell growth and protein production. The design of perfusion bioreactors will be reviewed in section 2.4.2.

2.4.1 Effect of shear stress on ECs

ECs respond to shear stress from blood flow for maintaining homeostasis of the circulatory system via angiogenesis and vascular remodelling (Ando and Yamamoto, 2009; Davies, 1995; Shi and Tarbell, 2011). Abnormal EC response to shear stress in turbulent or stagnant flow may lead to vascular diseases such as atherosclerosis (Ando and Yamamoto, 2009; Davies, 1995; Shi and Tarbell, 2011). Thus the type and intensity of shear stress can change the morphology, function and gene expression in ECs. In large arteries, the mean wall shear stress is approximately 20 – 40 dyn/cm² (Davies, 1995); and in capillaries where flow is laminar, the shear stress is typically 4 dyn/cm² (Shaik et al., 2009).
Shear stress can be mechanically transduced to ECs via ion channels (e.g. Ca\(^{2+}\) channel), G proteins, tyrosine kinase receptors (e.g. VEGF receptor), adhesive proteins (e.g. integrins), caveolae, rearrangement of cytoskeleton, conformational change in glycocalyx, and primary cilia (Ando and Yamamoto, 2009; Davies, 1995). In terms of adhesive proteins, shear stress activates integrins, which leads to the activation of various FA components such as FAK (Ando and Yamamoto, 2009; Davies, 1995). Upon exposure to laminar shear stress, ECs increase nitric oxide production for vascular remodelling (Ando and Yamamoto, 2009; Davies, 1995). The levels of anti-thrombotic proteins such as thrombomodulin and tissue-type plasminogen activator were shown to increase under a shear stress of 15 dyn/cm\(^2\) (Ando and Yamamoto, 2009). Enhanced production and phosphorylation of CD31 has been reported to occur within 30 seconds after exposure to shear stress, leading to the activation of ERK and EC proliferation; while the levels of V-CAM-1 and endothelin were decreased (Ando and Yamamoto, 2009). Shear stress also induces the production of various growth factors such as PDGF, FGF-2, TGF-β, IL-1, and IL-6 in ECs (Ando and Yamamoto, 2009; Davies, 1995). Laminar shear stress can inactivate reactive oxygen species (molecules that mediate vascular diseases and reperfusion injury) (Ando and Yamamoto, 2009).

In addition, studies have reported that shear stress promotes the differentiation of endothelial progenitor cells into mature ECs, thereby inducing angiogenesis (Ando and Yamamoto, 2009).

### 2.4.2 Perfusion bioreactors

For effective nutrient delivery and cell culture, many types of bioreactors such as the spinner flasks and rotating bioreactors have been developed. Rotating bioreactors contain cylindrical chambers where the cells or cell-seeded constructs are distributed and the culture medium is delivered to the cells or constructs via stirring (Gaspar et al., 2012). However, uniform mixing of medium in rotating bioreactors remains a challenge (Gaspar et al., 2012).

Alternatively, perfusion bioreactors are designed to facilitate uniform medium perfusion with controllable shear stress. In terms of perfusion bioreactor design, the simplest designs utilize a parallel circuit of perfusion chambers, where tissue-engineered constructs sit in parallel chambers, and a peristaltic pump is used for pumping the medium continuously or non-continuously from one or more medium reservoirs (Cho et al., 2008; Gaspar et al., 2012; Navarro et al., 2001; Raimondi et al., 2008; Silva et al., 2014; Zhao et al., 2009) (Fig. 2.8). The tissue-
engineered constructs often sit in separate, parallel channels in the chamber (Cho et al., 2008; Navarro et al., 2001; Raimondi et al., 2008; Silva et al., 2014), but they can also be placed in a series in one channel (Zhao et al., 2009). For example, a study by Raimondi et al. designed the perfusion chamber with three parallel channels and each channel is designated for one scaffold (Raimondi et al., 2008). Similarly, the work of Cho et al. placed only one scaffold per flow chamber; and two or more chambers are arranged in a parallel circuit with the peristaltic pump (Cho et al., 2008). It should be noted that the scaffolds used for perfusion bioreactors must be highly porous (i.e. 70 – 99%) with interconnected pores for directing medium perfusion (Gaspar et al., 2012).

**Figure 2.8.** The design of parallel circuit perfusion bioreactors generally consists of a peristaltic pump, gas exchange, parallel perfusion chambers that contains one or more tissue-engineered constructs, and medium reservoirs.

For the choice of flow rates, there is no consensus on the magnitude but the flow rate should match that in the physiological condition or disease model. For bone regeneration, the flow rates are typically low (0.01 – 1 mL/min) for enhancing cell growth, attachment and mineralization (Gaspar et al., 2012; Seitz et al., 2007; Silva et al., 2014). For example, the study by Seitz et al. used 0.0181 mL/min to cultivate human mesenchymal stem cells for 14 days such that they could differentiate toward osteoblastic cells (Seitz et al., 2007). Similarly, at 0.1 mL/min, adipose-derived stem cells showed osteogenic differentiation and increased proliferation when cultured in bioactive glass foam in a perfusion bioreactor (Silva et al., 2014). For vascular tissue regeneration, the flow rate is dependent on the size of the blood vessels (e.g. high flow rate for arteries vs. low flow rate for capillaries and veins) (Mcintire et al., 1998). For example, for
inducing angiogenesis, ECs and mesenchymal stem cells were co-cultured in 3D collagen gels under perfusion (5 mL/min) to allow for vascularization (Lee and Niklason, 2010). Alternatively, ECs cultured in thin collagen gels were assembled into cell sheets and a flow rate of 0.5 mL/min was used to promote EC migration and capillary formation (Sakaguchi et al., 2013). In terms of regenerating oral soft tissues, Mathes et al. used a flow rate of 0.6 mL/min to perfuse culture medium continuously through HGF-seeded collagen sponges (Mathes et al., 2010). Consequently, there should be an optimized range of flow rates for culturing a specific cell type or to promote specific biological events such as angiogenesis or differentiation.

Aside from the parallel arrangement of perfusion chambers, other styles of perfusion bioreactors have been designed. For example, disc-shaped bioreactors are developed in a way such that medium flow is inputted from the top and then dispersed radially to the cells (Malek et al., 1993; Ng et al., 2005). In tissue engineering of vascular grafts and trachea, where cells are seeded in a tubular scaffold, perfusion bioreactors often adopt a tubular design (Diamantouros et al., 2013; Tan et al., 2007; Touroo et al., 2013).

2.5 Angiogenesis in tissue-engineered constructs

Cell viability and optimal function of tissue-engineered constructs cannot be sustained via diffusion alone. A previous study showed that the diffusion of nutrients and oxygen from surrounding tissues is limited to only 150 sq. microns (Awwad et al., 1986). Blood vessels facilitate the transport of nutrients and oxygen via the vascular structure, which promotes cell migration, proliferation, differentiation and ECM production; as well as the delivery of inflammatory cells to defend the host against pathogens (Taba Jr. et al., 2005). The ingrowth of blood vessels has been shown to proceed very slowly after the tissue-engineered construct was implanted into the host, which resulted in necrosis in the centre of the construct. Gingival tissues are known to be highly vascular for facilitating transport of nutrients and metabolites (Moharamzadeh et al., 2007), as such, angiogenesis in the tissue-engineered gingiva is essential.

Angiogenesis is the formation of new blood vessels from ECs (Bae et al., 2012; Cross and Claesson-Welsh, 2001; Tae et al., 2007). It is considered as one of the most important requirements for the transport of oxygen and nutrients in a 3D scaffold (Hofmann et al., 2008). Angiogenesis has two phases: activation and resolution. It depends on the interactions between
cells and the ECM, which can be regulated by growth factors, transmembrane proteins and extracellular proteinases and their inhibitors (Ferrari et al., 2009).

The activation phase of angiogenesis can be determined by a number of characteristic events including an increase in vascular permeability, disassembly of the vessel wall, degradation of the basement membrane, cell migration, EC proliferation and lumen formation (Pepper, 2001). The activation phase is controlled positively by angiogenic factors (growth factors that favour angiogenesis) such as VEGF, acidic fibroblast growth factor (FGF-1) and FGF-2, TGF-β1, PDGF, hepatocyte growth factor, tumour necrosis factor-α (TNF-α), angiogenin and IL-8 (Cornelini et al., 2003; Ferrari et al., 2009; Folkman and Shing, 1992; Hughes, 2008; Smith and Martinez, 2006; Taba Jr. et al., 2005; Yamada et al., 2006; Yancopoulos et al., 2000).

The resolution phase of angiogenesis can be characterized by the inhibition of EC proliferation and migration, the reassembly of the vessel wall, and the reconstitution of the basement membrane, all of which are maintained by the dominance of negative angiogenic regulators (Pepper, 2001) such as TGF-β1 (Ferrari et al., 2009; Mallet et al., 2006), vWF (Lenting et al., 2012), IFN-α, platelet factor IV and IL-12 (Pepper, 1997). The balance between the positive and negative regulators is the key to achieve angiogenesis.

Angiogenesis in the tissue-engineered construct is a major challenge in the regeneration of oral mucosal tissues (Taba Jr. et al., 2005); and it partly depends on the introduction of angiogenic factors to the tissue-engineered construct, which could typically be released by exogenous delivery such as the use of synthetic polymeric scaffolds (Bae et al., 2012; Zisch et al., 2003) (section 2.5.2) or from cells that are co-cultured with ECs (Bae et al., 2012; Cornelini et al., 2003; Hofmann et al., 2008; Hughes, 2008; Smith and Martinez, 2006; Sukmana and Vermette, 2010; Taba Jr. et al., 2005; Yamada et al., 2006) (section 2.5.3). The quick establishment of the vascular network in the tissue-engineered construct and subsequent functional sustainability (e.g. ability to withstand shear stress and adequate burst strength (Taba Jr. et al., 2005)) in vivo remain to be addressed.

2.5.1 Angiogenic factors

Angiogenic factors are growth factors that promote angiogenesis. Some of the angiogenic factors that are commonly studied include VEGF, TGF-β1, FGF-2 and PDGF-B (Cornelini et al.,
2003; Ferrari et al., 2009; Hughes, 2008; Smith and Martinez, 2006; Taba Jr. et al., 2005; Yamada et al., 2006). In this thesis, VEGF, TGF-β1 and FGF-2 will be studied.

2.5.1.1 VEGF

VEGF plays a critical role in angiogenesis as it stimulates EC proliferation and migration (Chu et al., 2004; Kirkpatrick et al., 2011; Li et al., 2011), as well as protecting them from apoptosis (Ferrari et al., 2009; Koch et al., 2011), which is essential for angiogenesis. VEGF can be produced by cells such as fibroblasts and ECs themselves; and it promotes the sprouting of blood vessels (Bae et al., 2012; Liu et al., 2009). During embryogenesis, VEGF controls the development of vasculature (Koch et al., 2011). It has been shown to activate various kinase receptors such as VEGF-R1, which is needed for chemotaxis and inducing vascular permeability; and VEGF-R2, which is needed for EC proliferation and survival (Ferrari et al., 2009; Koch et al., 2011). VEGF has been reported to initiate the expression of proteolytic enzymes, which is needed for the proteolysis associated with the activation phase of angiogenesis (Pepper, 2001). VEGF can also induce vascular leakage, which is required for angiogenesis (Ferrara et al., 2003) and for inflammation and tumour development (Koch et al., 2011).

2.5.1.2 TGF-β1

Aside from its role in maintaining tissue homeostasis and myofibroblast differentiation, TGF-β1 is also an angiogenic factor. TGF-β1 can be produced by cells such as fibroblasts and ECs themselves; and it stabilizes the newly formed vasculature by inducing apoptosis in ECs (Ferrari et al., 2009; Liu et al., 2009) and inhibiting angiogenic sprouting (Mallet et al., 2006), thereby allowing maturation of nascent blood vessels (Bae et al., 2012; Dohle et al., 2011; Folkman and D'Amore, 1996; Iruela-Arispe and Dvorak, 1997). TGF-β has been shown to bind to TGF-β type II receptor, which then can recruit and phosphorylates activin-like kinase receptors to regulate EC proliferation and migration (Liu et al., 2009).

2.5.1.3 FGF-2

Aside from its antagonistic effect in myofibroblast differentiation, FGF-2 has been shown to promote cell proliferation and migration as well as angiogenesis (Cao et al., 2008; Dvorak et al., 2006; Lyons et al., 1991; Montesano et al., 1986; Taba Jr. et al., 2005) by stimulating EC survival, proliferation, and migration (Cross and Claesson-Welsh, 2001); as well as by regulating the
development of vascular network (Bae et al., 2012). FGF-2 is one of the twenty-three FGFs (Więdłocha and Sørensen, 2004), and two of the FGF receptors – FGFR-1 and FGFR-2 – are important for angiogenesis (Cross and Claesson-Welsh, 2001). FGF-2 has been shown to regulate angiogenesis through signalling pathways such as Ras-MEK-MAPK, Src family tyrosine kinases, PI3K, and the PLC pathway (Cross and Claesson-Welsh, 2001). In addition, FGF-2 can upregulate laminin in periodontal ligament cells, a protein that plays an important role in angiogenesis (Taba Jr. et al., 2005).

Studies reported that FGF-2 lacks cytoplasmic sequences for extracellular export, indicating that they need biological carriers or physical stimuli for secretion (Cross and Claesson-Welsh, 2001; Sterpetti et al., 1994). FGF-2 is also characterized to have a high affinity for heparan sulfate proteoglycans, which allows them to be stored in the ECM and be released in a regulated manner (Cross and Claesson-Welsh, 2001; Sterpetti et al., 1994).

2.5.1.4 Interplay between VEGF, TGF-β1, and FGF-2

VEGF production can be regulated by growth factors such as TGF-β1 (Asano-Kato et al., 2005), FGF-2 (Ferrari et al., 2009), IL-17 (Honorati et al., 2006), TNF-α (Nasu et al., 2007) and other factors (Ferrara et al., 2003). TGF-β1 and FGF-2 have been reported to induce VEGF expression in ECs (Fig. 2.9) (Ferrari et al., 2009). VEGF expression is tightly regulated by activin-like kinase receptors 1 and 5 in ECs (Shao et al., 2009), which are also receptors for TGF-β. Despite the regulation of VEGF production by TGF-β1 in ECs, VEGF has been shown to control TGF-β1 induction of capillary morphogenesis in vitro (Ferrari et al., 2009).

Moreover, VEGF and FGF-2 have been shown to have synergistic effects on angiogenesis, as their simultaneous presence has resulted in more rapid tubule formation in vitro and in vivo (Asahara et al., 1995; Davis et al., 2002; Goto et al., 1993; Pepper et al., 1992; Taba Jr. et al., 2005).
2.5.2 Delivery of angiogenic factors using scaffolds

Due to the instability of angiogenic factors when they are administered exogenously in vivo (Baiguera and Ribatti, 2013; Taba Jr. et al., 2005), they are commonly incorporated in a delivery vehicle such as polymeric scaffolds or microspheres. Functionalization of scaffolds with angiogenic factors has been shown to promote sprouting of capillaries within the tissue-engineered constructs. For example, PLGA scaffolds had been incorporated with VEGF to allow for slow release to the ECs in the scaffolds over 15 days of culture, which resulted in enhanced vascularization (Taba Jr. et al., 2005). Similarly, FGF-2 was embedded into PLGA microspheres, which are incorporated into porous alginate scaffolds for slow release (Perets et al., 2003). The FGF-2-releasing scaffold resulted in four-times more penetrating capillaries than the control scaffolds in vivo. Angiogenic factors can also be delivered directly to the cells using perfusion. For example, exogenous VEGF was pumped through a tissue-engineered construct using a perfusion system (Tan et al., 2007). Despite the success of the exogenous delivery of angiogenic factors in angiogenesis, it is not cost-effective (e.g. $346.50 CAD per 10 ug VEGF (Sigma-Aldrich, U.S.A.)), and it often requires high amount of angiogenic factors (e.g. infusion of 0.3 – 30 µg FGF-2 per kg body mass of patient (Simons et al., 2002)). The release of angiogenic
factors from scaffolds would also need to consider the effect of burst release and the effect of scaffold degradation on the release profile (Baiguera and Ribatti, 2013); as well as the inability to direct vessel growth (Bae et al., 2012).

2.5.3 Co-culture of ECs and fibroblasts

Many studies were conducted to investigate the choice of cell type(s) for inducing angiogenesis. In physiological conditions, ECs interact with one or more cell types in order to survive and proliferate (Battiston et al., 2014). ECs in monoculture fail to self-assemble into tube-like structures (Hashimoto and Kuroyanagi, 2008). For example, Hofmann et al. showed that human umbilical vein ECs (HUVECs) died rapidly after one week in monoculture, while the co-culture of ECs with human osteoblasts induced HUVEC maturation and promoted type IV collagen expression, suggesting the need of co-cultures for vascularization (Hofmann et al., 2008).

ECs have been co-cultured with various cell types such as smooth muscle cells (Battiston et al., 2014; Rose and Babensee, 2007; Shum-Tim et al., 1999; Taba Jr. et al., 2005), monocytes (Battiston et al., 2014; McDonald et al., 2011; Schubert et al., 2008), osteoblasts (Battiston et al., 2014; Shah et al., 2011), epiCs (Bae et al., 2012; Baiguera and Ribatti, 2013), fibroblasts (Bae et al., 2012; Battiston et al., 2014; Co et al., 2005; Hughes, 2008; Sorrell et al., 2007; Sukmana and Vermette, 2010), etc. to induce angiogenesis. These cell types have been shown to support angiogenesis via paracrine signalling or cell-cell contact (Battiston et al., 2014).

Fibroblasts in general provide an angiogenic response (Hughes, 2008; Sukmana and Vermette, 2010). They deposit ECM for tubulogenesis (Bae et al., 2012; Davis et al., 2002; Sorrell et al., 2007) and regulate EC activation and proliferation by secretion of various factors such as VEGF (Bae et al., 2012; Hughes, 2008; Yamada et al., 2006), TGF-β1 (Bae et al., 2012; Cornelini et al., 2003; Hughes, 2008; Smith and Martínez, 2006), FGF-2 (Taba Jr. et al., 2005), nitric oxide (Li and Chang, 2013), etc. In fact, the earliest studies in angiogenesis involved the co-culture of ECs with dermal fibroblasts for promoting vascularization in the skin (Battiston et al., 2014; Bishop et al., 1999). Hence the interaction between fibroblasts and ECs is important in angiogenesis.

The ECM is important for angiogenesis in that it mediates mechanical transduction to ECs with integrins such as β1-integrins and αvβ3 integrin (Davis et al., 2002), and it provides a reservoir
for angiogenic factors such as TGF-β1 and FGF-2 (Blumbach et al., 2010; Cross and Claesson-Welsh, 2001; Hinz, 2007; Sterpetti et al., 1994; Wipff and Hinz, 2009). The remodelling of ECM can also regulate angiogenesis by exposing certain peptides or releasing previously stored growth factors to the ECs (Sottile, 2004). Types I and III collagen have been shown to promote EC tube formation and stabilize blood vessels (Sottile, 2004); and ECs cultured on collagen-rich ECM can develop into capillaries in 48 hours (Davis et al., 2002). As fibroblasts mainly produce collagen (particularly type I collagen for HGFs) in addition to the angiogenic factors, this may well explain the enhanced angiogenic effects in EC-fibroblast co-cultures. For example, Guillemette et al. demonstrated that ECs cultured on a fibroblast-cell sheet resulted in the formation of capillary-like structures after 7-days (Guillemette et al., 2010). Moreover, by seeding HUVECs and dermal microvascular ECs onto confluent layers of fibroblasts, the “lawn” of fibroblasts provided collagen-rich ECM, which promoted ECs to form tube-like structures that are stable for up to five weeks (Sorrell et al., 2007).

EC-fibroblast co-cultures are more effective in providing ECs with angiogenic factors in a localized, regulated manner. A study by Sukmana et al. demonstrated that the EC-fibroblast co-culture was more effective in promoting EC tubulogenesis than the exogenous delivery of VEGF (2 ng/mL) or FGF-2 (20 ng/mL) to ECs (Sukmana and Vermette, 2010). Fibroblasts are capable of releasing multiple angiogenic factors to ECs as discussed above, and the matrix proteins derived from fibroblasts can have a synergistic angiogenic effect with the angiogenic factors (Newman et al., 2011). For example, Newman et al. showed that fibroblasts, when co-cultured with HUVECs, are capable of releasing 5-10 pg/mL of VEGF. However, when the same range of VEGF concentrations was delivered to the EC monoculture (i.e. in the absence of fibroblasts), ECs failed to sprout and form lumen-like structures (Newman et al., 2011). In addition, the cell-cell contacts between fibroblasts and ECs can induce angiogenesis (Baiguera and Ribatti, 2013), which further suggests the important presence and multi-factorial effect of fibroblasts in promoting vessel formation.

Even though many studies have shown some success in establishing vascularized structures in co-cultures on synthetic materials such as PLGA (Kaully et al., 2009), the culture conditions such as the choice of culture medium and cell seeding ratios are not defined. A study with human mesenchymal stem cells and HUVECs showed that different ratios of osteogenic supplements and endothelial growth factors in the culture medium can have different effects on
angiogenesis and cell proliferation (Correia et al., 2011). The work of Choong et al. used a 1:1 mixture of fibroblast medium and EC medium for the EC-fibroblast co-culture (Choong et al., 2006) while the study of Salem et al. only used the fibroblast medium (Dulbecco’s modified Eagle medium) (Salem et al., 2002).

A recent study has shown that in a 3D spheroid structure, an increase in EC proliferation and vessel-like formation was observed at EC-fibroblast ratios of 1:40 and 1:4 (i.e. more fibroblasts than ECs) (Kunz-Schughart et al., 2006). However, if there were more ECs than fibroblasts in the spheroid, a significant loss of the EC population was observed as early as 3 days of culture. Ma et al. also investigated the effect of cell seeding ratio on angiogenesis and bone mineralization, and their work showed that highest mineralization and moderate angiogenesis were observed at a 1:1 ratio of osteoprogenitor cells and ECs (Ma et al., 2011). When there were more HUVECs than osteoprogenitor cells, cell viability in the co-culture was low, indicating that the percentage of ECs in the co-culture should be kept below 50% to optimize cell viability. Consequently, there is no consensus in the literature on the optimal cell culture medium or cell seeding ratio in co-cultures for supporting cell proliferation and capillary formation.

Aside from co-culturing ECs with fibroblasts, endothelial progenitor cells (EPCs) have also been used in monocultures or co-cultures to promote vascularization (Aguirre et al., 2010; Kobayashi et al., 2008; Loibl et al., 2014; Suuronen et al., 2009). For example, a study showed that fibroblast sheets co-cultured with EPCs promoted angiogenesis and subsequently improved cardiac function of infarcted hearts (Kobayashi et al., 2008). The work of Suuronen et al. looked at methods for recruiting EPCs and culturing them in collagen-based scaffolds to promote vascularization in ischemic tissues (Suuronen et al., 2009). Lastly, EPCs co-cultured with mesenchymal stem cells can induce angiogenesis via paracrine signalling and direct cell-cell contact (Aguirre et al., 2010; Loibl et al., 2014).

2.6 Summary

Gingival tissue engineering represents a promising method for regenerating the lamina propria. A degradable PU hydrogel (D-PHI) has been shown to support the culture of various cell types in vitro and its degradation in vivo demonstrates that it is a suitable material for tissue growth. This thesis will assess the biocompatibility of D-PHI scaffolds with HGFs for gingival tissue
regeneration based on cell viability, proliferation and collagen production. As gingival lamina propria is highly vascular, a perfusion bioreactor will be designed to facilitate medium perfusion through the HGF-seeded scaffolds and to promote vessel formation in the co-culture system. The influence of the D-PHI material and the perfusion of medium on myofibroblast differentiation will be explored, which will provide new insights into the signalling pathways that are associated with the regulation of myofibroblast differentiation in a perfused system with D-PHI. ECs require shear stress for survival and proliferation, and the presence of fibroblasts in the co-culture with ECs is important for angiogenesis. By developing a perfused (dynamic) co-culture system with ECs and HGFs, this study will also investigate different co-culturing conditions (e.g. cell density, type of culture medium, cell seeding ratio) that can optimize cell proliferation, collagen production, clustering of ECs and angiogenic factor production, which are all key factors in building a highly perfused tissue-engineered construct for gingival tissue regeneration.
2.7 References


3.1 Foreword

[Gingival atrophy can] cause the breakdown of the tooth-supporting gingival tissue. In treatments aimed at gingival tissue regeneration, tissue engineering is preferred over the common treatments such as scaling. Perfused (dynamic) culture has been shown to increase cell growth in tissue-engineered scaffolds. Since gingival tissues are highly vascularized, it was desired to investigate the influence of perfusion on the function of human gingival fibroblasts (HGFs) when cultured in a degradable polyurethane scaffold (degradable/polar/hydrophobic/ionic polyurethane (D-PHI)) during the early culture phase (4 weeks) of engineering gingival tissues. It was observed that the growth of HGFs was continuous over 28 days in the dynamic culture (3-fold increase, p<0.05) while it was reduced after 14 days in the static culture (i.e. no flow condition). Cell metabolic activity, as measured by a WST-1 assay, and total protein production show that HGFs were in a different metabolic state in the dynamic vs. static culture. Observations from scanning electron microscopy and type I collagen (Col I) production measured by Western blotting suggest that medium perfusion significantly promoted collagen production in HGFs after the first four weeks of culture (p<0.05). The different proliferative and metabolic states for HGFs in the perfused scaffolds suggest a different cell phenotype which may favour tissue regeneration.

This chapter has been published in ‘Acta Biomaterialia’ as: Cheung JWC, Rose EE, Santerre JP. Perfused culture of gingival fibroblasts in a degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold leads to enhanced proliferation and metabolic activity. Acta Biomaterialia 2013; 9(6): 6867-6875. Modifications of text are indicated by “[ ]” and footnote.

Permission to reprint this manuscript has been granted from Elsevier.

*Modification of published text.
3.2 Introduction

[Gingival atrophy is highly prevalent (Albandar and Kingman, 1999; Kassab and Cohen, 2003; Sarfati et al., 2010; Susin et al., 2004) and is defined as the oral exposure of the root surface due to the recession of the gingiva (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Tonetti et al., 2014; Wennström, 1996). Current treatment for gingival atrophy often involves soft connective tissue grafts (Chambrone et al., 2010; Graziani et al., 2014; Harris, 1992; Tonetti et al., 2014; Tözüm et al., 2005). However, tissue grafts are associated with several important limitations such as donor site morbidity and tissue insufficiency (Camargo et al., 2001; Chambrone et al., 2010; Karring et al., 1975; Moharamzadeh et al., 2007; Paolantonio et al., 2002; Rastogi et al., 2009; Tonetti et al., 2014).] In treatments aimed at gingival tissue regeneration, human gingival fibroblasts (HGFs) contribute to the reconstruction of the lamina propria (gingival connective tissues) and mediate epithelial cell morphogenesis (Locke et al., 2008; Moharamzadeh et al., 2007; Mussig et al., 2008). Gingival tissues are known to be highly vascularized for facilitating transport of nutrients and metabolites (Melcher and Bowen, 1969; Moharamzadeh et al., 2007; Schroeder, 1986; Taba Jr. et al., 2005), as such, it would be anticipated that tissue-engineered constructs that are developed under perfusion would develop quicker and retain a more physiologically relevant phenotype than those cultured under static conditions.

HGFs have been commonly used to test the biocompatibility of dental implants (Jin et al., 2012; Ma et al., 2011), soft tissue constructs (Chung et al., 2009), and bone substitutes (Ruediger et al., 2012), due in part to their anatomical location in the gingival tissue. Some studies have been carried out to investigate the reconstruction of the gingival connective tissues (Blackwood et al., 2008; Hillmann et al., 1999; Mohammadi et al., 2007; Moharamzadeh et al., 2007; Moharamzadeh et al., 2008; Moscato et al., 2008; Mussig et al., 2008; Sachar et al., 2012;

*Modification of published text.*
Yamada et al., 2006) but due to the absence of perfused conditions, most cells could not migrate to or remain viable in the core of the constructs. HGFs in these non-perfused cultures were often stretched out extensively and formed a cell layer on the material’s surface with increasing collagen production (Hillmann et al., 1999; Mussig et al., 2008).

Many hydrogel-based scaffolds have been established in order to promote medium transport through the tissue constructs. For example, the work by Sachar et al., showed that HGFs which had been cultured in nanofibrous gelatin scaffolds demonstrated increased growth near the core of the scaffold after 14 days of culture (Sachar et al., 2012); however, the degradation profile of this material remained a challenge as gelatinous scaffolds generally have a fast degradation rate (e.g. >60 wt% after 4 weeks in vivo (Wang et al., 2008)), which can result in the collapse of the supporting structure before complete tissue reconstruction actually takes place (i.e. months). Similarly, studies with collagen gels (Ma et al., 2003) and fibrin gels (Ye et al., 2000) showed that these hydrogels degraded within days of culture, which limited their use in tissue reconstruction.

Previous studies using a degradable/polar/hydrophobic/ionic polyurethane (D-PHI) showed that the material was non-cytotoxic, improved the wettability of the scaffold for enhancing cell seeding (Sharifpoor et al., 2010), inhibited inflammation post-implantation (McBane et al., 2011), and had a degradation rate of 21 wt% in vivo after 100 days, with which the porosity increased to 87% in a retained structure (McBane et al., 2011). Aside from mechanical (Silvani et al., 2009) and electrical stimulation (Chiu et al., 2012) and the use of growth factors (Florczyk et al., 2012), it has been demonstrated that perfusion systems can improve cell proliferation and migration for skeletal and muscular tissue regeneration (Bancroft et al., 2002; Bettahalli et al., 2012; Du et al., 2008; Flaibani et al., 2009; Timmins et al., 2007; Zhao et al., 2009); however, very few studies to date have used perfusion bioreactors with synthetic degradable polymeric biomaterials for the regeneration of periodontal and/or gingival tissues. This study was carried out to assess HGF proliferation and metabolic activity on 2D D-PHI flat films as an acute screening for cell compatibility, and then assess the potential for cell proliferation and migration in a porous scaffold in perfused (dynamic) vs. non-perfused (static) culture. It is hypothesized that HGFs cultured on D-PHI scaffolds in a dynamic culture will proliferate faster and be more metabolically active than in the static culture.
3.3 Materials and methods

3.3.1 2D D-PHI flat film fabrication

D-PHI flat films were generated using a divinyl oligomer (DVO), synthesized in house by reacting polyhexamethylene carbonate diol (Sigma-Aldrich, United States of America) with lysine diisocyanate (Kyowa Yuka, Yokkaichi City, Japan) and 2-hydroxyethyl methacrylate (Sigma-Aldrich) in a 1:2:2 ratio (Sharifpoor et al., 2009). A free radical polymerization between DVO, methacrylic acid (Sigma-Aldrich) and methyl methacrylate (Sigma-Aldrich) in a 1:5:15 molar ratio using benzoyl peroxide (Sigma-Aldrich) as the initiator was carried out under dry conditions. After purging the curing oven with nitrogen, 50 µL of the polymer mixture was added to the wells of a 96-well polypropylene plate. The mixture was left to cure at 110°C for 24 hours.

3.3.2 3D D-PHI scaffold fabrication

D-PHI scaffolds were fabricated using the same free radical polymerization as that used in D-PHI films fabrication except that porogens were added. Poly(ethylene glycol) (Sigma-Aldrich, 10 wt%) and sodium bicarbonate (Sigma-Aldrich, 70 wt%) were used as the porogens, and the pore size of the final scaffolds (Sharifpoor et al., 2009) ranged from 30–250µm. The resulting mixture was molded into discs and cured at 110°C for 24 hours. The scaffolds were subjected to sonication in water for 14 days for porogen leaching. After leaching, the scaffolds were dehydrated by soaking in increasing concentrations of ethanol (30, 50, 70, 90, 95 and 100%) for one hour at each concentration (except 95%, overnight). Finally, the scaffolds were stored at room temperature in a dry environment until needed. Each scaffold had a diameter of 6 mm and a thickness of 2 mm.

3.3.3 Culture of HGF cell line and seeding

A human gingival fibroblast cell line (HGF-1, ATCC CRL-2014) developed by McAllister et al. (McAllister et al., 1993a; McAllister et al., 1993b) was purchased from American Tissue Culture Collection. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, GibcoBRL) containing 4.5g/L glucose, 4mM L-glutamine, 110mg/L sodium pyruvate and 3.7g/L NaHCO₃ supplemented with 100U/mL Penicillin/100μg Streptomycin (Sigma-Aldrich) and 10% fetal
bovine serum (Sigma-Aldrich) in a humidified atmosphere at 37°C with 5% CO₂. Medium was changed every 2 to 3 days.

Prior to seeding, the D-PHI flat films were soaked in 70% ethanol for 24 hours. After drying in the biosafety cabinet, they were conditioned in an incubator with DMEM for 24 hours. In the second phase of the work, 3D D-PHI scaffolds were treated by exposure to 70% ethanol for 24 hours and then dried under sterile laminar flow conditions for an additional 24 hours. The scaffolds were then pre-wetted with DMEM as described above for 24 hours. When cells reached 80-90% confluence, they were trypsinized and seeded onto the pre-conditioned scaffolds. 200 µL of HGF suspension (approximately 20000 cells/sample for 2D flat films and 40000 cells/sample for 3D scaffolds) were added. The seeded films and scaffolds were confined in the wells of a 96-well plate. Cells for both static and dynamic conditions were initially cultured for 24 hours at 37°C with 5% CO₂. For dynamic culture, the constructs were transferred to the bioreactors for perfusion for up to 28 days at 500 µL/min (day 1 of culture is defined as 24 hours after the initiation of the bioreactors). Medium was changed every 2 to 3 days for up to 14 days for the flat films and 28 days for the scaffolds in static and dynamic cultures.

3.3.4 Perfusion bioreactor

The bioreactor is used to perfuse medium through the HGF-resided scaffolds to increase cell proliferation and migration. The setup of the bioreactor is shown in Fig. 3.1a. There are three main parts to the bioreactor (Fig. 3.1b): (i) the top, (ii) the gasket, and (iii) the platform. A fully assembled bioreactor is shown in Fig. 3.1c. The peristaltic pump (Thermo Scientific, model no. 72-320-126) is a multi-channel system which can supply up to 8 bioreactor chambers. The flow rate (500 µL/min) was chosen to generate a laminar flow (Re < 1000 (Truskey et al., 2004)) through the bioreactor channels with minimal bubble accumulation in the chambers and shear stress of less than 10 dynes/cm², which is the wall shear stress in the capillaries (Navarro et al., 2001;Wong et al., 2012;Zhao et al., 2009). Oxygen was delivered to the samples through the perfused medium. Each bioreactor chamber contains 3 channels and each one can hold one D-PHI scaffold in the middle of the channel in a tight-fit manner (facilitated by the inherent swelling of the polymer (Sharifpoor et al., 2009;Sharifpoor et al., 2010)) to ensure the flow through each sample was laminar. Prior to dynamic culture, all parts of the bioreactor (excluding the platforms, peristaltic pump and pump head) were autoclaved. The platforms were soaked in
70% ethanol overnight followed by ultraviolet (UV) radiation for 4 hours. The pump and pump head were wiped with 70% ethanol and placed under UV radiation for 4 hours prior to setup. At a given time point of 1, 7, 14, and 28 days, the perfusion through the bioreactor of interest was terminated and the bioreactor samples (and also the static samples) were removed from the system while the remaining time point samples in both types of cultures continued until day 28.

**Figure 3.1.** (a) Schematic of the setup for the bioreactor chamber in one of the pump’s channels. (b) Parts of the bioreactor’s chamber: (A) the platform, (B) the gasket, (C) the top, (D) wing nuts, and (E) hex bolts. The locations of the three samples in the bioreactor are indicated by arrow heads. (c) A fully assembled bioreactor chamber.

### 3.3.5 DNA mass quantification

For 2D D-PHI flat films, at 1, 7, and 14 days, HGF DNA content was quantified. For 3D D-PHI scaffolds in static and dynamic cultures, DNA content was quantified at days 0 (24 hours after seeding), 1, 7, 14 and 28 of culture. Samples from both cultures were first taken out from the system and rinsed with PBS; and they were placed separately into microcentrifugue tubes. The samples were then minced and cells were lysed with cell lysis buffer (100 mM NaCl, 100 mM Tris-HCl, 25 mM EDTA, 0.1% SDS). The samples were placed in a 65°C water bath for 1 hour to allow cell lysis buffer to penetrate and lyse the cells, followed by centrifugation for 15 minutes at 8°C to allow the scaffold pieces to settle. Finally, cell lysates from each sample were added to a black polypropylene, flat-bottomed 96-well plate (Thermo Scientific) containing the DNA stain (Hoechst 33258, Sigma-Aldrich). The stain was read with a fluorescence microplate reader with the excitation wavelength set at 360 nm and the emission wavelength set at 460 nm (Bio-Tek® FL600). The fluorescence values were compared to the standard curve (calf thymus
DNA, Sigma-Aldrich) to obtain an estimate of DNA mass. The background fluorescence emitted from the scaffolds (i.e. controls with no cells seeded) was subtracted from the reading to obtain the actual value.

3.3.6 Water-soluble tetrazolium (WST)-1 assay

The metabolic activity of HGFs seeded in the samples was quantified using a water soluble tetrazolium-1 (WST-1) assay (Roche Diagnostics, Laval, Quebec, Canada). WST-1’s active reagent is sodium 5-(2, 4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium (Berridge et al., 2005). The samples were placed in a 96-well plate and rinsed with PBS; and 200 µL of DMEM with no phenol red was added to each sample. 20 µL of the WST reagent was added to each well and the wells were incubated at 37°C with 5% CO₂ for 30 minutes. The samples were placed on a shaker to mix for 1 minute and the solution in the wells was transferred to a new 96 well plate. The absorbance was read using a spectrophotometer (Molecular Devices Corp.) at 450 nm with a reference wavelength of 650 nm.

3.3.7 Histology

Cell growth and distribution in the 3D D-PHI scaffolds was analyzed using histology. Samples were rinsed using PBS and then fixed in 4% paraformaldehyde for 20 minutes. The samples were stored in 15% sucrose for one day, followed by storage in 30% sucrose until being paraffin-embedded. Sections with 20 µm thickness were cut from the centre of the D-PHI scaffolds. Sample preparation, paraffin-embedding and hematoxylin and eosin staining was done by the Histology Department of the Faculty of Dentistry (University of Toronto, Toronto, Canada).

3.3.8 Scanning electron microscopy (SEM)

Cell morphology on the surface of the 3D scaffold was examined using SEM. Samples were rinsed with PBS and then fixed in 3% glutaraldehyde in PBS for 24 hours. Prior to ethanol dehydration, samples were washed with PBS. Then, they were soaked in increasing concentrations of ethanol (30, 50, 70, 90, 95, and 100%) for one hour at each concentration (except 95% (overnight)) and maintained at room temperature to dehydrate. Samples then underwent critical point drying and the dried samples were sputter-coated with 12 nm platinum.
using an SC515 SEC Coating Unit (Polaron Equipment, Uckfield, UK). Samples were viewed at a voltage of 10 kV using a Hitachi 2500 SEM (Hitachi, Mito City, Japan).

3.3.9 Total protein measurement and Western blotting

The total amount of protein in each sample was determined using the Bradford assay (Bio-Rad, Canada). Briefly, cell lysates that were prepared for DNA mass quantification were mixed with the Bradford reagent (Bio-Rad) and the solutions were plated in a 96-well plate. The absorbance of the samples was measured with a spectrophotometer (Molecular Devices Corp.) at 595 nm and the absorbance values were compared to the standard curve (Protein Standard II, Bio-Rad) to obtain an estimate of total protein amount in the samples.

For Western blotting, sample volumes were loaded onto a 7% polyacrylamide gel for type I collagen (Col I) and a 12% polyacrylamide gel for vimentin (Vim), and then transferred onto the nitrocellulose membrane. Col I and Vim were determined using mouse anti-Col I and mouse anti-Vim respectively (monoclonal antibodies, Abcam) as the primary antibody and goat anti-mouse IgG (Thermo Scientific, Illinois, USA) as the secondary antibody. The staining substrate was Supersignal West Femto Maximum Sensitivity Substrate (PI-34094, Thermo Scientific, Illinois, USA). Images of the blot were captured using a Molecular Imager Bio-Rad ChemiDoc XRS (Bio-Rad, USA) and densitometry was carried out using the same apparatus. The images and the densitometry data were analyzed using Quantity One version 4.6.2. (Bio-Rad, USA), and the difference in the background intensities between groups was accounted for in the software. All Western blotting results were normalized to the day 0 time-point (24 hours after seeding). In addition, the data were also normalized to the Vim content in a given sample to determine the production relative to cell content, as Vim has been previously correlated to cell number (Swaney et al., 2005) (see Appendix C).

3.3.10 Statistical analysis

In this study, the number ‘n’ represents the number of biological replicates. When comparing two or more groups of data obtained in the above experiments, statistical analysis was performed using the SPSS program (version 20.0). T-tests were used to determine the statistical significance of any differences between the mean values of two groups. One-way Analysis of Variance (ANOVA) was used to determine the statistical significance in the difference among
the mean values of more than two groups. In these analyses, the overall variability among the groups was compared to the variability within the groups. When statistical significance was found among the groups, post-hoc analysis was used to determine which of the groups contributed to the statistical significance (Norman and Streiner, 2008). For post-hoc analysis, Tukey’s-b was used in the case of equal variances. Levene’s test of homogeneity of the variances was conducted. For analysis with one-way ANOVA, in the event that the variances were unequal, the unequal-variance version of ANOVA (Welch) was used and Dunnett’s T3 was used for post-hoc analysis. In all analyses, the significance threshold was set to $\alpha = 0.05$. All average values were presented in mean ± standard error (Norman and Streiner, 2008).

3.4 Results

3.4.1 Viability of HGF on 2D D-PHI flat films

DNA content (Fig. 3.2) showed that HGFs remained viable on the 2D D-PHI films throughout the culture period and there was a significant 1.7 fold increase in the HGF population over 14 days of culture ($p<0.05$). Similar results was observed in the WST-1 data (Fig. 3.2), which also showed a significant increase in the metabolic activity over 14 days ($p<0.05$).

![Figure 3.2](image)

Figure 3.2. HGF cell population and metabolic activity on 2D D-PHI flat films over a 14-days culture period were assessed by (a) DNA mass quantification and (b) WST-1 assay respectively ($n = 9$, ± standard error (SE)). Initial cell seeding density was 20000 cells/well. *Significantly higher value compared to day 1 ($p<0.05$). ‡Significantly higher value compared to day 7 ($p<0.05$).
3.4.2 HGF proliferation and metabolic activity in perfused 3D D-PHI scaffolds

Since HGF cell growth on the 2D D-PHI flat films showed that the material is biocompatible for HGF culture, the interaction between HGFs and a 3D scaffold for tissue engineering applications was then investigated with and without the effect of medium perfusion to promote cell proliferation and metabolic function.

HGFs were cultured on D-PHI in a medium-perfused system (dynamic culture), with both cell proliferation and metabolic activity being measured and compared with the static culture (no perfusion). DNA content (Fig. 3.3a) showed that over 28 days of culture, there were more HGFs in the dynamic vs. static culture at all time points. HGFs showed continued growth in the dynamic culture, with a significant 3-fold increase from day 1 to day 28 (p<0.05), whereas growth started to slow down in the D-PHI scaffold for the static culture after 14 days.

Similarly, the metabolic activity of HGFs in the D-PHI scaffolds, as measured by a WST-1 assay (Fig. 3.3b), increased significantly from day 1 to day 28 in the dynamic culture (p<0.01); whereas the metabolic activity reached a plateau in the static culture after 14 days, suggesting that cells were more active in the dynamic vs. static culture. This was supported by the trends observed for total protein production (Fig. 3.3c), which showed a significant increase over 28 days in D-PHI for the dynamic culture (p<0.05) but not in the static culture.
Figure 3.3. (a) HGF proliferation on D-PHI scaffolds in static (no perfusion) vs. dynamic (with perfusion) culture was measured by DNA contents. (b) The metabolic activity of the cells was assessed by (b) a WST-1 assay and (c) total protein production. (n = 9, ± SE, value was significantly different from each other, †: p<0.05, *: p<0.01, §: p≤0.001.).
In addition, cell infiltration into the D-PHI scaffolds was observed by histology (Fig. 3.4) and the results show that more cells were observed in the scaffold as culture time progressed in both types of culture; however, more cells were found in the inner regions of the scaffold in the dynamic culture, particularly at days 14 and 28.
Figure 3.4. Histology images of HGFs in 3D D-PHI scaffolds in static vs. dynamic culture at days 1, 7, 14, and 28. The control scaffold (no cells) is shown at the bottom. Cells were stained with hematoxylin and eosin (H&E) and the extent of their distribution indicated by the arrows. Scale bar = 500 µm.

3.4.3 Scanning electron micrographs of HGF on perfused D-PHI scaffolds

Using scanning electron microscopy (SEM), images of HGFs and extracellular matrix (ECM) production on the D-PHI scaffolds in both types of culture systems were recorded (Fig. 3.5). The non-seeded scaffold was porous and the pores appeared to be on the order of 30 to 250 µm in size. HGFs were spindle-shaped, with cell processes having a width of approximately 7 to 10 µm (indicated by arrows), and they appeared to form clusters throughout days 7 to 28 in the dynamic culture whereas they were more stretched in the static culture. At the 4000x magnification, strand-like structures (arrowheads) were observed in between the cell processes at day 7 for dynamic culture. Similar features existed throughout days 7 to 28 for the dynamic culture (expanded images not shown for days 14 and 28). These strand-like structures have the appearance of extracellular matrix due to their sub-micron features. Day 1 SEM images were not shown as the cells were sparsely spread out on the scaffold and had not fully distinguished themselves in either of the cultures.
Figure 3.5. Scanning electron micrographs of spindle-shaped HGFs (arrows) stretched out with their processes in the static culture (b – d) while they formed clusters in the dynamic culture (e – h) on D-PHI scaffolds. (a) No cells were seeded in the control scaffold. (e) A 4000-times magnification of the boxed area in (f), where strands of extracellular matrix (arrow heads) were observed.

3.4.4 Type I collagen (Col I) production

Since the total protein production increased significantly over 28 days of dynamic culture, it was hypothesized that the increase could possibly be co-related to collagen, particularly Col I, production. The results show that the accumulated Col I production (Fig. 3.6b) in the dynamic culture was greater than that of static culture for all time points, but was only significantly greater at day 7 (p<0.05). When the accumulated Col I production was normalized to the amount of Vim, which provides a loading control and an indication of cell population (Swaney et al., 2005), Fig. 3.6c shows that more Col I was produced relative to cell content in the dynamic culture when compared to the static culture on D-PHI, specifically at day 28 (p<0.05). Significantly lower levels of Col I production per cell were observed at days 7 and 14 in comparison to days 1 and 28 in the dynamic culture, suggesting the resorption and possibly
remodelling of the collagen matrix. As seen in Fig. 3.6b, the profile of Col I production is not co-related to the relatively linear increase in the total protein production shown in Fig. 3.2c.

**Figure 3.6.** Col I production in HGFs in D-PHI scaffolds over 28 days of culture: (a) Western blot image, Vim = vimentin, (b) Col I content normalized to value at day 0 (n = 6, ± SE), (c) Col I content normalized to vimentin content at corresponding time point, then normalized to value (Col I/Vim) at day 0 (n = 6, ± SE). This shows the amount of Col I produced relative to cell content at specific time points. * Significantly different from each other (p<0.05); ‡ significantly different from other time points in that culture condition (p<0.05).
3.5 Discussion

Tissue engineering has provided some promising results in regenerating damaged tissues and degradable polyurethanes are becoming of interest for use as elastomeric scaffolds for soft tissue engineering (Liu et al., 2008). Medium perfusion has been shown to increase cell growth in the tissue-engineered scaffolds (Blackwood et al., 2008; Hillmann et al., 1999; Mohammadi et al., 2007; Moharamzadeh et al., 2007; Moharamzadeh et al., 2008; Moscato et al., 2008; Mussig et al., 2008; Navarro et al., 2001; Sachar et al., 2012; Yamada et al., 2006) and it is of intrinsic interest to determine if the changes resulting from medium perfusion can be differentiated from static conditions when new synthetic biomaterials are introduced into a system given that the cellular reaction to a biomaterial can sometimes dominate the biological response. In a study carried out by Navarro et al., the population of human oral keratinocytes cultured on a perfused scaffold made of collagen and chondroitin sulfate showed 88% more growth than that on the non-perfused structures (Navarro et al., 2001). Perfused scaffolds have also been used to increase the density of human mesenchymal stem cells in a 35-day culture (Zhao et al., 2009), which resulted in an increase in the cells’ osteogenic ability, as shown by the greater expression of osteonectin.

Since the gingival connective tissue is highly vascularized and populated by $2 \times 10^8$ fibroblasts per cubic centimetre (Schroeder, 1986), it would be anticipated that any tissue engineering approach for such tissues would rely on the scaffold being perfused. In view of this, the present study developed a perfusion bioreactor and investigated the effect of medium perfusion (dynamic culture) on HGF proliferation and metabolic function on the D-PHI material, a recently developed elastomeric polyurethane hydrogel. It was demonstrated that D-PHI is biocompatible with HGFs and that the dynamic culture enhanced HGF proliferation, metabolic activity, and infiltration in the scaffold. As well, the total protein production and Col I production were promoted by dynamic culture.
3.5.1 Dynamic culture enhanced HGF proliferation, metabolic activity, and infiltration in the scaffold

HGFs remained viable on the D-PHI materials (Fig. 3.2). D-PHI has been shown to be a biocompatible material with other cell types such as human coronary smooth muscle cells (Sharifpoor et al., 2010; Sharifpoor et al., 2011) and has been demonstrated to down-regulate monocytic activity (McBane et al., 2009; McBane et al., 2011). However, the reaction of fibroblasts with this material had not been previously reported on. More specifically, it was desired to determine if the cell-biomaterial interactions would limit the benefits of a perfusion system with respect to enhanced cellular responses such as proliferation.

It was shown that cell proliferation continuously increased throughout the dynamic culture period (Fig. 3.3a); whereas it was reduced in the static culture after 14 days. This trend was also reflected in the metabolic activity (Fig. 3.3b) and the total protein production (Fig. 3.3c), despite the less prominent differences observed in this latter parameter, and that there were no significant differences in these two parameters between static and dynamic cultures at any given time point. While statistical difference between the static and dynamic conditions for WST and protein production was not found for a given time point, it is perhaps more important to recognize that the static condition showed no change in time for metabolic activity whereas the dynamic culture showed a strong statistical difference between day 1 and day 28. Likewise for protein production, as static culture showed no statistical change over time after 7 days, the dynamic culture showed a statistical difference between day 7 and day 28. These data suggest that the perfused D-PHI scaffold has enabled HGF to exist in a more proliferative state. In a study with C2C12 muscle precursor cells cultured on 3D collagen scaffolds, it was shown that there was an increase in the cell fractions in the proliferative phases (S/G2/M) under medium perfusion in long-term cultures (Flaibani et al., 2009). Even though the culture period is different for those cells and the HGFs studied here, both the HGF cell line and C2C12 cells have a mesenchymal origin; as such, more HGFs in the dynamic culture are hypothesized to be in the proliferative phase. Cells in the proliferative phase can undergo cell division and multiply themselves. Proliferative cells are desired in tissue regeneration as they are more capable of repopulating the tissue construct by multiplying themselves and form a tissue-like structure. A similar study with C2C12 cells also showed an enhancement of cell proliferation (Bettahalli et al., 2012) when they were cultured in perfused scaffolds which led to increased permeation of
nutrients and oxygen throughout the porous structure. The open-pore structures of the D-PHI scaffolds may have further contributed to increased mass transport in the perfused system as the high porosity and pore interconnectivity in D-PHI scaffolds have been shown to maximize the potential for the diffusion of nutrients and the migration of vascular smooth muscle cells (Sharifpoor et al., 2010). As well, an in vivo study with D-PHI scaffolds showed that the porous structure of D-PHI was maintained and extensive tissue formation was observed through the porous material at 100 days, while a porous structure of PLGA scaffolds collapsed and tissue formation was only observed around the peripheral of the scaffold (McBane et al., 2011).

The histology images (Fig. 3.4) showed a most visible difference between the perfused vs. non-perfused culture on the D-PHI scaffold. Cell infiltration was greater in the scaffold for the dynamic vs. static culture. It has been shown that perfusion bioreactors can increase cell distribution throughout the 3D scaffolds (Bancroft et al., 2002; Bettahalli et al., 2012). All of the above data indicate that the D-PHI scaffold enabled enhanced cellular responses for HGFs under medium perfusion, which involved enhanced cell proliferation and infiltration. This is anticipated to be beneficial for generating a tissue substitute for damaged gingival connective tissue as it is expected that when combined with other tissue-enabling cells (e.g. endothelial cells), cell infiltration could be easily achieved and microvessels could be rapidly regenerated in the D-PHI scaffold. The results from this study, in combination with the low inflammatory nature of D-PHI (McBane et al., 2011), show this material to be very attractive for gingival tissue engineering.

3.5.2 Dynamic culture promoted Col I production in HGF

To generate a functional substitute for the gingival connective tissues, HGFs not only need to remain viable and demonstrate continuous growth in the material, but they also have to have the ability to produce collagen as the latter provides the structural support needed for HGFs (Lodish et al., 2003). The SEM images (Fig. 3.5) showed that strands of ECM appear to be present in the perfused scaffolds as early as 7 days. Since the lamina propria has an abundant network of Col I (Hillmann et al., 1999), its production was measured in both types of cultures and the data were reported in Fig. 3.6. The accumulated Col I production (Fig. 3.6b) and Col I production relative to cell content (proportional to vimentin (Swaney et al., 2005), Fig. 3.6c) both showed that more Col I was produced in the dynamic culture, suggesting that medium perfusion not only changed
the metabolic state of HGF, but it stimulated the cells to produce more collagen. Medium perfusion has been shown to increase type III procollagen content in bone mesenchymal stromal cells for regenerating the meniscus (Liu et al., 2012). It is interesting to see that the decrease in Col I production at days 7 and 14 in the dynamic culture did not parallel the total protein production at the same time points. This indicates that throughout the 28-day dynamic culture period, resorption of Col I and production of other proteins might have occurred in the perfused D-PHI scaffolds between days 7 and 14. The work of Hinz et al. have shown that fibroblasts can be differentiated into myofibroblasts, a contractile phenotype of fibroblasts, when they are mechanically stimulated, resulting in an upregulation of α-smooth muscle actin, matrix metalloproteinases (i.e. increased ECM remodelling), etc (Hinz, 2010). Whether HGFs in the current system have differentiated into myofibroblasts between days 7 and 14 remains to be investigated. Mechanical stimulation is also known to be an important regulator in cell response and integrin β1 has a critical role in mechanotransduction, which can regulate collagen production (Liu et al., 2009; Liu et al., 2010). As such it is hypothesized that medium perfusion may have contributed to a mechanical stimulus of the cells, which activated integrin β1 and caused a downstream effect on collagen production.

Collagen production could also be affected by the production of various growth factors such as the transforming growth factor (TGF)-β1 (Hayashida et al., 2007). The production of these growth factors may be upregulated or downregulated by mechanical stimulation, which can activate or inhibit certain molecular pathways (e.g. Smad 2/3 (Hinz, 2007), FAK/mTOR (Dalla Costa et al., 2010)) and cause different cell responses (e.g. myofibroblast differentiation (Hinz, 2007)). Hence, it will be necessary to further investigate the above molecular pathways and growth factor production to further understand how perfusion changes HGF response cultured in D-PHI scaffolds.

As a result, the different metabolic state and increased Col I production suggested that HGFs in the dynamic culture had a different phenotype than those in the static culture, which is anticipated to be beneficial for gingival tissue reconstruction as the lamina propria is densely populated by fibroblasts in a collagenous matrix.
3.6 Conclusion

We have shown that a synthetic degradable polyurethane hydrogel (D-PHI), when undergoing perfusion, enables enhanced HGF proliferation, metabolic activity and Col I production, which suggests a different cell phenotype. Further characterization of HGFs in the perfused system, including an investigation of the production of growth factors and other phenotypic markers (e.g. TGF-β1, α-SMA) is required in order to better understand how this phenotype is preserved within the D-PHI material.

3.7 Acknowledgments

This study was supported by a Natural Sciences and Engineering Research Council (NSERC) Discovery grant (360520), NSERC Alexander Graham Bell Canada Graduate Scholarships (CGS D3), Ontario Graduate Scholarship Program, and Canadian Institutes of Health Research-CellSignals Training Fellowship (TGF-53877).
3.8 References


Chapter 4
Establishing a gingival fibroblast phenotype in a perfused degradable polyurethane scaffold: mediation by TGF-β1, FGF-2, β1-integrin, and focal adhesion kinase

4.1 Foreword

Medium perfusion has been shown to enhance cell proliferation and matrix protein production. In more recent work, under perfusion, a degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold was shown to enhance growth and production of collagen by human gingival fibroblasts (HGFs). However, the nature of the HGFs cultured in the perfused D-PHI scaffolds, and the mechanisms by which medium perfusion activates these cells to facilitate proliferation and collagen production are not defined. The current study sought to investigate HGF interaction within the D-PHI scaffolds under perfusion by examining the production and the spatial distribution of α-smooth muscle actin (α-SMA) and type I collagen (Col I), the secretion of transforming growth factor (TGF)-β1 and basic fibroblast growth factor (FGF-2) in the conditioned medium, with a goal of defining the mechanistic pathways affecting the production of these markers in the dynamic culture. It was found that the perfused D-PHI scaffold shifted the HGF phenotype from myofibroblast-like (upregulation of α-SMA) to fibroblast-like (downregulation of α-SMA) over the course of 28 days. Both TGF-β1 and FGF-2 were significantly greater in the dynamic vs. static culture at day 1. Although TGF-β1 has been often reported to increase α-SMA and collagen expression, the D-PHI material and significant high level of FGF-2 at day 1 of dynamic culture appear to play a role in regulating α-SMA production while allowing HGFs to increase Col I production. β1-integrin production was increased and focal adhesion kinase (FAK) was activated 2 hours after HGFs were exposed to medium perfusion, which may have in part promoted cell growth, α-SMA and Col I production in the early dynamic culture. Consequently, the D-PHI material and medium perfusion has modulated fibroblast phenotype, and enhanced cell growth and Col I production through the coordinated actions of TGF-β1, FGF-2, β1-integrin and FAK.

This chapter has been published in ‘Biomaterials’ as: Cheung JWC\textsuperscript{1}, McCulloch CAG\textsuperscript{2}, Santerre JP\textsuperscript{1,2}. Establishing a gingival fibroblast phenotype in a perfused degradable...
polyurethane scaffold: mediation by TGF-β1, FGF-2, β1-integrin, and focal adhesion kinase. *Biomaterials* 2014;35(38):10025-10032. Modifications of text are indicated by “[*]” and footnote. **Figure 4.7** and **Supplementary Figure 4.1** were updated.

Permission to reprint this manuscript has been granted from Elsevier.

1Institute of Biomaterials and Biomedical Engineering, University of Toronto, 124 Edward Street, Room 461, Toronto, Ontario, Canada M5G 1G6.

2Faculty of Dentistry, University of Toronto, 124 Edward Street, Room 464D, Toronto, Ontario, Canada M5G 1G6.

4.2 Introduction

[Gingival atrophy is highly prevalent (Albandar and Kingman, 1999; Kassab and Cohen, 2003; Sarfati et al., 2010; Susin et al., 2004) and it is defined as an exposure of the root surface due to gingival recession, which can cause root sensitivity and tooth loss (Chambrone et al., 2010; Kassab and Cohen, 2003; Tonetti et al., 2014). It also has a negative impact on the quality of life with regards to impaired aesthetics of the smile (Chambrone et al., 2010; Tonetti et al., 2014).] In aging society, there has been interest in using tissue engineering approaches to provide autologous supply of tissues for restoring lost [gingival lamina propria] (Locke et al., 2008; Moharamzadeh et al., 2007; Mussig et al., 2008). [Human gingival fibroblasts (HGFs) are involved in the repair of the lamina propria of the gingiva (Locke et al., 2008; Moharamzadeh et al., 2007; Mussig et al., 2008).] Three-dimensional synthetic scaffolds such as poly(lactic-co-glycolic acid) (PLGA, 70/30) have been widely used in tissue engineering for constructing a vascular graft (Wen et al., 2007); porous polycaprolactone scaffolds have been used to enhance chondrocyte proliferation and cartilaginous extracellular matrix production (Meretoja et al., 2012). However, the availability of elastomeric polymers such as degradable polyurethane (PU) has several appealing physical properties for soft tissue regeneration where biomechanical forces may play an important role in determining tissue structure (Sharifpoor et al., 2011).

The impact of biomechanical forces arising from the material structure and medium perfusion within the scaffold are important considerations for tissue engineering designs, as noted in data

---

*Modification of published text.*
relating to material modulus (Galie et al., 2012; Hinz, 2007; Liu et al., 2010; Tomasek et al., 2002) and [flow stimuli such as shear stress] (Galie et al., 2012; Ng et al., 2005). Notably, fibroblasts cultured on soft polydimethylsiloxane (PDMS) (elastic modulus = 9.6 kPa) do not differentiate into myofibroblasts, while those cultured on a stiffer PDMS surface (780 kPa) exhibit myofibroblast differentiation and stress fiber formation (Goffin et al., 2006). Further, flow-induced shear stress can augment cell growth (Navarro et al., 2001), enhance growth factor secretion (Galie et al., 2012), and increase collagen production in the scaffolds (Ng et al., 2005). In addition, [flow] can modulate the conversion of fibroblasts to myofibroblasts in vascular remodelling and wound healing (Shi et al., 2007). Myofibroblasts, characterized mainly by the expression of α-smooth muscle actin (α-SMA), display enhanced contractility and are abundant in early stages of healing wounds (Galie et al., 2012; Hinz, 2007; Hinz, 2010). The prolonged presence of myofibroblasts in healing tissues is undesirable because of their association with scarring and fibrosis (Hinz, 2007; Hinz, 2010; Tomasek et al., 2002).

Recent work focusing on the development of elastomeric tissue engineering scaffolds has described PU elastomeric hydrogels (degradable/polar/hydrophobic/ionic polyurethane (D-PHI)), which elicit a low inflammatory response post-implantation compared to PLGA (McBane et al., 2011). D-PHI scaffolds exhibited a degradation rate of 21 wt.% 100 days after implantation in vivo and up to 87% increased porosity while retaining structural form (McBane et al., 2011). In an in vivo subcutaneous mouse model, D-PHI scaffolds demonstrated increased tissue in-growth and micro-vessel formation (McDonald et al., 2011).

Preliminary examination of a dynamic flow system that incorporates D-PHI scaffolds in perfused culture has shown enhanced proliferation and production of collagen by HGFs over 28 days (Cheung et al., 2013). However, the interaction of HGFs with the D-PHI scaffold in perfused condition requires further assessment, including the cell phenotype that is defined by the expression of α-SMA. Moreover, the signaling pathways by which the D-PHI scaffold and medium perfusion activates HGFs to facilitate proliferation and expression of type I collagen (Col I) are not defined.

HGFs mainly express Col I, which can be enhanced by transforming growth factor (TGF)-β

*Modification of published text.
(Galie et al., 2012; Hayashida et al., 2007; Horan et al., 2008; Tomasek et al., 2002). TGF-β isoforms also promote myofibroblast differentiation via the Smad signaling pathway (Galie et al., 2012; Hinz, 2007; Hinz, 2010; Horan et al., 2008; Tomasek et al., 2002). This process can be inhibited by basic fibroblast growth factor (FGF-2) (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). Focal adhesion proteins such as β1-integrin and focal adhesion kinase (FAK) have been shown to influence myofibroblast differentiation and collagen production (Supplementary Fig. 4.1) (Dalla Costa et al., 2010; Langholz et al., 1995; Liu et al., 2009; Liu et al., 2010; Surazynski et al., 2005).

Supplementary Figure 4.1. A schematic diagram outlining the proposed mechanisms that are involved in the modulation of type I collagen (Col I) and α-smooth muscle actin (α-SMA) expression (myofibroblast differentiation). [Flow-induced shear stress can act as one of the mechanical stimuli]. Transforming growth factor (TGF)-β1 is believed to be a regulator of collagen production and it is a potent inducer of α-SMA expression via the Smad signaling pathway (Galie et al., 2012; Hinz, 2007; Hinz, 2010; Horan et al., 2008; Tomasek et al., 2002), however this process can be inhibited by basic fibroblast growth factor (FGF-2), which has been shown to inhibit Smad nuclear translocation, as well as downregulating α-SMA expression at the transcription level (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). [Flow] has been reported to stimulate focal adhesion kinase (FAK) signaling via β1-integrin (Galie et

*Modification of published text.*
Collagen production has also been shown to be mediated by β1-integrin (Langholz et al., 1995; Liu et al., 2009; Liu et al., 2010) and FAK (Dalla Costa et al., 2010; Surazynski et al., 2005). Both integrins and FAK are associated with myofibroblast differentiation (i.e. α-SMA production) (Hinz, 2010).

This study sought to investigate the influence of D-PHI elastomeric scaffolds on HGF cell behaviour under medium perfusion. The production and localization of cells expressing α-SMA and Col I, the secretion of TGF-β1 and FGF-2 into the medium, and the signaling pathways that affect the production of these proteins over 28-day culture periods were examined. It is hypothesized that the combination of the D-PHI scaffold and medium perfusion (dynamic culture) modulates fibroblast phenotype through the coordinated actions of TGF-β1 and FGF-2 in response to [flow]. Finally, it is hypothesized that the increased Col I production by cells cultured on perfused D-PHI scaffolds (Cheung et al., 2013) is mediated by the β1-integrin and FAK. An improved understanding of the mechanisms underlying fibroblast-myofibroblast transition in dynamic culture could enable rational developments for generating a functional, engineered tissue.

4.3 Materials and methods

4.3.1 D-PHI film and scaffold fabrication

Cell responses on the 2D D-PHI films were investigated in order to examine the material’s effect on HGFs without the influence of flow or the 3D architecture, and compared with the reference material 2D tissue culture polystyrene (TCPS; 96-well plate, Sarstedt, Canada).

2D D-PHI films and 3D scaffolds were fabricated using a divinyl oligomer (DVO), which was synthesized in house by reacting polyhexamethylene carbonate diol (Sigma-Aldrich, United States of America) with lysine diisocyanate (Kyowa Yuka, Yokkaichi City, Japan) and 2-hydroxyethyl methacrylate (Sigma-Aldrich) in a 1:2:2 ratio (Cheung et al., 2013; Sharifpoor et al., 2009). The DVO, methacrylic acid (Sigma-Aldrich) and methyl methacrylate (Sigma-Aldrich) were then reacted via free radical polymerization in a 1:5:15 molar ratio under dry conditions using benzoyl peroxide (Sigma-Aldrich) as the initiator.

*Modification of published text.
For D-PHI films, the resin was casted into 96-well plates made of polypropylene. For D-PHI scaffolds, poly(ethylene glycol) (Sigma-Aldrich, 10 wt.%) and sodium bicarbonate (Sigma-Aldrich, 70 wt.%) were introduced as the porogens. The resin was molded into circular discs. For both films and scaffolds, the casted resin and the molded discs were cured at 110°C for 24h under dry conditions. The resulting scaffolds were subjected to sonication in distilled water for 14 days for porogen leaching. Each scaffold had a diameter of 6 mm and a thickness of 2 mm with 80% porosity (Sharifpoor et al., 2009).

4.3.2 Culture of HGF cell line and setup of perfusion (dynamic) culture

A human gingival fibroblast cell line (HGF-1, ATCC CRL-2014, originally developed by McAllister et al. (McAllister et al., 1993a; McAllister et al., 1993b)) was used. Cells were cultured at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; GibcoBRL) supplemented with 100 U/mL penicillin/100 µg of streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (Sigma-Aldrich). Prior to seeding, the D-PHI films and scaffolds were treated with 70% ethanol overnight, followed by drying under sterile laminar flow conditions for 24h. The films and scaffolds were then pre-wetted with sterile DMEM for 24h at 37°C with 5% CO₂ in a sterile, humidified environment. When cells reached 80-90% confluence, HGFs were trypsinized and seeded onto the pre-conditioned films or scaffolds at 20,000 cells (for films) or 40,000 cells per sample (for scaffolds). The seeded samples were confined in the wells of a 96-well plate and were initially cultured for 24h at 37°C with 5% CO₂ in static conditions.

For D-PHI films, the samples were fixed for immunofluorescence after 24h. For D-PHI scaffolds, following 24h of incubation (denoted as day 0), the samples were separated into static culture (without medium perfusion, in 96-well plates) and dynamic culture (with medium perfusion) for up to 28 days. A custom-built bioreactor was used to perfuse medium through the seeded scaffolds in the dynamic culture, thereby providing a differential transport of nutrients and metabolites as well as flow stimulus to the cells. The bioreactor system used a peristaltic pump; and the bioreactor chamber contains as many as three D-PHI scaffolds which are arranged in a parallel circuit (Cheung et al., 2013). Medium was changed every 2 to 3 days for both groups.
4.3.3 Immunohistochemistry and immunofluorescence

Samples (two-dimensional (2D) D-PHI films after 24h static culture and three-dimensional (3D) D-PHI scaffolds at day 28 static and dynamic cultures) were rinsed with Dulbecco’s-phosphate buffered saline (D-PBS, pH 7.4), fixed in 4% paraformaldehyde, dehydrated, paraffin embedded, and sectioned at 20 µm thickness [to preserve scaffold’s porous structure] (histology service, Faculty of Dentistry, University of Toronto, Canada).

Immunofluorescence staining for α-SMA and Col I was conducted to estimate protein expression in cells cultured on the D-PHI film samples and tissue culture polystyrene (TCPS). Staining for fibroblast surface protein (FSP) and Col I was performed to observe extracellular Col I production in the perfused and non-perfused 3D D-PHI scaffolds. The film samples were incubated with 0.1% Trypsin-CaCl₂ (Sigma-Aldrich) at 37°C for 30 min for antigen-unmasking. Following an initial blocking in 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS at 37°C for 20 min, the sections were incubated overnight at 4°C in 0.3% Triton X-100 containing mouse antibody to α-SMA (Abcam, diluted 1:25) and rabbit antibody to Col I (Abcam, diluted 1:50). The samples were subjected to a second blocking in 10% goat serum for 30 min at room temperature, and were then incubated in 10% goat serum containing Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Invitrogen, diluted 1:200) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrogen, diluted 1:200). Hoechst 33342 (Invitrogen, H3570, diluted 1:1000) was used to label DNA in nuclei. For 3D D-PHI scaffolds, the above steps were applied to deparaffinized and rehydrated scaffold sections. A mouse antibody to FSP (Abcam, diluted 1:300) and the same Col I antibody were used, followed by mounting with PermaFluor (Thermo Scientific). [The Hoechst stain also stained the porous scaffold sections. This background in the scaffold was minimized with the attempt to avoid diminishing the signal for observing the cell nuclei. The cell nuclei can be distinguished from the scaffold sections by the oval shapes of the nuclei (see Appendix C).] All samples were viewed with a fluorescence microscope (DM-IRE2, Leica Microsystems, Germany).

Immunohistochemical staining of α-SMA and Col I was performed to examine the spatial distribution of cells expressing these two proteins in 3D perfused vs. non-perfused D-PHI scaffolds at the end of the 28-day culture period. Deparaffinized and rehydrated 20 µm-thick

*Modification of published text.
sections were incubated with 0.01M sodium citrate buffer (pH 6.0, 10 min, 85-95°C) for antigen retrieval, which was followed by a 1-hour blocking in 10% rabbit serum and 1% BSA at room temperature. Sections were incubated overnight at 4°C in primary antibody solution (1.5% goat serum, 0.1% BSA) containing either mouse anti-α-SMA (1:75) or mouse anti-Col I (1:200) monoclonal antibody (Abcam), followed by incubation with rabbit anti-mouse IgG horseradish peroxidase-conjugated antibody (1:100 for α-SMA and 1:200 for Col I, 1h). Diaminobenzidine (DAB) peroxidase substrate (Vector labs, SK-4100) was applied to detect positive staining cells. The sections were counterstained with hematoxylin (Vector labs, H-3404) and viewed by light microscopy (Olympus BX51-TF microscope, 4x magnification). For both immunofluorescence and immunohistochemical analyses, three independent experiments were performed in triplicate for each time point and condition. Control samples consisted of cell-scaffold sections without primary antibodies and cell-free scaffold sections with both primary and secondary antibody to assess background fluorescence (Sharifpoor et al., 2011).

4.3.4 Western blotting

Total protein in each tissue-scaffold was estimated with the Bradford assay (Bio-Rad, Canada) as described (McBane et al., 2012). Samples (5 µg) were loaded onto 12% polyacrylamide gels for analysis of proteins of interest; α-SMA and vimentin (Vim) were assessed using mouse monoclonal antibodies to α-SMA and Vim (Abcam). A goat anti-mouse IgG (Thermo Scientific, Illinois, USA) was used as the secondary antibody. Images of the blot were obtained (ChemiDoc XRS; Bio-Rad, Mississauga, ON) and densitometry was conducted using the same instrumentation. The images and the densitometry data were analyzed using Quantity One version 4.6.2 (Bio-Rad). Differences in the background intensities between groups were adjusted with the software. All Western blotting results were normalized to the Vim content in a given sample in order to estimate protein production relative to cell number (Swaney et al., 2005) (see Appendix C) and then normalized to the day 0 time point (24 h after seeding).

For measurements of β1-integrin, phosphorylated FAK (pFAK) and total FAK, mouse anti-β1-integrin (clone 12G10, Millipore, USA), mouse anti-pFAK at Tyr-397 (Santa Cruz Biotechnology, USA), and mouse anti-FAK (Santa Cruz Biotechnology) were used respectively as primary antibodies and the same goat anti-mouse IgG was used as the secondary antibody. Since β1-integrin and FAK have been reported to be activated at highest levels within hours of
cultural (Schlaepfer et al., 1999), cell lysates with β1-integrin, pFAK and FAK were recovered 2h after the initiation of the bioreactor. The amount of β1-integrin was normalized to Vim and the amount of pFAK was normalized to total FAK. For all measurements using Western blotting, based on previous experiments, three independent experiments were performed to enable statistical analysis.

4.3.5 TGF-β1 Enzyme linked immunosorbent assay (ELISA)

Conditioned medium from the static and dynamic cultures were analyzed for TGF-β1 production by using commercial human TGF-β1 ELISA kit (R&D Systems Inc., USA). Conditioned medium was collected at day 0 (24 hours after seeding), 1, 7, 14 and 28 of culture. Due to the large sample volume, for samples derived from dynamic culture, 10-mL conditioned medium was concentrated to 500 µL using Amicon Ultra-15 centrifugal filters (with 10kDa cut-off, Millipore, USA). Prior to the TGF-β1 ELISA, TGF-β1 in each sample was activated by the addition of 1 N hydrochloric acid, followed by acid-neutralization with 1.2 N sodium hydrochloride/0.5 M HEPES to achieve pH 7.2 – 7.6. With the acid treatment, the assay measures both active and latent TGF-β1. The absorbance of the samples was measured using a VersaMax tunable microplate reader (Molecular Devices Corp.) with the wavelength set to 450 nm and background at 540 nm, and compared to the standard curve to quantify the amount of growth factor in the samples. Background absorbance emitted from the controls (i.e. no cells seeded) was subtracted from the reading to obtain the final value. For all measurements, based on previous experiments, three independent experiments were performed to enable statistical analysis.

4.3.6 FGF-2 ELISA

Conditioned medium from the static and dynamic cultures were analyzed for FGF-2 production by using commercial human FGF ELISA kit (R&D Systems Inc., USA). Similar to TGF-β1 ELISA, conditioned medium was collected at day 0 (24 hours after seeding), 1, 7, 14 and 28 of culture. For samples derived from dynamic culture, the conditioned medium was concentrated to 500 µL using the same centrifugal filters as described in Section 4.3.5. The absorbance of the samples was measured using the same microplate reader at 450 nm (background at 540 nm), and compared to the standard curve to quantify the amount of FGF-2 in the samples. Background absorbance emitted from the controls (i.e. no cells seeded) was subtracted from the reading to
obtain the final value. For all measurements, based on previous experiments, two independent experiments were performed to enable statistical analysis.

4.3.7 Inhibition of the Smad pathway

Initially, HGFs seeded in perfused and non-perfused D-PHI scaffolds were allowed to culture in 96-well plate for 24h in DMEM. In dynamic culture, the seeded scaffolds were transferred to the bioreactor and cultured with treated (DMEM containing 3 µM of (2E)-1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl)-propenone hydrochloride (SIS3, Sigma-Aldrich)) (Jinnin et al., 2006) or untreated medium for 1h. SIS3 is a Smad2/3 inhibitor. The static culture samples remained in the 96-well plate and cultured for 1h in the changed media (treated/untreated). The scaffold samples were then treated with cell lysis buffer (100 mM NaCl, 100 mM Tris–HCl, 25 mM EDTA, 0.1% SDS, 1:100 protease/phosphatase inhibitor) to extract DNA and proteins. α-SMA was measured using Western blotting and the data was normalized to Vim and to values measured on day 0. For all measurements, based on previous experiments, three independent experiments were performed to enable statistical analysis.

4.3.8 Inhibition of β1-integrin

We examined whether the β1-integrin was involved in α-SMA production by treatment with an inhibiting antibody (4 µg/mL; clone MAb4B4, Beckman Coulter, Mississauga, ON) (Langholz et al., 1995), which was added at 40 µg/mL to static and dynamic cultures immediately after cell seeding. The antibody and the medium were replenished every other day (Langholz et al., 1995). At days 1 and 14, α-SMA production was examined by Western blotting and the data were normalized to values obtained at day 0. For all measurements, based on previous experiments, two experiments with three samples were performed to enable statistical analysis.

4.3.9 Statistical analysis

In this study, the number ‘n’ represents the number of biological replicates. Statistical analysis was performed using the SPSS program (version 20.0). Student’s t-test was used to determine the statistical significance of any differences between the mean values of two groups. In the case of more than two groups, one-way ANOVA was used. When statistical significance was found, post-hoc analysis was used to determine which of the groups contributed to the statistical
significance (Norman and Streiner, 2008). Tukey’s b was used in the case of equal variances. Levene’s test of homogeneity of the variances was also conducted. For one-way ANOVA, in the event that the variances were unequal, the unequal-variance version of ANOVA (Welch) was used and Dunnett’s T3 was applied for post-hoc analysis. In all analyses, the significance threshold was set to $\alpha = 0.05$. All average values are presented as mean ± standard error (Norman and Streiner, 2008).

4.4 Results

4.4.1 D-PHI inhibits myofibroblast differentiation

Prior to studying the interaction of HGFs in a perfused 3D system, HGFs were initially cultured on 2D D-PHI films for 24 hours to examine the effect of the material on $\alpha$-SMA and Col I production without the influence of flow or the 3D architecture, and to compare it with the reference material 2D TCPS. After 24 hours of static culture, HGFs on both D-PHI films and TCPS express Col I (Fig. 4.1a and 4.1d). Most of the HGFs on D-PHI films did not stain for $\alpha$-SMA (Fig. 4.1b), while most HGFs cultured on TCPS were $\alpha$-SMA positive (Fig. 4.1e). These results indicate that compared to the culture of cells on standard TCPS, the culture on D-PHI decreases $\alpha$-SMA expression and inhibits myofibroblast differentiation in HGFs, suggesting that D-PHI is a material which can potentially be used for gingival tissue regeneration.
Figure 4.1. Immunofluorescence images of HGFs cultured on (a – c) D-PHI films and (d – f) TCPS after 24 hours of static culture. HGFs were stained for (a, d) Col I (red) and (b, e) α-SMA (green), with the nuclei stained with Hoechst (c, f, blue). Scale bar = 60 µm.

4.4.2 Dynamic culture modulates α-SMA and Col I production over time

Previous data showed that in 3D D-PHI scaffolds under perfusion, HGFs exhibit ~3-fold increase in cell proliferation (Cheung et al., 2013), suggesting that medium perfusion may influence cell behaviour. The expression of α-SMA was reduced in both static and dynamic culture over 28 days of culture (Fig. 4.2), indicating that for HGFs, culture on 3D D-PHI inhibits myofibroblast differentiation. At day 14, α-SMA expression was greater in the perfused vs. non-perfused scaffolds (p<0.05), however the level of expression was lower than that of day 1 culture (p<0.05).

Figure 4.2. α-SMA production in perfused (dynamic) vs. non-perfused (static) D-PHI scaffold over 28 days of culture. n = 9, ±standard error (SE). *Significantly different from each other (p<0.05).

Immunohistochemistry images show that, at day 28 of dynamic culture, Col I was well distributed in the 3D perfused, 2 mm-thick scaffold (Fig. 4.3b) when compared with the non-perfused scaffold (Fig. 4.3a), where Col I appeared predominantly near the top of the scaffold. Staining of α-SMA showed that there was more α-SMA-positive cell infiltration in the perfused
(Fig. 4.3d) vs. non-perfused scaffolds (Fig. 4.3c). The DAB peroxidase substrate (brown),
which was used to detect positive staining cells, did not bind non-specifically with the D-PHI
scaffold (Supplementary Fig. 4.2a). [Immunofluorescence imaging of the 3D scaffold sections
(Fig. 4.3e and 4.3f) showed that some Col I content appeared in locations where HGFs (as
stained by FSP and Hoechst) were not present. Since the unsecreted and extracellularly
deposited Col I can be recognized by the Col I antibody, the separation of some of the Col I
stains from the FSP stains suggest that] extracellular Col I deposition occurred in both culture
conditions. Supplementary data (Supplementary Fig. 4.2b) show that the secondary antibodies
did not bind with the D-PHI scaffold while Hoechst 33342 was bound.

*Modification of published text.
Figure 4.3. Immunohistochemistry images of HGFs cultured in (a, c) non-perfused (static) and (b, d) perfused (dynamic) D-PHI scaffolds at day 28 of culture. HGFs were stained for (a – b) Col I and (c – d) α-SMA. The area of low Col I content in (a) is indicated below the dotted line. Immunofluorescence images of HGFs cultured in (e) non-perfused and (f) perfused D-PHI
scaffolds. HGFs were stained for FSP (green, indicated with white arrow heads) and Col I (red), with the nuclei and scaffolds stained with Hoechst (blue). (a – d) Scale bar = 500 µm, (e – f) Scale bar = 60 µm.

**Supplementary Figure 4.2.** Control images of D-PHI scaffolds with HGFs in static culture stained without the primary antibodies for (a) immunohistochemistry and (b) immunofluorescence. Non-specific binding of the DAB peroxidase substrate (in (a)) and the secondary antibodies (in (b)) was not observed. The cell nuclei and scaffolds were stained with (a) hematoxylin and (b) Hoechst (blue). (a) Scale bar = 500 µm, (b) Scale bar = 60 µm.

Taken together, the Western blotting and localization data showing decreased α-SMA expression and enhanced Col I distribution by HGFs in the perfused D-PHI scaffold indicate that the combination of the D-PHI material and medium perfusion inhibit myofibroblast differentiation while supporting collagen production.

4.4.3 Both TGF-β1 and FGF-2 were increased in the dynamic culture

The mechanisms by which α-SMA and Col I production was regulated by perfusion condition were investigated by examining the concentration of growth factors in the conditioned medium from static and dynamic cultures. The concentration of TGF-β1, a potent inducer of α-SMA, was ~6-fold greater in dynamic vs. static cultures throughout the 28-day culture period (p<0.01;
Fig. 4.4). Notably, the level of FGF-2, a growth factor that may antagonize the effect of TGF-β1 in regulating α-SMA expression (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999), was also higher in the dynamic culture at day 1 when compared with static culture (p<0.05; Fig. 4.5). However the production decreased over time of culture.

**Figure 4.4.** The production of TGF-β1 from HGFs cultured in static and dynamic cultures. n = 9, ±SE. *Significantly different from each other (p<0.01).

**Figure 4.5.** The production of FGF-2 from HGFs cultured in static and dynamic cultures. n = 6, ±SE. ‡Significantly different from each other (p<0.05).
The implication of Smad2/3 associated with the effect of perfusion on myofibroblast differentiation was examined since TGF-β1-driven expression of α-SMA involves the Smad2/3 signalling pathway (Galie et al., 2012; Hinz, 2007; Hinz, 2010; Horan et al., 2008; Tomasek et al., 2002). Inhibition of Smad3 by SIS3 showed no statistical change in α-SMA production for either static or dynamic cultures (p = 0.52 and p = 0.69 respectively; Supplementary Fig. 4.3).

**Supplementary Figure 4.3.** α-SMA content (normalized to Vim) of HGFs cultured in perfused (D0 Dyn) or non-perfused (D0 Stat) D-PHI scaffolds, inhibited with 3µM of SIS3. HGFs cultured in untreated medium (SIS3-) was used as negative control. Data was obtained at day 0 of culture (D0, i.e. 24 hours after seeding) after treatment with or without SIS3. n = 9, ±SE.
4.4.4 Level of total β1-integrin was increased and FAK was activated in the dynamic culture

[Mechanical stimuli produced by medium perfusion]* may be transduced to cells via focal adhesion, in which integrins are key components (Galie et al., 2012; Ng et al., 2005). Since binding of the β1-integrin by matrix ligands is associated with α-SMA and collagen expression (Langholz et al., 1995; Liu et al., 2009; Liu et al., 2010), the levels of total β1-integrin in static and dynamic cultures was examined. In Fig. 4.6, the total β1-integrin production was greater in the dynamic vs. static culture (p<0.05). Since inhibition of myofibroblast differentiation was observed after day 1 of culture and there was a difference in α-SMA production between the static and dynamic cultures at day 14, antibody-mediated inhibition of the β1-integrin over the first 14 days of culture was carried out (Fig. 4.7). The data showed that the inhibition was associated with decreased α-SMA production at day 14 in the perfused D-PHI scaffold (p<0.01). The α-SMA levels were statistically similar with or without β1-integrin inhibition in the static culture at day 14. The results indicate that the modulation of α-SMA and Col I expression observed in Figs. 4.2 and 4.3 may be associated with the β1-integrin. Further, there was a higher level of pFAK in cells cultured in perfused compared with non-perfused scaffolds (p<0.05; Fig. 4.6), supporting the notion that focal adhesions are involved in the signalling process by which D-PHI scaffolds interact with HGFs and modulate the expression of α-SMA and Col I.

*Modification of published text.
Figure 4.6. The total β1-integrin production and FAK phosphorylation (pFAK) was measured 2h after the initiation of the dynamic culture. For static culture, the samples remained in the 96-well plate and were taken out at the same time as those in the dynamic culture. For β1-integrin, the results were normalized to Vim; for pFAK, the results were normalized to total FAK. n = 9, ±SE. *Significantly different from static culture (p<0.05).
Figure 4.7. α-SMA content at days 1 and 14 with or without antibody-mediated inhibition of β1-integrin in static and dynamic cultures. Data were normalized to day 0 values. ‘+’: with antibody; ‘-’: without antibody. Significantly different from each other (*: p<0.05; ň: p<0.001). n = 6, ±SE.

4.5 Discussion

The current study investigated HGF interaction within the perfused D-PHI scaffolds and defined some of the mechanistic pathways affecting the production of key markers in the dynamic culture. The results from this study suggest that on the D-PHI material, HGFs retain their responsiveness to medium perfusion, thereby establishing a fibroblast phenotype favouring tissue regeneration through the coordinated actions of TGF-β1, FGF-2, β1-integrin and FAK.

4.5.1 Perfused D-PHI scaffolds inhibit myofibroblast differentiation

The contractile nature of myofibroblasts in normal wound healing is desirable for wound closure and contraction of the nascent collagen matrix (Hinz, 2010); while in fibrotic diseases such as lung fibrosis, the persistence of myofibroblasts may lead to tissue and organ dysfunction (Hinz, 2010; Tomasek et al., 2002). In tissue regeneration, the initial presence of myofibroblasts is desired for wound contraction (i.e. α-SMA positive), increased collagen production, and growth factor secretion (e.g. TGF-β) for promoting tissue repair and vasculogenesis (Hinz, 2010; Tomasek et al., 2002). Beyond the acute phase of connective tissue regeneration, the
myofibroblast population is expected to be replaced by fibroblasts so that the tissues can be
restored to their natural state (i.e. collagogenous but non-contractile) (Tomasek et al., 2002). [Mechanical stimuli have] been shown to modulate the fibroblast-myofibroblast transition (Shi et al., 2007). Hence it is notable that the D-PHI material positively affects the response of HGFs (phenotypically and functionally) to [mechanical stimuli provided by medium perfusion such as shear stress].

Recently, in addition to the delivery of exogenous growth factors such as FGF-2 (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999), the design of biomaterials has increasingly focused on suppressing myofibroblast differentiation. For example, Acharya et al. conjugated silk fibroin with lactose to fabricate scaffolds that inhibit myofibroblast differentiation, thereby promoting more attachment of fibroblasts than myofibroblasts (Acharya et al., 2008). With regards to substrate compliance, Dreier et al. found that a more compliant 2D, collagen-coated polyacrylamide substrate (4 – 71 kPa) can inhibit TGF-β-induced myofibroblast differentiation (Dreier et al., 2013), which further emphasizes the unique character of the biomaterial to influence the cell’s phenotype. However, the degradation rate of silk fibroin and collagen matrices in vivo can be rapid (17 days to a year for silk fibroin (Cao and Wang, 2009) and 10 – 20 days for collagen (Laurent, 1987)), and the biodegradation of the scaffolds made of these materials can greatly alter their structural integrity for tissue reconstruction.

The present study demonstrated lower α-SMA expression by HGFs on 2D D-PHI films when compared with TCPS after 24 hours of culture. This dominant feature of D-PHI is retained with or without perfusion within the 3D D-PHI scaffolds, as α-SMA expression was decreased in both static and dynamic cultures with 3D D-PHI scaffolds over 28 days. The reported data indicate that the D-PHI material promotes loss of the myofibroblastic phenotype in HGFs as early as 24 hours after culture on the material, thus an explicit trend in α-SMA production was not observed over the 28-day culture period. It should be noted that although the immunohistochemical staining showed more α-SMA-positive cells in the dynamic (perfused) culture when compared with static culture at day 28, the Western blotting data conveyed that the level of α-SMA at the latter time point was significantly lower when compared with day 1 of dynamic culture. When compared with the previously reported silk and collagogenous biomaterials,

*Modification of published text.
D-PHI showed similar ability to inhibit myofibroblast differentiation, and it exhibited a slower degradation rate than those materials (i.e. 21 wt.% 100 days after implantation in vivo (McBane et al., 2011)) while retaining the porous structure in the scaffold after degradation.

In response to flow, fibroblast proliferation and collagen production was increased when cultured under perfusion (at 600 µL/min) (Mathes et al., 2010). Flow can also mechanically stimulate cells to release greater levels of FGF-2 on fibronectin-coated polystyrene (Sterpetti et al., 1994), thereby potentially inhibiting myofibroblast differentiation. Within the 3D D-PHI scaffolds, flow stimulus induced similar cell response in HGFs over the 28-day culture when compared with the above studies. The inhibition of myofibroblast differentiation in HGFs on D-PHI is beneficial for tissue repair as natural soft connective tissues are non-contractile (Tomasek et al., 2002).

Medium perfusion can enhance collagen production from fibroblasts cultured in collagen sponges (Ng et al., 2005). It has been shown that Col I production was greater in perfused vs. non-perfused 3D D-PHI scaffolds at day 28 (Cheung et al., 2013). The increase of Col I content in the perfused culture and its enhanced extracellular deposition throughout the perfused scaffold by day 28 further suggested that HGFs were phenotypically fibroblastic, which is a desired state for gingival tissue repair as the regeneration of gingival connective tissues require collagen production without fibrotic tissue formation.

4.5.2 High levels of TGF-β1 and FGF-2 in perfused culture regulates α-SMA production

TGF-β1 is an important growth factor that regulates cell growth, differentiation, motility, vasculogenesis, and extracellular matrix production (Mallet et al., 2006; Washio et al., 2011). High levels of TGF-β1 can induce myofibroblast differentiation (Hinz, 2007; Hinz, 2010). Studies have shown that [perfusion] can stimulate TGF-β1 expression in endothelial cells (Lum et al., 2000; Ohno et al., 1995); thus it is anticipated that HGFs in the perfused D-PHI scaffold can be stimulated by perfusion to produce high levels of TGF-β1. Indeed, Fig. 4.4 supports that TGF-β1 levels were significantly increased in the perfused D-PHI scaffolds. While [perfusion] and α-SMA expression have been linked to TGF-β1 in some studies (Hinz, 2007; Lum et al.,

*Modification of published text.*
high α-SMA expression at day 1 was not observed in the perfused culture (despite elevated TGF-β1 level) when compared to the non-perfused condition, and nor was it observed later at 28 days in the culture. It is speculated that the observed α-SMA expression might have been regulated by other factors such as specific cell-material interactions with D-PHI or a combination of material response with other growth factors (e.g. FGF-2), which explains the temporal difference between perfused and non-perfused conditions on this specific biomaterial.

FGF-2 has been shown to decrease α-SMA production (Akasaka et al., 2007; Ishiguro et al., 2009; Khouw et al., 1999). Under perfusion, FGF-2 release can be augmented, which can inhibit the formation of myofibroblast (Sterpetti et al., 1994). By introducing 0.44 ng/mL of FGF-2 to the fibroblast culture, Khouw et al. showed the suppression of TGF-β-induced myofibroblast differentiation in vitro (Khouw et al., 1999). Also, by introducing FGF-2 to open skin wounds, α-SMA expression and the myofibroblast population were decreased in the wound areas (Ishiguro et al., 2009). Fig. 4.5 demonstrated that the concentration of FGF-2 was significantly higher in the dynamic vs. static culture in most of the culture period, particularly at day 1. However the decreasing FGF-2 levels over the 28-day culture period suggest that decreased α-SMA expression was influenced not only by the elevated FGF-2 concentration but also by other factors, potentially including the D-PHI material itself.

Previous studies showed that TGF-β1 induced Col I production in dermal (Washio et al., 2011) and atrial fibroblasts (Tsai et al., 2011). As medium perfusion can enhance TGF-β1 production (Galie et al., 2012), the elevated levels of TGF-β1 observed in the dynamic culture may well explain the increased Col I production in the dynamic vs. the static culture.

Given that TGF-β1 was increased in the dynamic culture, it is speculated that the Smad signalling pathway was activated (Galie et al., 2012; Hinz, 2007; Hinz, 2010; Horan et al., 2008; Tomasek et al., 2002). However, the intentional inhibition of Smad3 at day 0 did not alter α-SMA expression for either type of culture relative to their respective controls, concluding that the Smad pathway may not be the prominent signaling pathway driving the observed findings in the perfused culture on D-PHI scaffolds. Kretzschmar et al. has reported that FGF-2 can activate the ERK 1/2 signaling pathway (Kretzschmar et al., 1999), which can inhibit TGF-β/Smad signaling, thereby suppressing α-SMA expression (Ishiguro et al., 2009). ERK 1/2 activation
can occur within the first 5 minutes of FGF-2 stimulation (Ishiguro et al., 2009). Hence, it is possible that a higher FGF-2 level in the dynamic vs. static culture at early culture time may have inhibited the Smad pathway via the ERK 1/2 pathway, irrespective of the observed high TGF-β1 level. [TGF-β1 can also induce α-SMA expression independent of Smad (Tomasek et al., 2005).]“

4.5.3 β1-integrin and FAK may mediate α-SMA and Col I expression in the dynamic culture

Proteins such as integrins and focal adhesion kinase (FAK) that are enriched in focal adhesions, are essential to the survival of anchorage-dependent cells, and affect proliferation, migration, and protein production (Li and Hua, 2008; Schlaepfer et al., 1999). Aside from TGF-β1 and FGF-2, β1-integrin and FAK can influence α-SMA and Col I expression (Hinz, 2010). β1-integrins are responsible for collagen binding (Langholz et al., 1995). Knocking out β1-integrin has been shown to result in decreased collagen expression and delayed wound healing (Liu et al., 2009; Liu et al., 2010), suggesting its relevant role in the regulation of collagen synthesis. Integrins are also major players in mechanotransduction (Chan et al., 2009; Hinz, 2010; Tomasek et al., 2002; Wipff and Hinz, 2009). FAK is widely known to be co-localized with integrins at the cell adhesion sites in fibroblasts (Schlaepfer et al., 1999) and it is closely related to β1-integrin via linkage proteins such as talin (Schlaepfer et al., 1999; Sieg et al., 2000). Consequently FAK is believed to be related to integrin-stimulated signaling events and is a downstream signaling molecule of integrin activation (Tomasek et al., 2002). Further, [interstitial flow]“ can stimulate FAK-ERK signaling via the β1-integrin, which in turn influences the proliferation and orientation of fibroblasts on collagen matrices (Galie et al., 2012; Ng et al., 2005).

As active integrin-FAK signaling has been reported to occur within hours of culture (Schlaepfer et al., 1999), the total β1-integrin production and phosphorylation of FAK were measured after two hours of culture. **Fig. 4.6** shows that β1-integrin and pFAK were significantly increased in the early perfused culture in the D-PHI scaffold when compared with the non-perfused culture, indicating that [mechanical stimuli from medium perfusion may have been transduced]“ to HGFs via focal adhesion, thus promoting collagen production and myofibroblast differentiation

*Modification of published text.*
in the early culture time. It should be noted that there are also non-integrin stimuli that may increase FAK phosphorylation such as growth factors (e.g. VEGF and PDGF) and other actin-related proteins (e.g. Rho) (Schlaepfer et al., 1999; Sieg et al., 2000).

The decrease in α-SMA expression by the intentional antibody-mediated inhibition of the β1-integrin further supports that β1-integrin mediates α-SMA expression in the dynamic culture. It is interesting to note that the early inhibition of β1-integrin did not affect α-SMA expression at day 1 but did so at a later time point (day 14). This would indicate that fibroblasts possibly used different adhesion proteins or more than one types of integrin for cell attachment over the course of the whole culture period in D-PHI scaffolds. Along with β1-integrin, it has been shown that αvβ3 and αvβ5 integrins are also present in supermature focal adhesion, which is one of the characteristics of myofibroblasts (Hinz, 2010; Tomasek et al., 2002). Thus these two types of integrins may act as alternative mechanosensors for myofibroblasts at early time points when β1-integrin was inhibited. Over the course of the 28-day dynamic culture period, the loss of the myofibroblast phenotype in HGFs could potentially lead to the disappearance of supermature focal adhesion, thereby changing the composition of focal adhesion structures (e.g. types of integrins for adhesion), and this could be further investigated in on-going work.

4.6 Conclusion

In summary, the dynamic culture system with D-PHI scaffolds inhibited myofibroblast differentiation while enhancing Col I distribution in the scaffold over 28 days of culture. High levels of TGF-β1 are believed to increase α-SMA expression, but the D-PHI material and higher FGF-2 level observed in the day 1 of dynamic culture might have regulated α-SMA production, thereby mediating the phenotypic change in HGFs. The changes in α-SMA and Col I expression in the perfused D-PHI scaffolds may also be mediated by increased total β1-integrin production and phosphorylation of FAK, indicating that flow stimulus was mechanically transduced to HGFs via focal adhesion. The perfused D-PHI scaffolds aid in establishing a gingival fibroblast phenotype which favours the potential generation of a functional tissue-engineered construct that may have future use in the repair of periodontal wounds.
4.7 Acknowledgments

This study was supported by a Natural Sciences and Engineering Research Council (NSERC) Discovery grant (360520), NSERC Alexander Graham Bell Canada Graduate Scholarships (CGS D3), Ontario Graduate Scholarship Program, and Canadian Institutes of Health Research-CellSignals Training Fellowship (STP-53877).
4.8 References


Chapter 5
Pro-angiogenic character of endothelial cells and gingival fibroblasts co-cultures in perfused degradable polyurethane (D-PHI) scaffolds

5.1 Foreword

Gingival atrophy manifests as exposure of the tooth root surface because of recession of the gingiva, a condition that affects >20% of adults and leads to increased root sensitivity and ultimately, tooth loss. Tissue engineering approaches that employ novel synthetic polymeric scaffolds are being considered for rebuilding the gingival lamina propria lost in the atrophic process. Specifically, polyurethane hydrogels (degradable/polar/hydrophobic/ionic polyurethane (D-PHI)) can enhance the proliferation of human gingival fibroblasts (HGFs) and collagen production in a perfusion system. However, few studies have assessed the potential of synthetic block co-polyurethanes to initiate blood vessel formation in an in vitro bioreactor system. As the gingival lamina propria is highly vascular, a co-culture system of human umbilical vein endothelial cells (HUVECs) with HGFs was used in perfused D-PHI scaffolds to determine the feasibility of initiating vascularization. Culture conditions were optimized for driving co-cultures towards the desired tissue-engineered construct. HUVEC-HGF co-culture in perfused D-PHI scaffolds with a cell seeding density of at least 80,000 cells/ scaffold in a 50/50 mix of HUVEC and HGF media (by vol.) exhibited enhanced cell growth and increased VEGF and FGF-2 production, as well as reduced myofibroblast differentiation. A greater fibroblast proportion (seeding ratio of 1:2) in the co-culture resulted in HUVEC cluster formations and increased TGF-β1 and FGF-2 production. The combined pro-angiogenic effects provided by these culture conditions are anticipated to be important in the development of a highly vascularized tissue-engineered construct for regenerating the gingival lamina propria and possibly other soft tissues.

This chapter has been published in ‘Tissue Engineering Part A’ as: Cheung JWC\textsuperscript{1}, Jain D\textsuperscript{1}, McCulloch CAG\textsuperscript{2}, Santerre JP\textsuperscript{1,2}. Pro-angiogenic character of endothelial cells and gingival fibroblasts co-cultures in perfused degradable polyurethane (D-PHI) scaffolds. Tissue Eng Part A 2015. (Article in press).
5.2 Introduction

Gingival atrophy is a high prevalence disorder (Albandar and Kingman, 1999; Kassab and Cohen, 2003; Sarfati et al., 2010; Susin et al., 2004) that is associated with multiple factors including tooth malalignment, alveolar bone dehiscences, chronic trauma, aging and smoking (Camargo et al., 2001; Chambrone et al., 2010; Graziani et al., 2014; Wennström, 1996). The exposed root surfaces are clinically associated with increased root sensitivity and root caries (Chambrone et al., 2010; Kassab and Cohen, 2003; Tonetti et al., 2014), and negatively impact the quality of life in regard to aesthetics (Chambrone et al., 2010; Tonetti et al., 2014). Current treatment for gingival atrophy often utilizes soft connective tissue grafts (Chambrone et al., 2010; Graziani et al., 2014; Harris, 1992; Tonetti et al., 2014; Tözüm et al., 2005) but their utilization is often limited by donor site morbidity and lack of sufficient donor tissue (Camargo et al., 2001; Chambrone et al., 2010; Karring et al., 1975; Moharamzadeh et al., 2007; Paolantonio et al., 2002; Rastogi et al., 2009; Tonetti et al., 2014). To rebuild the lost gingival lamina propria, tissue engineering strategies are potential alternative treatment approaches.

Over the past decade, there has been considerable interest in the use of degradable polymeric scaffolds for tissue engineering (Aframian et al., 2002; Blackwood et al., 2008; Buurma et al., 1999; Bäumchen et al., 2009; Dhandayuthapani et al., 2011; Meretoja et al., 2012; Williams et al., 2005). In addition to the more traditional poly(lactic-co-glycolic acid) (PLGA) materials (Aframian et al., 2002; Blackwood et al., 2008; Buurma et al., 1999; Bäumchen et al., 2009; Dhandayuthapani et al., 2011), recent work has focused on developing novel polymers and co-polymers that minimize or control the release of lactic acid, a pro-inflammatory stimulant (Yang et al., 2001; Yang et al., 2005). The authors’ recent work has explored degradable polyurethane hydrogels such as the degradable/polar/hydrophobic/ionic polyurethane (D-PHI), which has been shown to reduce the release of inflammatory cytokines from monocytes.
(McBane et al., 2011a), and promote the growth of smooth muscle cells in a contractile phenotype under cyclic mechanical strain (Sharifpoor et al., 2011). D-PHI scaffolds also display good biocompatibility in vivo (McBane et al., 2011b), and enhance endothelial cell (EC) incorporation into capillaries that are formed in subcutaneous mouse implants (McDonald et al., 2011). For potential gingival tissue regeneration, D-PHI scaffolds enhanced the proliferation of human gingival fibroblasts (HGFs) and collagen production under medium perfusion (Cheung et al., 2013), as well as inhibiting myofibroblast differentiation (Cheung et al., 2014).

The gingival lamina propria is highly vascular (Moharamzadeh et al., 2007). The diffusion of nutrition and oxygen from surrounding tissues is limited to only 150 µm² (Awwad et al., 1986) and the in-growth of blood vessels from the host to implanted scaffolds was very slow, which resulted in necrosis at the center of the construct. Accordingly, perfusion systems and methods to promote vascularization should be considered at the time of engineering the tissue constructs.

ECs are essential for building and maintaining tissue vasculature, and the percent survival and proliferation of ECs are increased when exposed to appropriate shear stress (Ando and Yamamoto, 2009; Davies, 1995). Hence, perfused cultures not only can facilitate metabolic exchange, but they can provide mechanical cues (e.g. flow-induced shear stress) to ECs for inducing vascularization.

The co-culture of ECs and fibroblasts within specific synthetic biomaterials (e.g. PLGA (Kaully et al., 2009)) has been shown to promote capillary network formation. Fibroblasts generally can enhance angiogenesis by depositing a collagen-rich extracellular matrix (ECM) for EC tubulogenesis (Bae et al., 2012; Davis et al., 2002; Sorrell et al., 2007), by growth factor release such as vascular endothelial growth factor (VEGF) (Bae et al., 2012; Hughes, 2008; Yamada et al., 2006), transforming growth factor (TGF)-β1 (Bae et al., 2012; Cornelini et al., 2003; Hughes, 2008; Smith and Martínez, 2006), basic fibroblast growth factor (FGF-2) (Taba Jr. et al., 2005), nitric oxide (Li and Chang, 2013), and others, and by cell-cell contact (Baiguera and Ribatti, 2013). Conceivably, the co-culture of ECs with fibroblasts in an appropriate biomaterial scaffold could serve as a signal-delivery system for angiogenesis without the use of exogenous biochemical stimuli.
Despite the success in establishing vascularized structures with co-cultures, specific culture conditions (e.g. choice of culture medium, cell seeding ratio) are not well defined and the influence of specific biomaterials on these cultures needs to be further investigated. Culture conditions must be systemically established for each unique cell source and the biomaterial of interest. The aim of the current study is to investigate the effect of perfusion, cell seeding density, culture medium and cell seeding ratio on the co-culture of HGFs with HUVECs in the D-PHI scaffolds. The end point is to identify a set of favourable conditions needed to promote cell proliferation, and structures that would be conducive for capillary formation and the production of angiogenic factors. It is hypothesized that at a cell seeding density of 80,000 cells/D-PHI scaffold (12.95 mg, 80% porosity), cells cultured in a 50/50 mix (by volume) of the HUVEC and HGF media and seeded at a greater proportion of HGFs within the perfused D-PHI scaffolds will enhance cell proliferation, promote HUVEC clustering/structural formation, and increase angiogenic factor production. All of these parameters are key factors associated with the development of a functional, highly vascularized soft tissue-engineered construct for gingival tissue regeneration.

5.3 Materials and methods

5.3.1 D-PHI scaffold fabrication

D-PHI porous scaffolds were fabricated using a divinyl oligomer (DVO), which was synthesized in house by reacting polyhexamethylene carbonate diol (Sigma-Aldrich, United States of America) with lysine diisocyanate (Kyowa Yuka, Yokkaichi City, Japan) and 2-hydroxyethyl methacrylate (Sigma-Aldrich) in a 1:2:2 ratio (Sharifpoor et al., 2009). The DVO was then reacted with methacrylic acid (Sigma-Aldrich) and methyl methacrylate (Sigma-Aldrich) in a free radical polymerization at a 1:5:15 molar ratio under dry conditions, with benzoyl peroxide (Sigma-Aldrich) as the initiator (Sharifpoor et al., 2009). Poly(ethylene glycol) (Sigma-Aldrich, 10 wt.%) and sodium bicarbonate (Sigma-Aldrich, 70 wt.%, particle size of 105 – 420 µm) were used as the porogens. The polymer resin was molded into circular discs, which were cured at 110°C for 24h under dry conditions. The resulting scaffolds were subjected to sonication in distilled water for 14 days in order to leach porogens. Each scaffold had a diameter of 6 mm, a thickness of 2 mm, and an average mass of 12.95 mg with 80% porosity (Sharifpoor et al., 2009).
5.3.2 Culture of HGF and HUVEC cell lines and perfused (dynamic) co-culture

HGFs (HGF-1 cell line, ATCC CRL-2014) were cultured in Dulbecco’s modified Eagle Medium (DMEM; GibcoBRL) supplemented with 100 U/mL penicillin/100 µg of streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS, Sigma-Aldrich). For HUVEC culture, a HUVEC cell line (HUV-EC-C, ATCC CRL-1730) was used. The cells were cultured in Kaighn's Modification of Ham's F-12 Medium (F-12 K Medium) supplemented with 10% FBS, 100 U/mL penicillin/100 µg of streptomycin, 0.1 mg/mL heparin (Sigma-Aldrich), and 0.05 mg/mL EC growth supplement (Sigma-Aldrich).

Prior to seeding, the D-PHI scaffolds were treated with 70% ethanol overnight, followed by drying under sterile laminar flow conditions for 24h. The scaffolds were then pre-wetted with sterile culture medium for 24h at 37°C with 5% CO₂ in a sterile, humidified environment. When HUVECs (passage 3-4) and HGFs (passage 4-6) reached 80-90% confluence, they were trypsinized, mixed and seeded onto the pre-conditioned scaffolds. Unless otherwise stated, HUVECs and HGFs were seeded at a 1:1 ratio. The seeded scaffolds were initially cultured for 24h in a 96-well plate (static condition) at 37°C with 5% CO₂. Following 24h of incubation (denoted as day 0), the scaffolds were transferred to the custom-built bioreactors (Cheung et al., 2013), which were perfused at 500 µL/min for up to 28 days (dynamic co-culture). The bioreactor system used a peristaltic pump; and the bioreactor chamber contains slots for three D-PHI scaffolds which are arranged in a parallel circuit. Medium was changed every 2 to 3 days. Seeded scaffolds cultured without flow were used in some experiments for comparison with perfused culture.

5.3.3 VEGF enzyme-linked immunosorbent assay (ELISA) for HGF monoculture study

To determine the angiogenic potential of HGFs in perfused D-PHI scaffold, conditioned medium from static and dynamic HGF monocultures (seeding density: 40,000 cells/scaffold) were analyzed for VEGF release by commercial human VEGF ELISA kit (R&D Systems Inc., USA). This ELISA kit measures VEGF-A isoforms VEGF_{165} and VEGF_{121}. Conditioned medium was collected at day 0 (24 hours after seeding) and at different time points up to day 28 of culture. Due to the potential for dilution of signal with the large sample volume of the dynamic cultures, 10 mL of conditioned medium was concentrated to 500 µL using Amicon
Ultra-15 centrifugal filters (with 10kDa cut-off, Millipore, USA). The absorbance of the samples was measured using a VersaMax tunable microplate reader (Molecular Devices Corp.) with the wavelength set to 450 nm and background at 540 nm, and compared to a standard curve in order to quantify the amount of growth factor in the samples. Background absorbance emitted from the controls (i.e. no cells seeded) was subtracted from the reading to obtain the reported value. The production levels were normalized to day 0 values. For all measurements, based on previous studies, three independent experiments (with three repeats each) were performed to enable statistical analysis. The measurement of growth factors in HUVEC monoculture was not included in this study as, based on previous experiments (unpublished data), HUVECs typically do not survive beyond day 7 of culture without the stimulus of co-culture with other cell types.

5.3.4 Cell seeding density

A mixture of HUVECs and HGFs (1:1 ratio) was seeded at 40,000 cells (i.e. 20,000 HUVECs and 20,000 HGFs) or 80,000 cells (i.e. 40,000 HUVECs and 40,000 HGFs) per scaffold for comparison. The lower seeding density was selected to benchmark against previous monoculture studies of HGFs (Cheung et al., 2013; Cheung et al., 2014). The cell-seeded scaffolds were cultured under medium perfusion for up to 28 days.

5.3.5 DNA quantification

DNA content was quantified at day 0 (24 hours after seeding) and defined time points up to day 28 of the co-culture. Samples were rinsed with phosphate buffer saline (PBS) and placed separately into micro-centrifuge tubes. The samples were then minced and cells were lysed with cell lysis buffer (100 mM NaCl, 100 mM Tris-HCl, 25 mM EDTA, 0.1% SDS). The samples were placed in a 65°C water bath for 1 hour to allow cell lysis buffer to penetrate and lyse the cells, followed by centrifugation for 15 minutes at 8°C to allow the scaffold pieces to settle. Finally, cell lysates from each sample were added to a black polypropylene, flat-bottomed 96-well plate (Thermo Scientific) containing the DNA dye Hoechst 33258 (Sigma-Aldrich), which was quantified with a fluorescence microplate reader (excitation wavelength = 360 nm; emission wavelength = 460 nm; Bio-Tek® FL600). The fluorescence values were compared to a standard curve (calf thymus DNA, Sigma-Aldrich) to estimate DNA content. The background fluorescence emitted from the scaffolds (i.e. controls with no cells seeded) was subtracted from the reading to obtain the reported value. For all measurements, based on previous studies, three
independent experiments were performed to enable statistical analysis (n = 9 for cell seeding density and culture medium studies, n = 8 for cell seeding ratio study).

5.3.6 Cell culture medium

Two types of culture media were compared. HUVECs and HGFs were seeded at 80,000 cells per scaffold. The cell-seeded scaffolds were either cultured under perfusion in a 50/50 mix (by volume) of F-12 K medium and DMEM, or in DMEM only for up to 28 days. The choice of culture medium was selected based on previous studies (Choong et al., 2006; Salem et al., 2002). In a previous study, HGFs displayed abnormal morphologies (i.e. not spindle-shaped) when cultured in F-12 K medium alone (Supplementary Fig. 5.1); hence this type of medium was not used by itself for comparison.

Supplementary Figure 5.1. The monoculture of HGFs on D-PHI (40,000 cells/ sample) cultured in F-12 K medium only. HGFs were stained for FSP (green). The morphologies of HGFs were abnormal (i.e. not spindle-shaped). Scale = 60 µm.

5.3.7 Water soluble tetrazolium assay

The metabolic activity of co-culture samples was quantified using a water soluble tetrazolium-1 (WST-1) assay (Roche Diagnostics, Laval, QC). The samples were placed in a 96-well plate and rinsed with PBS. DMEM, with no phenol red, was first added to each sample, followed by the addition of the WST reagent. The samples were incubated at 37°C with 5% CO₂ for 30 minutes, and then the well-plate was placed on a shaker to mix gently for 1 minute. The solution in the wells was transferred to a new 96-well plate. The absorbance was read with a spectrophotometer (Molecular Devices Corp.) at 450 nm with a reference wavelength of 650 nm. For all
measurements, based on previous studies, three independent experiments were performed to enable statistical analysis (n = 9 for culture medium study, n = 8 for cell seeding ratio study).

5.3.8 Immunofluorescence

Samples from the day 28 co-cultures were rinsed with Dulbecco’s-PBS (pH 7.4), fixed in 4% paraformaldehyde, dehydrated, paraffin embedded, and sectioned at 20 µm thickness to preserve the scaffold’s porous structure (Faculty of Dentistry, University of Toronto, ON).

Immunofluorescence staining for von Willebrand factor (vWF), cluster of differentiation 31 (CD31), fibroblast surface protein (FSP), type I collagen (Col I), α-smooth muscle actin (α-SMA), and caspase 3 was carried out. The 20 µm-thick sections of scaffold were deparaffinized and rehydrated, followed by incubation with 0.1% trypsin-CaCl₂ (Sigma-Aldrich) at 37°C for 30 min for antigen-unmasking. Following an initial blocking in 3% bovine serum albumin in PBS at 37°C for 20 min, the sections were incubated overnight at 4°C in 0.3% Triton X-100 containing a combination of mouse anti-FSP (Abcam, diluted 1:300), rabbit anti-vWF (Abcam, diluted 1:100), rabbit anti-CD31 (Abcam, diluted 1:50), mouse anti-α-SMA (Abcam, diluted 1:25), rabbit anti-Col I (Abcam, diluted 1:100), and/or rabbit anti-caspase 3 (New England Biolabs, diluted 1:200). The samples were subjected to a second blocking in 10% goat serum albumin for 30 min at room temperature, and were then incubated in 10% goat serum containing Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, diluted 1:200) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrogen, diluted 1:200). Hoechst 33342 (Invitrogen, H3570, diluted 1:1000) was used to label DNA in nuclei but it also stained the porous scaffold. The background staining of scaffolds was minimized to enhance visualization of cell nuclei, which were readily distinguished on the basis of their oval shapes (see Appendix C). Scaffold sections were mounted with PermaFluor (Thermo Scientific). All samples were viewed with a fluorescence microscope (DM-IRE2, Leica Microsystems, Germany).

For image capture, counting of α-SMA-positive cells and quantification of Col I coverage, three independent experiments were performed in triplicate for each time point and condition. For HUVEC and HGF counting in the immunofluorescence images, two to three regions of interest were imaged per scaffold section, for up to a total of four sections for each time point and condition to enable statistical analysis.
Control samples consisted of cell-scaffold sections without primary antibodies and cell-free scaffold sections with both primary and secondary antibody to assess background fluorescence (Sharifpoor et al., 2011). For generating a caspase 3-positive control (i.e. an apoptotic sample), HGF monoculture was treated with 1µM of staurosporine (Sigma) for 4 hours to induce apoptosis (Kainulainen et al., 2002).

5.3.9 HUVEC and HGF counting in immunofluorescence images

Images from day 0 and day 28 co-culture samples in each condition were stained for CD31 (HUVECs) and FSP (HGFs), and counterstained with Hoechst 33342. Cell counts were performed using the ImageJ software (version 1.47). Only cells that were positive for both CD31 and Hoechst or FSP and Hoechst were counted. Regions of interest were chosen from the outer to middle locations of the scaffold sections (where most cells were located) and nine entire images (under same magnification (5x)) were counted for each condition (see section 5.3.8 for image capture and sample size). The regions of interest were also magnified at 15x to facilitate counting.

5.3.10 Quantification of α-SMA-positive cells (myofibroblasts) and type I collagen (Col I) coverage in immunofluorescence images

Myofibroblasts in the co-cultures of each condition were stained for α-SMA (Cheung et al., 2014; Hinz, 2007). α-SMA-positive cells, which were also positive for Hoechst, in the day 0 (24 hours after seeding) and day 28 co-culture samples in each condition were counted with the ImageJ software (version 1.47). Cell counts at day 28 were normalized to day 0 values.

For quantification of Col I coverage at days 0 and 28 of co-culture for each condition, Col I stained images were binary masked with ImageJ, which was followed by area measurement using the same software. The area was normalized to the amount of cell nuclei counted in the same image.

For both analyses, regions of interest were chosen from outer to middle locations of the scaffold sections (where most cells were located) and nine entire images (under same magnification (5x)) were used for quantification for each condition (see section 5.3.8 for image capture and sample size). The regions of interest were also magnified 15x to facilitate counting.
5.3.11 ELISAs for co-culture studies

Conditioned medium from each time point and condition in the HUVEC-HGF co-cultures was analyzed for VEGF, TGF-β1 and FGF-2 production by commercial human VEGF, TGF-β1 and FGF-2 ELISA kits respectively (R&D Systems Inc., USA). Similar to HGF monoculture studies, the conditioned medium from the dynamic cultures was concentrated to 500 µL using the same centrifugal filters as described in section 5.3.3. Prior to the TGF-β1 ELISA, TGF-β1 in each sample was activated by the addition of 1 N hydrochloric acid, followed by acid-neutralization with 1.2 N sodium hydrochloride/0.5 M HEPES to achieve pH 7.2 – 7.6. With the acid treatment, the assay measures both active and latent TGF-β1. The absorbance of the samples was measured using the same microplate reader at 450 nm (background at 540 nm), and compared to a standard curve in order to quantify the amount of growth factor in the samples. Background absorbance emitted from the controls (i.e. no cells seeded) was subtracted from the reading to obtain the reported value. The production levels were normalized to day 0 values. For all measurements, based on previous studies, three independent experiments (with three repeats each) were performed to enable statistical analysis.

5.3.12 Cell seeding ratio

For investigating the effect of seeding ratio, mixtures of HUVECs and HGFs at ratios 2:1, 1:1, or 1:2 (HUVEC:HGF) were seeded into the D-PHI scaffolds at 80,000 cells per scaffold. The ratios were selected based on the studies of Kunz-Schughart et al. and Ma et al., in which angiogenesis was observed when there was an equal amount or greater proportion of fibroblasts in the co-cultures (Kunz-Schughart et al., 2006; Ma et al., 2011). The cell-seeded scaffolds were cultured under perfusion in the 50/50 mix medium (chosen based on the study described in section 5.3.6) for up to 28 days.

5.3.13 Statistical analysis

In this study, the number ‘n’ represents the number of biological replicates. Statistical analysis was performed using the SPSS program (version 20.0). Student’s t-test was used to determine the statistical significance of any differences between the mean values of two groups. In the case of more than two groups, one-way ANOVA was used. When statistical significance was found, post-hoc analysis was used to determine which of the groups contributed to the statistical
significance (Norman and Streiner, 2008). Tukey’s b was used in the case of equal variances. Levene’s test of homogeneity of the variances was also conducted. For one-way ANOVA, in the event that the variances were unequal, the unequal-variance version of ANOVA (Welch) was used and Dunnett’s T3 was applied for post-hoc analysis. In all analyses, the significance threshold was set to $\alpha = 0.05$. All average values are presented as mean $\pm$ standard error (Norman and Streiner, 2008). Unless otherwise stated, all experiments were repeated in triplicates, and there were three repeat samples for each condition studied.

5.4 Results

5.4.1 Enhanced VEGF production by HGFs in perfused D-PHI scaffolds

Since perfusion has been previously shown to enhance the production of TGF-$\beta$1 in D-PHI scaffolds with HGF monoculture (Cheung et al., 2014), it was examined whether the scaffold or perfused condition stimulated the generation of factors that would enable EC culture. The levels of VEGF produced by HGFs in perfused D-PHI scaffolds were significantly greater than the non-perfused scaffolds at all time points in the 28-day culture (Fig. 5.1; $p<0.01$ for days 1 and 7; $p<0.05$ for days 14 and 28). The elevated VEGF levels in the dynamic culture indicate that HGFs could potentially provide favourable conditions for the culture of ECs and capillary formation in the perfused D-PHI scaffold.

![Figure 5.1. VEGF production from HGF monoculture in static or dynamic condition. n = 9, ± standard error (SE). Significantly different from each other (*p<0.05, †p<0.01).](image-url)
5.4.2 Effect of cell seeding density

Over the 28-day culture period, measurements of increasing DNA content (Fig. 5.2) were consistent with cell proliferation in cultures for both seeding densities (40,000 and 80,000 cells/scaffold). A seeding density of 80,000 cells/scaffold resulted in a significantly greater population at days 14 and 28 than that of 40,000 cells/scaffold (p<0.05 and p<0.01 respectively) in the perfused D-PHI scaffolds. These data indicated that the higher seeding density would be more favourable to use in the subsequent studies.

Figure 5.2. DNA content of two different cell seeding densities at various time points. Total number of cells are expressed in cells/scaffold. Cells were cultured under perfusion at a HUVEC:HGF ratio of 1:1, in 50/50 mix medium. n = 9, ±SE. Significantly greater than co-culture with 40,000 cells (*p<0.05, Ńp<0.01).

5.4.3 Effect of culture medium type

The HUVEC-HGF co-culture was characterized based on culture medium type. The samples were either cultured in a 50/50 mix (by vol.) of F-12 K medium and DMEM, or in DMEM only. DNA content (Fig. 5.3a) in the co-culture with the 50/50 mix medium showed a significant 3.1-fold increase in cell population over 28 days of culture (p<0.01) while the DMEM condition showed no statistical increase. The metabolic activity of cells cultured in both types of medium (Fig. 5.3b) decreased significantly over 28 days (p<0.05). The cells in the co-culture for both conditions were stained for the apoptotic marker, caspase 3 (Figs. 5.4d and 5.4h), and were
negative when compared with the positive control (Fig. 5.4o), suggesting that there was minimal apoptosis for both media types at day 28, despite the low metabolic activity.

![Graph showing DNA content and metabolic activity](image)

**Figure 5.3.** (a) DNA content and (b) metabolic activity (as measured by WST-1 assay) of HUVEC-HGF co-cultures in 50/50 mix medium or DMEM only at various time points. Cells were cultured under perfusion at a HUVEC:HGF ratio of 1:1 with a seeding density of 80,000 cells/scaffold. n = 9, ±SE. Significantly different from each other (*p<0.05, ňp<0.01).

HUVECs and HGFs were stained for vWF (Figs. 5.4a and 5.4e) and FSP (Figs. 5.4b and 5.4f) respectively. The immunofluorescence images indicated that both media types maintained the growth of HUVECs and HGFs in the perfused D-PHI scaffold at day 28 of co-culture. However, no conclusions could be drawn with regards to the formation of lumen-like structures from HUVECs in these images. **Fig. 5.4n** shows that the secondary antibodies did not non-
specifically bind with the D-PHI scaffold while Hoechst 33342 was bound. Hence a higher magnification (15x) was used for subsequent quantification and cell counting.

Staining for Col I (Figs. 5.4i and 5.4k) and α-SMA (Figs. 5.4j and 5.4m) and subsequent quantification (Figs. 5.4p and 5.4q) shows that there is a trend of more Col I production over 28 days of co-culture in the 50/50 mix condition (p = 0.078) when compared with DMEM. There were significantly fewer α-SMA-positive cells (i.e. myofibroblasts) in the 50/50 mix vs. the DMEM condition at day 28 of culture (p<0.05), suggesting the modulation of myofibroblast differentiation. The decreased abundance of myofibroblasts in the 50/50 condition may explain the low metabolic activity observed at day 28 since myofibroblasts have been reported to have high metabolic activity (Blumbach et al., 2010; Hinz, 2007).
Figure 5.4. Immunofluorescence images of HUVEC-HGF co-cultures at day 28 of culture in (a) – (d), (i) – (j) 50/50 mix medium, or (e) – (h), (k) – (m) DMEM only. Cells were cultured under perfusion at a HUVEC:HGF ratio of 1:1 with a seeding density of 80,000 cells/ 12.95 mg scaffold. Cells were stained for von Willebrand factor (vWF, (a, e)), fibroblast surface protein (FSP, (b, f)), cell nuclei (Hoechst 33342; c, g), caspase 3 (d, h), type I collagen (Col I, (i, k)), and α-smooth muscle actin (α-SMA, (j, m)). Hoechst 33342 also stained the D-PHI scaffolds (negative control with no primary antibodies (n)), hence a higher magnification (15x) was used to distinguish between scaffold sections and cell nuclei. HGFs were treated with staurosporine to induce apoptosis (i.e. caspase 3 activation), which was used as a positive control (o). The top
of the scaffold is indicated by a dotted line. Scale = 60 µm. (p) The area of Col I (per cell) in the scaffold sections in 50/50 mix medium vs. DMEM at days 0 and 28 of culture. (q) The number of myofibroblasts (i.e. α-SMA+ cells) in 50/50 mix medium vs. DMEM at day 28 of culture, normalized to day 0 values (denoted as 1 (dotted line)). For (p) and (q), n = 9, ±SE. Significantly different from each other (*p<0.05).

The proportion of HUVECs to HGFs in the co-culture throughout the 28-day period was assessed by counting each cell type in the immunofluorescence images (Supplementary Fig. 5.2). The results showed that the proportion remained at a 1:1 ratio in both types of media throughout the co-culture period.

**Supplementary Figure 5.2.** The proportion of HUVECs to HGFs in the perfused co-culture over 28 days in either 50/50 mix medium or DMEM. Cells were cultured at a HUVEC:HGF ratio of 1:1 with a seeding density of 80,000 cells/ scaffold. n = 9, ±SE.

VEGF (day 28) and FGF-2 (days 14 and 28) levels were significantly greater in the 50/50 mix condition when compared with DMEM alone (p<0.05, Figs. 5.5a and 5.5b). The levels of TGF-β1 were similar in both media types for all time points (Fig. 5.5c).
Figure 5.5. (a) VEGF, (b) FGF-2, (c) TGF-β1 release from the HUVEC-HGF perfused coculture (HUVEC:HGF = 1:1, at seeding density of 80,000 cells/ scaffold) in either 50/50 mix medium or DMEM. n = 9, ±SE. Significantly different from each other (*p<0.05).
The enhanced cell proliferation, reduced myofibroblast differentiation, and increased VEGF and FGF-2 production suggest that the 50/50 mix medium is superior to DMEM for HUVEC-HGF co-cultures.

5.4.4 Effect of cell seeding ratio

The HUVEC-HGF co-cultures were also characterized based on cell seeding ratio (2:1, 1:1, and 1:2 (HUVEC:HGF)). As seen in Fig. 5.6a, the DNA content at day 28 of culture for ratios 1:1 and 1:2 was greater than that at ratio 2:1 (p<0.05). A ratio of 1:2 (HUVEC: HGF) resulted in a drop in the metabolic activity after day 1 (Fig. 5.6b) but this level remained constant at subsequent time points up to day 28 of culture. This latter value was greater than that in the other two ratios (p<0.05), suggesting a greater proportion of HGFs had resulted in greater metabolic activity while still yielding similar DNA content when compared to the 1:1 ratio.
Figure 5.6. (a) DNA content and (b) metabolic activity of HUVEC-HGF perfused co-cultures in 50/50 mix medium at various time points. HUVECs and HGFs were seeded at ratios 2:1, 1:1, or 1:2 (HUVEC:HGF), with a seeding density of 80,000 cells/ scaffold. n = 8, ±SE. Significantly different from each other (*p<0.05, ‡p<0.01).

Examination of immunofluorescence images (Fig. 5.7) showed that HUVECs formed more and larger clusters/ structures in the ratio 1:2 (HUVEC:HGF, Fig. 5.7c, Fig. C.4 in Appendix C (example of higher magnification)) when compared with the ratios 2:1 (Fig. 5.7a) and 1:1 (Fig. 5.7b). HGFs appeared to be localized near HUVECs in the D-PHI scaffold, particularly for the 1:1 and 1:2 ratios (Figs. 5.7d to 5.7f and 5.7g to 5.7i). Similar Col I production was observed in the scaffold sections for all three ratios at day 0 (data not shown), as well as at day 28 (Figs. 5.7j to 7m and 5.7q). Myofibroblasts were present in all three ratios at day 28 of culture (Fig. 5.7n to 5.7p), but the myofibroblast population had decreased when compared with day 0 (Fig. 5.7r).
Figure 5.7. Immunofluorescence images of HUVEC-HGF co-cultures at day 28 of culture at seeding ratios (a, d, g, j, n) 2:1, (b, e, h, k, o) 1:1, or (c, f, i, m, p) 1:2 (HUVEC:HGF). Cells were cultured under perfusion in 50/50 mix medium with a seeding density of 80,000 cells/scaffold. Cells were stained for vWF (a) – (c), FSP (d) – (f), Col I (j) – (m), and α-SMA (n) – (p). Cell nuclei were stained with Hoechst 33342, which also non-specifically stained the D-PHI scaffolds (g) – (i), hence a higher magnification (15x) was used to distinguish between cell nuclei and scaffold sections. HUVEC clustering/structural formations were indicated by white circles. The top of the scaffold is denoted by a dotted line. Scale = 60 µm. (q) The area covered by Col I in the scaffold sections at all three ratios for days 0 and 28 of culture, normalized to the amount of cells. (r) The number of myofibroblasts at all three ratios at day 28 of culture, normalized to day 0 values (denoted as 1 (dotted line)). For (q) and (r), n = 9, ±SE.

The proportion of HUVECs to HGFs was greater at ratio 2:1 (~60%) when compared with ratios 1:1 (~50%) and 1:2 (~45%) at day 0 of the co-culture (p<0.05, Fig. 5.8a). However, at day 28 of culture, the proportion of HUVECs to HGFs at the ratio 2:1 had decreased to ~50%, while the other two ratios remained at a similar percentage to their respective day 0 (Fig. 5.8b), suggesting decreased percent of HUVEC survival at 2:1.
Figure 5.8. The proportion of HUVECs to HGFs in the perfused co-culture at (a) day 0 and (b) day 28 with seeding ratios 2:1, 1:1 or 1:2 (HUVEC:HGF). Cells were cultured in 50/50 mix medium with a seeding density of 80,000 cells/scaffold. n = 9, ±SE. Significantly different from each other (*p<0.05, ‡p<0.01).

For angiogenic factors, VEGF production was the greatest for the 2:1 ratio at the later culture period (p<0.05, Fig. 5.9a), followed by the 1:2 ratio and then the 1:1 ratio which was significantly lower than that of the other conditions (p<0.05). FGF-2 (Fig. 5.9b) and TGF-β1 production (Fig. 5.9c) was significantly greater for the 1:2 ratio at day 28 of culture when compared with the other two ratios (p<0.05).
Figure 5.9. (a) VEGF, (b) FGF-2, (c) TGF-β1 release from the HUVEC-HGF perfused coculture (in 50/50 mix medium, at seeding density of 80,000 cells/scaffold) at seeding ratios 2:1, 1:1, or 1:2 (HUVEC:HGF). n = 9, ±SE. Significantly different from each other (*p<0.05, ǂp<0.01).
The results suggest that the ratio 1:2 may favour increased cell proliferation, enhanced HUVEC percent survival, promotion of HUVEC clustering, and upregulation of angiogenic factors.

5.5 Discussion

The current study explored different culture conditions that would favour the co-culture of HUVEC-HGF and promote the development of a vascularized tissue-engineered construct within D-PHI; these constructs could be used for the repair of the gingival lamina propria. The results show that a perfused culture system, in combination with the D-PHI scaffold, with greater cell seeding density and a higher proportion of HGFs vs. HUVECs for seeding in the co-culture within a 50/50 mix medium, promoted cell proliferation, HUVEC clustering, and angiogenic factor release.

The co-culture of ECs with fibroblasts in scaffolds such as those made of PLGA, poly(ethylene terephthalate), and collagen, can be effective in inducing vascularization in tissue regeneration processes (Bae et al., 2012; Battiston et al., 2014; Davis et al., 2002; Kaully et al., 2009; Sorrell et al., 2007; Sukmana and Vermette, 2010) because fibroblasts have been shown to deposit collagen-rich ECM and release specific angiogenic factors (e.g. VEGF, TGF-β1), both of which are essential to EC tubulogenesis (Bae et al., 2012; Davis et al., 2002; Hughes, 2008; Sorrell et al., 2007; Taba Jr. et al., 2005). Newman et al. showed that fibroblasts, when co-cultured with HUVECs in fibrin gels, are capable of releasing 5-10 pg/mL of VEGF. However, when the same range of VEGF concentrations was delivered to the EC monoculture exogenously (i.e. in the absence of fibroblasts), ECs failed to sprout and form lumen-like structures, which indicates that the angiogenic effect of fibroblasts in the co-culture is multi-factorial (Newman et al., 2011). The results from this study demonstrate the ability of D-PHI to support the angiogenic nature of fibroblasts cultured under perfusion and in combination with ECs. These findings suggest the potential of using this biomaterial with these culture strategies to induce early capillary formation and expand opportunities for gingival tissue engineering.

Cell responses can vary depending on the cell seeding population (Battiston et al., 2014). Cell seeding density can affect cell distribution in a tissue engineering scaffold, thereby influencing cell-cell interaction and cell maturation (Chiu et al., 2004). For example, greater cell density can
result in enhanced heterotypic interactions between hepatocytes and liver epithelial cells but also can lead to decreased albumin secretion (Bhatia et al., 1999). A higher cell population in the co-culture can promote EC migration when co-cultured with breast cancer cells (Chung et al., 2009). The results in this study (Fig. 5.2) indicate that a higher cell seeding density in the biomaterial scaffold may be more favourable for HUVEC-HGF co-cultures in perfused D-PHI constructs.

In co-cultures with ECs, researchers have used different culture media but there is no defined medium type. A study by Kolbe et al. switched from an osteogenic medium, which was used in the monoculture of mesenchymal stem cells, to an endothelial growth medium for the co-culture in order to optimize EC growth and tubulogenesis (Kolbe et al., 2011). Choong et al. used a 50/50 mixture (by vol.) of EC medium and fibroblast medium for inducing angiogenesis in polycaprolactone scaffolds (Choong et al., 2006), while Salem et al. did not change the medium type and used DMEM for the EC-fibroblast co-culture on fibronectin-coated silicon nitride films (Salem et al., 2002).

The current study examined whether a mixture of culture media exerted a positive effect on cell proliferation and angiogenesis when compared with only one type of medium, when cells were cultured in the synthetic D-PHI scaffold. The DNA data indicated that a 50/50 mix of F-12 K medium and DMEM (by vol.) augmented the co-culture growth significantly when compared with DMEM only. The decreased metabolic activity at day 28 for both media types may be explained by the drop in the myofibroblast population (Fig. 5.4q), as these cells are metabolically very active cells (Blumbach et al., 2010;Hinz, 2007). The significantly lower myofibroblast population in the 50/50 vs. DMEM condition indicates that the former medium potentially negatively interferes with myofibroblast differentiation more effectively than the DMEM. Myofibroblasts are contractile and they can produce high ECM content while facilitating tissue remodelling during acute, normal wound healing; but their prolonged presence at high density is often associated with fibrosis (Blumbach et al., 2010;Galie et al., 2012;Hinz, 2006;Hinz, 2007;Horan et al., 2008;Tomasek et al., 2002;Wipff and Hinz, 2009). As soft tissues like the gingival lamina propria are non-contractile (Tomasek et al., 2002), minimal myofibroblast presence is desired for gingival tissue regeneration.
Under the effect of the 50/50 mix medium and D-PHI, VEGF and FGF-2 levels were greater at day 28 of culture when compared with DMEM only. VEGF and FGF-2 are reported to have a positive synergistic effect on angiogenesis, as their simultaneous presence has resulted in more rapid tubule formation in tissue constructs both *in vitro* and *in vivo* (Asahara et al., 1995; Davis et al., 2002; Goto et al., 1993; Pepper et al., 1992; Taba Jr. et al., 2005). It is also possible that the HUVEC-HGF co-culture contributed to the increase in VEGF as it has been shown in EC-osteoblast co-cultures that VEGF levels increase significantly in the co-culture when compared to the monoculture of ECs (Santos and Reis, 2010).

Despite the potential pro-angiogenic effect of the 50/50 mix medium, capillary formation was not observed in the immunofluorescence images. The levels of TGF-β, an angiogenic factor that is required for nascent blood vessel stabilization (Ferrari et al., 2009; Liu et al., 2009) and maturation (Bae et al., 2012; Dohle et al., 2011; Folkman and D’Amore, 1996; Iruela-Arispe and Dvorak, 1997), remained similar between the two media types throughout the 28-day co-culture. This suggests that cell seeding density and culture medium may not be the only factors affecting the HUVEC-HGF co-culture.

Many studies have shown that cell seeding ratio plays a key role in determining the angiogenic outcomes in co-cultures containing ECs. A recent study has shown that in a three-dimensional spheroid structure, an increase in EC proliferation and capillary formation was observed at EC-fibroblast ratios of 1:40 and 1:4 (i.e. more fibroblasts than ECs) (Kunz-Schughart et al., 2006). If there were more ECs than fibroblasts in the spheroid, a significant loss of the EC population was observed as early as 3 days of culture. Similarly, Ma et al. showed that when there were more HUVECs than osteoprogenitor cells in the co-culture, cell viability was low, indicating that the percentage of ECs in the co-culture should be kept below 50% to optimize cell viability (Ma et al., 2011). Excessive ECs in the co-culture can also impede blood vessel growth and expansion of vasculature in the scaffold (Rao et al., 2012). In the current study, similar trends were observed as suggested by the enhancement in DNA content and metabolic activity for the ratio 1:2 (HUVEC:HGF) in D-PHI when compared to the other two ratios, indicating that a greater proportion of HGFs favours cell proliferation in the co-culture.

Moreover, HUVECs formed large clusters near HGFs only in the ratio 1:2 (*Fig. 5.7c, 5.7f, and 5.7i*), which may be a prelude to capillary formation (Ingber and Folkman, 1989), and there is a
higher HUVEC percent survival in the D-PHI scaffold for this ratio. These data further support the importance of the cell seeding ratio in regulating the cellular response in EC-fibroblast co-culture within synthetic elastomeric scaffolds.

The greater FGF-2 and TGF-β1 production at day 28 for the 1:2 ratio in D-PHI suggests that the increased number of HGFs may have contributed to the upregulation of both angiogenic factors when cultured in this biomaterial. For example, high TGF-β1 levels have been shown to promote capillary formation (Senger and Davis, 2011). Interestingly, VEGF production was the greatest for the 2:1 ratio but no HUVEC clusters were observed in the immunofluorescence images. It should be noted that VEGF has been reported to be an angiogenic factor that promotes blood vessel sprouting, but the newly formed blood vessels in a matrix require stabilization by the presence of other angiogenic factors such as TGF-β1 (Bae et al., 2012; Dohle et al., 2011; Ferrari et al., 2009; Folkman and D’Amore, 1996; Iruela-Arispe and Dvorak, 1997; Liu et al., 2009). Blood vessels that are exposed to high doses of VEGF have been reported to be malformed and leaky (Santos and Reis, 2010). Hence, the unique combination of HUVECs and HGFs within biomaterial scaffolds such as D-PHI, that facilitates not only the release of pro-angiogenic factors but also effectively influences EC structural organization, will be a crucial determinant in developing highly perfused tissues.

All of these data indicated that a greater proportion of HGFs in the co-culture during initial seeding within the D-PHI scaffold is required for HUVEC survival and increased TGF-β1 release, as these conditions may contribute to the clustering of ECs. The mechanisms by which the increased HGF proportion affects the survival of HUVECs and their clustering near HGFs (e.g. intercellular cross-talk) within the synthetic scaffold still require further investigation.

5.6 Conclusion

In summary, the current study demonstrated the enhanced angiogenic potential of HGFs when cultured in the D-PHI scaffolds under perfusion. HUVEC-HGF co-culture in perfused D-PHI scaffolds with a cell seeding density of at least 80,000 cells/12.95 mg scaffold (80% porosity) and a greater proportion of HGFs vs. HUVECs (seeding ratio of 1:2) in a 50/50 mix of HUVEC and HGF media (by vol.) exhibited enhanced cell proliferation, HUVEC clustering, reduced myofibroblast differentiation, and increased angiogenic factor production. The combination of
these pro-angiogenic culture conditions can potentially be used to produce a tissue-engineered construct for regenerating a vascularized gingival connective tissue.

5.7 Acknowledgments

This study was supported by a Natural Sciences and Engineering Research Council (NSERC) Discovery grant (360520), NSERC Alexander Graham Bell Canada Graduate Scholarships (CGS D3), Ontario Graduate Scholarship Program, and Canadian Institutes of Health Research-CellSignals Training Fellowship (STP-53877).
5.8 References


Sorrell, J. M., Baber, M. A. and Caplan, A. I. (2007). A self-assembled fibroblast-endothelial cell co-culture system that supports in vitro vasculogenesis by both human umbilical vein


Chapter 6
Conclusion

6.1 Summary

The current thesis established a highly perfused tissue-engineered construct using a dynamic co-culture system in vitro with HGFs, ECs, and D-PHI scaffolds. The perfused construct showed enhanced cell proliferation and infiltration, contained high collagen content, disabled myofibroblast differentiation and displayed early signs of HUVEC cluster formation, which physically and biologically modeled phenotypic characteristics of the gingival lamina propria.

D-PHI scaffolds have been shown to be biocompatible with various cell types (McBane et al., 2011a; McBane et al., 2011b; McDonald et al., 2011; Sharifpoor et al., 2009; Sharifpoor et al., 2010; Sharifpoor et al., 2011). Perfused culture can enhance cell proliferation by facilitating cells’ metabolic exchange and providing mechanical stimuli to cells in the tissue-engineered scaffolds (Du et al., 2008; Navarro et al., 2001; Timmins et al., 2007; Zhao et al., 2009). In Chapter 3, the main objective was to synthesize D-PHI scaffold (80% porosity, 2 mm thickness) and assess its biocompatibility with HGFs based on cell viability, proliferation and collagen production, as well as to design and develop a perfusion bioreactor system for facilitating medium perfusion (dynamic culture) through HGF-seeded D-PHI scaffolds such that cell proliferation and collagen production are enhanced. To address this objective, D-PHI porous scaffolds were synthesized from lysine-based polycarbonate DVO and acrylate monomers, with sodium bicarbonate and polyethylene glycol as porogens (Sharifpoor et al., 2009). HGF cell viability and proliferation were investigated on 2D D-PHI films over a 14-day static culture. For medium perfusion (dynamic culture), a perfusion bioreactor system was designed and built, which consisted of a parallel circuit of perfusion chambers, a peristaltic pump, and medium reservoirs. Similar to the 2D studies, HGF cell viability and proliferation in 3D D-PHI porous scaffolds were assessed in dynamic (500 µL/min) vs. static culture for up to 28 days by measuring DNA content and metabolic activity. HGF cell morphology and spatial localization of HGFs in the perfused D-PHI scaffolds were also examined. Furthermore, Col I production from HGFs was measured and normalized to Vim content. It was observed that HGF growth was continuous on the 2D D-PHI films over 14 days of static culture. When cultured in the 3D D-PHI scaffolds, HGF growth was
augmented by 3-fold over 28 days in the dynamic culture while it was reduced after 14 days in the static culture. Cell metabolic activity and total protein production show that HGFs in the perfused scaffolds were in a different metabolic state than those in the non-perfused ones. As observed with SEM, HGFs were less stretched and formed clusters in the perfused scaffolds. Greater cell distribution was observed in the perfused scaffolds as compared to non-perfused ones in the histology images. Furthermore, medium perfusion significantly promoted collagen production in HGFs after 28 days of culture. These data suggest that the metabolic, phenotypic and differentiation state of HGFs were altered in dynamic cultures, thereby possibly favouring tissue regeneration.

The nature of the HGFs cultured in the perfused D-PHI scaffolds, and the mechanisms by which medium perfusion activates these cells to facilitate proliferation and Col I production are not defined. Therefore, in Chapter 4, the main objective was to investigate the interactions of HGFs with D-PHI scaffolds under medium perfusion by characterizing the cell phenotypic change and examining signalling pathways that are activated during dynamic culture. In this study, the production of α-SMA, one of the distinctive myofibroblast markers (Hinz, 2007), and Col I by HGFs in the perfused vs. non-perfused D-PHI scaffolds was measured. The spatial localization of these two markers in the perfused D-PHI scaffolds was also observed. Furthermore, the release of pro-fibrotic growth factor, TGF-β1 (Blumbach et al., 2010; Galie et al., 2012; Hinz et al., 2001; Hinz, 2006; Hinz, 2007; Horan et al., 2008; Wipff and Hinz, 2009), and one of its antagonists, FGF-2 (Akasaka et al., 2007; Ishiguro et al., 2009; Khoury et al., 1999), in the culture medium was measured. The mechanistic pathways affecting the production of the aforementioned markers were examined by intentional inhibition of the Smad pathway (Blumbach et al., 2010; Hinz, 2007), by measuring the total β1-integrin production and FAK phosphorylation (i.e. focal adhesion-related pathways), and by intentional inhibition of β1-integrin. It was found that the perfused D-PHI scaffold disabled myofibroblast differentiation while increasing Col I production over the course of 28 days. Both TGF-β1 and FGF-2 were significantly greater in the dynamic vs. static culture at day 1. Although TGF-β1 has been often reported to increase α-SMA and collagen production (Hinz, 2007), the D-PHI material and significant high level of FGF-2 at day 1 of dynamic culture appear to play a role in regulating α-SMA production while allowing HGFs to increase Col I production. β1-integrin production and FAK phosphorylation were increased 2 hours after HGFs were exposed to medium perfusion,
indicating that flow stimulus was mechanically transduced to HGFs via focal adhesion. This may have in part promoted cell proliferation, α-SMA and Col I production in the early dynamic culture. Consequently, medium perfusion and the D-PHI material has modulated fibroblast phenotype, and enhanced cell proliferation and Col I production through the coordinated actions of TGF-β1, FGF-2, β1-integrin and FAK.

In healthy gingival tissues, the lamina propria is highly vascular and has been modelled by a co-culture of ECs and gingival fibroblasts (Moharamzadeh et al., 2007; Saxena, 2008; Taba Jr. et al., 2005). Co-culture of ECs with fibroblasts can provide strong angiogenic stimuli (Baiguera and Ribatti, 2013; Davis et al., 2002; Guillemette et al., 2010; Newman et al., 2011; Sorrell et al., 2007; Sukmana and Vermette, 2010) but the culture conditions are not well defined. Thus the main objective of Chapter 5 was to establish a perfused co-culture system with HGFs and ECs by incorporating ECs and HGFs simultaneously in D-PHI scaffolds using the perfusion bioreactor system that was described in Chapter 3, and to select optimal culture conditions (cell seeding density, type of culture medium, and cell seeding ratio) based on cell proliferation, EC cluster formation, angiogenic factor production, and Col I production. In this study, the production of the angiogenic factor, VEGF, in HGF monoculture was measured in perfused vs. non-perfused D-PHI scaffolds to demonstrate the angiogenic potential of HGFs cultured under medium perfusion. A perfused HUVEC-HGF co-culture system was established within the D-PHI scaffolds. The effects of medium perfusion and cell seeding density on the co-culture were investigated based on cell growth. The effects of culture medium (50/50 mix by volume of F-12 K medium and DMEM vs. DMEM only) and seeding ratios (2:1, 1:1, 1:2 (HUVEC:HGF)) were examined based on cell proliferation, metabolic activity, HUVEC clustering, α-SMA and Col I production, the proportion of HUVECs to HGFs over time, and angiogenic factor (VEGF, TGF-β1, FGF-2) production in the 28-day dynamic co-culture. It was observed that VEGF production from HGFs in the monoculture was enhanced within the perfused D-PHI scaffold. HUVEC-HGF co-culture in perfused D-PHI scaffolds with a cell seeding density of at least 80,000 cells/12.95 mg scaffold (80% porosity) in a 50/50 mix of HUVEC and HGF media (by vol.) exhibited enhanced cell proliferation, disabled myofibroblast differentiation, as well as increased VEGF and FGF-2 production. A greater fibroblast proportion (seeding ratio of 1:2) in the co-culture resulted in HUVEC clustering (prelude to capillary formation) and increased TGF-β1 and FGF-2 production. At this time, the mechanisms by which increased HGF proportion affects the
survival of HUVECs and their clustering near HGFs (e.g. intercellular cross-talk) within the perfused scaffolds are not well defined and future work will be required to further explore the related signalling pathways.

6.2 Scientific contributions

Chapter 3 demonstrated the potential of D-PHI as a scaffold material for gingival tissue regeneration. Synthetic materials are preferred over natural ones due to the reproducibility of synthetic polymers and their versatility in terms of controlling their properties (e.g. elastic modulus, degradation rate, microstructure, etc.) (Benatti et al., 2007;Dhandayuthapani et al., 2011). Like other synthetic materials, the physical and chemical properties of polyurethane hydrogels such as D-PHI can be tailored for cell culture and tissue regeneration (Saxena, 2008;Yang et al., 2009). D-PHI has a slower degradation rate in vivo than other synthetic materials such as PLGA and the structural integrity of D-PHI was not altered by biodegradation (McBane et al., 2011b). Furthermore, the current thesis emphasized the need for medium perfusion in tissue engineering approaches for vascularized tissues such as the gingival lamina propria. Medium perfusion can facilitate the transport of nutrients and wastes in a tissue-engineered construct and provide mechanical stimuli to cells, which encouraged cell proliferation and protein production (Galie et al., 2012;Mcintire et al., 1998;Navarro et al., 2001;Ng et al., 2005;Sterpetti et al., 1994;Zhao et al., 2009). In this study, the perfused culture with D-PHI scaffolds showed increased HGF proliferation, metabolic activity, cell infiltration in the scaffold, and Col I production as compared with the static culture. The above observation suggested that HGFs in the perfused culture were in a different phenotypic state than those in the static culture, which may favour gingival tissue reconstruction as the lamina propria is densely populated by fibroblasts in a collagenous matrix.

The work conducted in Chapter 4 contributed to a better understanding of the phenotype of HGFs in the perfused D-PHI scaffolds and the mechanistic pathways involved in the phenotypic change. Specifically, it highlighted the influence of perfused D-PHI scaffolds in establishing a gingival fibroblast phenotype that favours the potential generation of a functional tissue-engineered construct for gingival tissue repair. The decrease in α-SMA production from HGFs on 2D D-PHI films and 3D D-PHI scaffolds, with or without perfusion, suggested that the D-PHI material not only promoted cell growth and collagen production, but it disabled
myofibroblast differentiation. Recently, the design of biomaterials has increasingly focused on suppressing myofibroblast differentiation (Acharya et al., 2008; Dreier et al., 2013) and D-PHI can be a promising material for use in this purpose. In tissue regeneration, the initial presence of myofibroblasts is desired for promoting tissue repair and vasculogenesis (Hinz, 2010; Tomasek et al., 2002). However, the prolonged presence of myofibroblasts may lead to tissue and organ dysfunction (Hinz, 2010; Tomasek et al., 2002). This study demonstrated that medium perfusion can stimulate myofibroblast differentiation in the early culture time. The phenotypic change may have triggered increased cell proliferation and collagen production, which is important for soft tissue regeneration. The coordinated actions of growth factors (TGF-β1 and FGF-2) and focal adhesion components (β1-integrin and possibly FAK) in modulating the HGF phenotype in perfused D-PHI exhibited that mechanical stimuli provided by perfusion can be transduced to cells, thereby affecting cell behaviour. The knowledge can enable rational developments of culture systems for generating a functional, engineered tissue.

Another unique aspect of this work is the recognition of various optimal culture conditions for the HUVEC-HGF co-culture, which has been demonstrated to favour HUVEC clustering, cell proliferation and angiogenic factor production in the D-PHI scaffold (Chapter 5). Co-culture of fibroblasts and ECs have shown promising results in establishing vascular network in the tissue engineering scaffold (Kaully et al., 2009), but the culture conditions are not well defined (Choong et al., 2006; Kunz-Schughart et al., 2006; Ma et al., 2011; Salem et al., 2002). Fibroblasts have been shown to promote angiogenesis by depositing collagen-rich ECM and releasing angiogenic factors (Baiguera and Ribatti, 2013; Davis et al., 2002; Guillemette et al., 2010; Newman et al., 2011; Sorrell et al., 2007; Sukmana and Vermette, 2010). The work in this chapter confirmed the angiogenic potential of HGFs, particularly in perfused condition. Furthermore, it demonstrated the effect of culture medium in enhancing cell proliferation and disabling myofibroblast differentiation, and emphasized the crucial role of the cell seeding ratio (i.e. greater fibroblast seeding proportion) in inducing EC tubulogenesis. Similar to the work of Kunz-Schughart et al., greater fibroblast ratio in the current thesis maintained HUVEC survival and stimulated HUVEC clustering near HGFs, which may be a preliminary event for capillary formation (Ingber and Folkman, 1989). Using this fundamental information and combining it with other pro-angiogenic culture conditions examined in this thesis, a functional and highly
perfused tissue-engineered construct could potentially be established for gingival tissue regeneration.

6.3 Recommendations and future perspectives

1. The seeding method used in the current thesis relies on the diffusion of cell suspension from the top of the D-PHI scaffold. As shown in the earlier culture time points (Chapters 3 and 4), most of the HGFs were located near the top of the scaffold in both perfused and non-perfused conditions. For more efficient seeding, cell suspensions can be seeded on both sides of the scaffold (Volkmer et al., 2008). Furthermore, a perfusion system can be applied to pump the cell suspension through the scaffold. For example, Wendt et al. demonstrated that seeding by perfusion improved cell viability by ~20% and enhanced more uniform cell distribution when compared with static seeding (Wendt et al., 2003). Therefore, the bioreactor system developed in this thesis can potentially be used for cell seeding.

2. Based on the findings from the co-culture studies (Chapter 5), cell seeding density can affect cell proliferation and cell-cell interaction. As natural gingival lamina propria has 2 x 10^8 fibroblasts per cubic centimetre (Schroeder, 1986), it is possible that increasing the cell seeding density in the HGF monoculture and the HUVEC-HGF co-culture can accelerate cell growth by enhancing the amount of cell-cell interaction. For example, Baumchen et al. seeded 5 x 10^5 HGFs in PGA scaffold (6 mm diameter, 85-95% porosity) to promote cell proliferation for tissue regeneration (Bäumchen et al., 2009). The cell seeding density in that study was greater than that in the current thesis by an order of magnitude. Furthermore, greater cell density have been reported to promote EC migration in co-cultures (Chung et al., 2009). There should be an optimal range of cell seeding density for promoting desired cell responses.

3. In this thesis, the HUVEC-HGF co-culture was established by mixing the HUVEC and HGF cell suspensions together before seeding and allowing the cells to rearrange themselves in the scaffold to form vascular structures. As it has been shown that confluent layers of fibroblasts can provide improved cellular cross-talk and collagen-rich ECM for EC tubulogenesis (Sorrell et al., 2007), sequential seeding (i.e. HGFs seeded before HUVECs) can be applied in the co-culture system. HGFs can be seeded first into the D-PHI scaffold and be cultured for a week
(under perfusion) in order to amplify the HGF population (i.e. to generate more cellular cross-talk), and to provide a collagen-rich environment for angiogenesis.

4. The flow rate used in the HGF dynamic monoculture and HUVEC-HGF dynamic co-culture was fixed at 500 µL/min. It is important to investigate the effect of flow rate and the magnitude of flow-induced shear stress on both monoculture and co-culture. It has been previously shown that at 8 dyn/cm², fibroblasts differentiated into myofibroblasts after 24 h; while at shear stresses between 9 and 13 dyn/cm², myofibroblast differentiation was inhibited (Sterpetti et al., 1994). There can be an optimal range of flow rates and flow-induced shear stress which regulates myofibroblast differentiation without impeding fibroblast proliferation. Furthermore, since ECs respond differently to the magnitude of shear stress (Ando and Yamamoto, 2009;Davies, 1995), the HUVEC and HGF response to a range of flow rates and shear stress should be explored. To investigate this, the flow rate in the bioreactor system developed in this thesis can be adjusted and varied from the minimal physiological value (e.g. 10-100 µL/min (Gaspar et al., 2012;Seitz et al., 2007;Silva et al., 2014)) to a larger flow rate (e.g. 5 mL/min (Lee and Niklason, 2010)). Shear stress can be adjusted by varying the viscosity of the medium (e.g. adding Dextran) without changing the flow rate. The optimal range of flow rates and shear stresses can be identified based on cell proliferation (measured by DNA content), inhibition of myofibroblast differentiation (measured by immunoblotting for α-SMA production), HUVEC cluster formation (observed with immunofluorescence), and Col I production (measured by immunoblotting and immunofluorescence).

5. As demonstrated in Chapter 4, mechanical stimuli from perfusion can be transduced to HGFs via focal adhesion proteins such as FAK, thereby mediating myofibroblast differentiation. The current thesis looked at the production of β1-integrin and the phosphorylation of FAK but the other integrin-related downstream signaling pathways were not investigated. For example, p38 (Sato et al., 2002;Wang et al., 2006) and c-Jun NH(2)-terminal kinase (MacKenna et al., 1998;Wang et al., 2006) are some of the integrin-mediated pathways. As well, FAK downstream signaling pathways such as mitogen activated protein kinase (MAPK)/ ERK (Saleem et al., 2009;Surazynski et al., 2005) were not explored. These signaling proteins can be measured by Western blotting and the results can be compared between perfused and non-perfused cultures.
6. Based on the findings from this thesis, perfused culture can induce growth factor production such as VEGF, TGF-β1, and FGF-2 in cells. Perfusion has been shown to increase the production of PDGF in aortic smooth muscle cells and the conditioned medium from these cells stimulated the growth of 3T3 fibroblasts (Sterpetti et al., 1994). PDGF is associated with the inhibition of myofibroblast differentiation (Hecker et al., 2011) and it is one of the angiogenic factors (Ando and Yamamoto, 2009; Davies, 1995; Hughes, 2008). It is important to determine if the perfused culture can enhance PDGF production and examine if PDGF is involved in promoting HGF growth in the monoculture as well as HUVEC cluster formation in the co-culture at ratio 1:2 (HUVEC:HGF). PDGF levels throughout the culture can be measured by ELISAs at various time points and compared between conditions. To investigate the influence of PDGF on HGF growth, an anti-PDGF antibody can be used to inhibit PDGF binding to cells (Sterpetti et al., 1994) and HGF growth can be measured by DNA content after the intentional inhibition. Similarly, the same blocking procedure can be used to observe HUVEC cluster formation upon the inhibition.

7. In addition to the pro-angiogenic culture conditions used in this thesis, hypoxia can be introduced to the system to induce angiogenesis. Hypoxia refers to a localized reduction of oxygen content to approximately 6% or below (Hadjipanayi and Schilling, 2013). Hypoxia has been reported to be associated with tumour angiogenesis (Shahneh et al., 2013), and recently, it has been used in tissue regeneration as hypoxic stress can stimulate a plethora of angiogenic factors such as VEGF, PDGF, etc. (Cheema et al., 2008; Hadjipanayi et al., 2010; Hadjipanayi and Schilling, 2013). For example, in a culture with ~3% oxygen, human dermal fibroblasts produced elevated levels of VEGF (Cheema et al., 2008). Moreover, by co-culturing the hypoxia-induced fibroblasts with ECs in 3D collagen matrix, EC migration and tubulogenesis was observed in vitro. After implantation, the hypoxia-treated tissue construct directed vessel ingrowth from the host after one week, which was a week quicker than the non-hypoxia treated construct (Hadjipanayi et al., 2010). Consequently, the HUVEC-HGF co-culture can be cultured in hypoxic condition by lowering the oxygen level to below 6% in the perfusion system.

8. Despite the HUVEC cluster formation observed with greater fibroblast proportion in the HUVEC-HGF co-culture, the amount of clusters was limited and the structures seemed incomplete. In the work of Gibot et al., the fibroblast-epithelial cell-HUVEC tri-culture was grown for 6 weeks in vitro, which resulted in pre-formed vascular network in the tissue
construct (Gibot et al., 2010). Therefore, it is possible to extend the dynamic co-culture period to beyond 28 days to allow for more collagen deposition from HGFs, as well as more time for HUVEC assembly and rearrangement into capillaries.

9. In this thesis, greater fibroblast proportion in the HUVEC-HGF co-culture exhibited HUVEC clustering and increased TGF-β1 and FGF-2 production. It is speculated that the observed cluster formation at this ratio is related to the enhanced production of these two angiogenic factors, but the related mechanistic pathways remain to be investigated. Upon binding to TGF-β1, activin-like kinase receptors have been reported to be phosphorylated in order to regulate EC proliferation and migration (Liu et al., 2009). Furthermore, FGF-2-binding can activate various pathways such as Ras-MEK-MAPK, Src family tyrosine kinases, PI3K, and the PLC pathway (Cross and Claesson-Welsh, 2001). Thus, the activation of the above signaling proteins at ratios 2:1, 1:1, and 1:2 (HUVEC:HGF) should be examined using methods such as Western blotting.

10. After a tissue-engineered construct is established in vitro, it is necessary to test the construct in an in vivo model. An athymic rat model can be applied by implanting the tissue scaffold in a gingival defect (Dan et al., 2014), or subcutaneously in dorsal dermal pouches (Buurma et al., 1999; Laschke et al., 2009). In the in vivo model, the size of the D-PHI scaffold may need to be reduced since the dimensions of wounds in gingival tissues are small (United States Centers for Disease Control and Prevention, 2007). The interaction between the tissue scaffold and the host can be observed using histology (H&E stain). Analysis of the implanted tissue scaffold can be carried out at various time points up to 2 months after implantation (Izumi et al., 2003; Suuronen et al., 2009), and compared with pre-operative conditions. Vascularization in the implant can be observed via capillary density (histology and immunofluorescence staining), and the perfusion of a visible dye or laser Doppler perfusion analysis (Suuronen et al., 2009) can be used to verify that the newly formed blood vessels are functional. Collagen production can be observed and quantified using Masson’s Trichrome (McBane et al., 2011b) or picrosirius red stain (also for observing collagen fibril arrangement). Furthermore, the construct’s interaction with the host epithelium can be observed by immunostaining for the cytokeratin family (Locke et al., 2008; Mackenzie et al., 1991). The integration of the implanted tissue construct into the environment and the establishment of a functional vascular network in the construct determine the success of the construct in vivo.
6.4 References


Sorrell, J. M., Baber, M. A. and Caplan, A. I. (2007). A self-assembled fibroblast-endothelial cell co-culture system that supports in vitro vasculogenesis by both human umbilical vein


Appendix A
Characterization of collagen expression from human gingival fibroblasts on a degradable/polar/hydrophobic/ionic polyurethane

A.1 Introduction

In gingival tissue engineering, several scaffold materials have been investigated. The present study focuses on poly(lactic-co-glycolic acid) (PLGA) and degradable/polar/hydrophobic/ionic polyurethane (D-PHI). Previous studies have demonstrated that human gingival fibroblasts (HGFs) remained viable and proliferated in three-dimensional (3D) D-PHI scaffolds (Cheung et al., 2013). However, the functionality of HGFs, as determined by the production of collagen (a major HGF phenotypic marker (Schroeder, 1986)), remained to be investigated. Therefore, the present study characterized collagen expression on D-PHI, PLGA, and the standard control (tissue culture polystyrene (TCPS)), at days 1, 7, and 14, using picrosirius red staining. Picrosirius red stains for total collagen production. To normalize the collagen data between conditions, DNA quantification was also performed in order to measure cell population. The objective of this study is to determine whether HGFs maintain their capability to produce collagen when they are cultured on D-PHI. Another objective is to compare the level of total collagen expression from HGFs seeded onto D-PHI films with those seeded onto PLGA films and TCPS. PLGA is an FDA-approved biomaterial (Moharamzadeh et al., 2007) and TCPS is a material commonly used in cell culture, thus these two substrates serve as appropriate comparisons. It is hypothesized that HGFs seeded onto D-PHI films will produce increasing collagen content due to increased cell growth. Moreover, we hypothesize that total collagen expression from HGFs cultured on D-PHI films will be comparable to that from HGFs cultured on PLGA films but less than that from HGFs cultured on TCPS. (This work was done by summer student, Hans Shih, under my supervision.)
A.2 Materials and methods

A.2.1 HGF cell culture

A.2.1.1 Cell line and culture medium

The cells used for the experiments were HGF-1 from ATCC (American Tissue Culture Collection, Manassas, VA). The culture medium in which the cells were kept was Dulbecco’s modified Eagle medium (DMEM) (GibcoBRL) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% antibiotics (Penicillin-Streptomycin, Sigma-Aldrich).

A.2.1.2 Cell seeding

Prior to cell seeding, all three substrates (D-PHI, TCPS, PLGA) were conditioned in 200μL of culture medium for 24h. The culture medium was then aspirated just before cell seeding. In order to obtain the cells for seeding, cells were trypsinized using 0.25% trypsin-EDTA for 5min at 37°C. Following incubation, the trypsin was deactivated by adding culture medium. The amount of cells in the suspension was estimated using Trypan Blue and a hemacytometer. The HGFs were then centrifuged at 1200-1400rpm for 1 min, after which, the supernatant was aspirated. The cell pellet was resuspended at a specific concentration in order to achieve the desired cell seeding density. The cells were then transferred into the wells containing each of the three types of substrates (D-PHI, TCPS, PLGA) in triplicates. The cell-seeded substrates were kept in the 96-well plates for incubation overnight at 37°C and 5% CO₂. The medium was replaced after 24h of incubation and subsequently every 2 to 3 days.

Wells with no cells seeded were used as negative controls.

A.2.2 PLGA films

A.2.2.1 PLGA coverslips preparation

In order to fabricate the 2D film using PLGA, pellets of PLGA were dissolved in chloroform and then casted onto round glass coverslips of 5mm in diameter (Electron Microscopy Sciences, Hatfield, PA). Before casting, the coverslips were soaked in 5-8% chromic acid solution overnight for cleaning. The chromic acid was then rinsed off and the coverslips were sonicated 3 times for 10min each, with 3 times of washes in between with distilled water. The coverslips
were left to dry on an angle on plastic Petri dishes at 50°C. After the coverslips were dried, they were ready for casting.

A.2.2.2 PLGA films preparation

PLGA pellets (lactide-to-glycolide ratio of 75:25, Sigma-Aldrich) were dissolved in chloroform in order to achieve 5 wt% (wt/wt). The PLGA solution was left to dissolve overnight in the chloroform in a sealed environment. After PLGA was completely dissolved, the PLGA solution was casted onto the pre-conditioned coverslips by loading 3-4 drops of solution onto the coverslips. Following casting, the PLGA-coated coverslips were left in a dry environment to allow the chloroform to evaporate overnight. After 24 hours, the coated coverslips were placed into a vacuum oven with a pressure of -30inHg (-101kPa) for 3h at room temperature for further solvent evaporation. Finally, the coated coverslips were dried at 45°C for another 3 hours to ensure that all residual chloroform was evaporated.

The PLGA films (casted onto the coverslips) were placed into the wells of a 96-well TCPS plate. The 96-well plate containing the PLGA films were then brought into a sterile environment. The films were exposed to UV light for 90min to allow for sterilization. Following sterilization, the films were ready for cell seeding.

A.2.3 D-PHI films preparation

D-PHI films were fabricated using free radical polymerization with divinyl oligomer, methyl methacrylate, and methacrylic acid at a molar ratio of 1:15:5 (Cheung et al., 2013). Benzyol peroxide was used as the initiator. The polymer resin was then mixed at room temperature overnight. After the mixture has been thoroughly mixed, 50μL of the mixture was added into the wells of a 96-well polypropylene plate. Prior to curing, the oven was purged of moisture by means of nitrogen purging. The polypropylene plate was then placed into the 110°C oven for curing for 24h under nitrogen. After curing, the polypropylene plate was removed from the oven and allowed to cool at room temperature for 24h. The D-PHI films were sterilized by placing them in 70% ethanol for 24h. The films were left to dry for 24h under sterile conditions.
A.2.4 Collagen assay

The collagen assay was based on the study of Xu et al. (Xu et al., 2007). Briefly, the films were rinsed with PBS twice. Methanol at -20°C was added into each sample and control in order to fix the cells for staining. The cells were kept in the methanol for 24h at -20°C. After 24h of fixing, the methanol was removed and the samples were rinsed with PBS. The samples were incubated in picrosirius red stain (Sirius Red 0.1% in Saturated Picric Acid, Electron Microscopy Sciences, Hatfield, PA) for 1h at room temperature. The stain selectively binds to all types of collagen and thus, allows the quantification of total collagen. After 1h, the samples were rinsed with 0.1% acetic acid 3 times in order to remove unbound picrosirius red stain. 0.1N NaOH was then added into the sample wells in order to solubilize the stain bound to the collagen to allow for absorbance reading. The sample well-plate was placed on a shaker for 1h. Following complete dissolution of the stain, the solution in the wells with the D-PHI and PLGA films was transferred to another well-plate. The absorbance was read at 540nm using a spectrophotometer (VERSAmax microplate reader, Molecular Devices).

A.2.5 DNA quantification

The sample wells were rinsed with PBS twice. The PLGA films were transferred to another well in order to isolate the cells on the PLGA films solely. Cell lysis buffer (1.25mL of 1% Triton X-100 in PBS, 21.25mL of PBS, 2.5mL of 100mM EDTA) was added to each sample and control well. The samples/controls were left in the lysis buffer for 1h 50min on ice and then were sonicated for 10min. The cell lysates were collected. Cell lysates from each sample were added to a black polypropylene, flat-bottomed 96-well plate (Thermo Scientific) containing the DNA dye Hoechst 33258 (Sigma-Aldrich). The stain was read with a fluorescence microplate reader with the excitation wavelength set at 360 nm and the emission wavelength set at 460 nm (BioTek® FL600). The fluorescence values were compared to a standard curve (calf thymus DNA, Sigma-Aldrich) to obtain an estimate of DNA content. The background fluorescence emitted from the scaffolds (i.e. controls with no cells seeded) was subtracted from the reading to obtain the actual value.

In order to determine cell seeding efficiency, cell suspensions were collected into microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 2400rpm, at 4°C, for 5min, after which, the supernatant was aspirated. The cell pellet was then incubated in cell lysis buffer
for 1h. The cell lysates then underwent the same procedure as described above to obtain an estimate of DNA content in the cell suspension. The cell seeding efficiency was calculated by dividing the DNA content of samples from day 0 by the DNA content of the cell suspension.

A.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was performed for statistical analysis using SPSS (version 17.0). p<0.05 was considered statistically significant.

A.3 Results

A.3.1 Collagen assay

In order to assess the behavior of HGFs (in terms of collagen expression) when they are seeded at different seeding densities, HGFs were seeded at various cell densities onto TCPS. As illustrated in Fig. A.1, the collagen expression by HGFs seeded onto TCPS increases over a 14-day period for lower cell seeding densities (20000 cells/well and below). However, at higher cell seeding densities (80000 cells/well and 40000 cells/well), total collagen expression did not increase over a 14-day period.
**Figure A.1.** Collagen expression over 14 days by HGFs seeded at various densities onto TCPS. Values are reported as mean ± standard error (SE). * indicates significantly different from day 1 (p<0.05). ± indicates significantly different from day 7 (p<0.05). n = 3.

To allow for assessment of the behavior of HGFs when they are cultured on D-PHI, PLGA, and TCPS, HGFs were seeded onto these three types of substrates at a cell seeding density of 20000 cells/well. The rationale for using cell seeding density of 20000 cells/well will be discussed later. As shown in **Figs. A.2**, the collagen expression by the HGFs increased significantly over a 14 day period for all three substrates (p<0.05).

![Collagen expression over 14 days by HGFs seeded at various densities onto TCPS.](image)

**Figure A.2.** Collagen expression over 14 days by HGFs seeded at a cell seeding density of 20000 cells/well onto three different substrates. Values are reported as mean ± SE. * indicates significantly different from day 1 (p<0.05). ± indicates significantly different from day 7 (p<0.05). n = 3.

A.3.2 HGF cell growth

In order to assess the behavior of HGFs (in terms of cell growth) when they are seeded at a higher cell density, the HGFs were seeded at a cell density of 40000 cells/well onto TCPS. As shown in **Fig. A.3**, HGF population was significantly decreased over a 14 day period at this seeding density.
Since HGFs showed decrease in cell population and collagen production over 14 days when seeded at a cell density of 40000 cells/well, a lower cell seeding density (20000 cells/well) was used for subsequent experiments. To assess the proliferation of HGFs on the three different substrates, the HGFs were seeded at 20000 cells/well onto TCPS, D-PHI, and PLGA. It was found that the growth of HGFs was increased over 14 days on all three substrates (Fig. A.4).
**Figure A.4.** DNA content of HGFs seeded at a cell density of 20000 cells/well onto three different substrates, over 14 days. Values are reported as mean ± SE. * indicates significantly different from day 1 (p<0.05). n = 12 for TCPS, n = 9 for D-PHI and PLGA.

### A.3.3 Cell seeding efficiency

The cell seeding efficiency was determined by comparing the DNA content of day 0 samples (on all three substrates) to that of the cell suspensions. As shown in **Fig. A.5**, cell seeding efficiency was found to be comparable among all three substrates.

![Cell Seeding Efficiency Graph](image)

**Figure A.5.** Cell seeding efficiency for HGFs seeded onto three different substrates. Values are reported as mean ± SE. n = 12 for TCPS, n = 9 for D-PHI and PLGA.

### A.3.4 Normalized collagen content

As shown in **Fig. A.6**, the amount of collagen (per DNA content) produced by HGFs on TCPS did not change over the 14-day culture. On D-PHI films, HGFs produced significantly more collagen than those on TCPS at day 14 (p<0.05). On PLGA, the collagen production by HGFs increased significantly from day 1 to day 14 (p<0.05).
A.4 Discussion

This study determined the appropriate cell seeding density to be used in investigating the effects of TCPS, D-PHI, and PLGA on HGFs, by measuring collagen production in HGFs on TCPS at various cell seeding densities. As illustrated in Fig. A.1, at 80000 cells/well, collagen production decreased over culture time; and at 40000 cells/well, collagen production did not increase over the 14-day culture. This suggests that HGFs may have been over-confluent, which caused cell death and inhibition in cell growth, thus affecting collagen production. In order to assess the feasibility of this explanation, DNA quantification was performed for HGFs seeded at 40000 cells/well on TCPS (Fig. A.3). It was found that DNA content was significantly decreased over the 14-day culture, which was indicative of cell death. At a cell seeding density of 20000 cells/well, the DNA content was increased over 14 days (Fig. A.4). Thus, a cell seeding density of 20000 cells/well was used for subsequent experiments that involved all three types of substrates.
As shown in Fig. A.2, HGFs were still capable of producing collagen when they were cultured on D-PHI. Also, over a 14-day culture period, similar increase in total collagen production was observed for D-PHI, TCPS and PLGA. This suggests that HGFs produce comparable amount of collagen on D-PHI when compared with PLGA, which is a commonly used biomaterial in biomedical devices. The results also indicate that D-PHI can potentially be used in gingival tissue regeneration as the gingival lamina propria is collagenous.

When collagen production from HGFs on the three substrates was normalized to the DNA content, it was found that the increase in collagen production over 14 days on TCPS was simply due to an increase in the number of cells (Fig. A.6). On D-PHI films, although there was an increase in the normalized amount of collagen at day 14, this increase was not statistically significant when compared with day 1 and day 7. However, when compared with TCPS at day 14, this value (on D-PHI) was significantly greater. The greater normalized collagen production may be explained by the fact that HGFs were more activated to produce collagen when they were cultured on D-PHI vs. TCPS in long-term culture. Further investigation will be required to explore the effect of D-PHI on HGF behaviour. The normalized collagen production in HGFs cultured on PLGA showed steady increase over the 14-day culture, which indicates that HGFs may become more activated to produce collagen when they were cultured on PLGA.

A.5 Conclusion

It was found that HGFs seeded on D-PHI films were still capable of producing collagen, and thus, provided evidence that HGFs remained functional in terms of collagen production when cultured on this novel biomaterial. Also, it was found that HGFs were able to proliferate over the 14-day culture on the D-PHI films at 20000 cells/well. Lastly, HGFs may be more activated to produce collagen at day 14 on D-PHI films when compared with TCPS.
A.6 References


Appendix B
Assessment of human umbilical vein endothelial cell growth and migration on degradable/polar/hydrophobic/ionic polyurethane (D-PHI) scaffold

B.1 Introduction
Previous studies with vascular endothelial cells (ECs) seeded on collagen matrix showed that ECs may form a monolayer on the surface and some of them migrated into the scaffold to form tubular structures (Davis et al., 2002). While the migration and tubular formation is desired, monolayer formation on the surface could hinder nutrient diffusion into the scaffold, hence there is a need to optimize seeding strategies for ECs in the D-PHI materials so as to optimize their uptake within the scaffolds rather than their predominance at the outer surface. In this study, we are interested in improving cell seeding within the D-PHI scaffolds (using a vacuum cell seeding technique) and promoting the growth and migration of HUVECs, in synthetic polymeric materials (D-PHI). Another objective is to investigate the effect of medium perfusion (dynamic culture) on the growth of HUVECs. It is hypothesized that seeding of HUVECs on D-PHI scaffolds using a vacuum pump will improve cell penetration in the scaffold and the dynamic culture will improve cell proliferation. (This work was done by Arezou Ossareh (for undergraduate thesis) under my supervision.)

B.2 Materials and methods

B.2.1 D-PHI scaffolds fabrication
D-PHI scaffolds were fabricated via free radical polymerization which involved the mixing of a lysine-based divinyl oligomer (DVO) with methacrylic acid and methyl methacrylate in a 1:5:15 ratio (Sharifpoor et al., 2010). Poly(ethylene glycol) and sodium bicarbonate were used as the porogens (Sharifpoor et al., 2010) and the pore size (Sharifpoor et al., 2010) ranged from 30–250μm with 79 ± 3% porosity. The resulting mixture was molded into discs and cured at 110 °C for 24 hours. The scaffolds were subjected to sonication in water for 14 days for porogen leaching. After leaching, the scaffolds were soaked in increasing concentrations of ethanol (30, 35, 50, 70, 90, 95 and 100 %) at room temperature to dehydrate. Finally, the scaffolds were
stored at room temperature in a dry environment until needed. Each scaffold has a diameter of 6 mm and a thickness of 2 mm.

B.2.2 Culture of HUVECs

HUVEC cell lines from American Tissue Culture Collection (ATCC, CRL-1730) were used. Cells were grown in T-75 flasks in Kaighn’s modification of Ham’s F-12 medium (F-12 K medium; Cedarlane, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Sigma) and 100U/mL penicillin/100μg streptomycin (Sigma), 0.1mg/mL heparin (Sigma), 0.05 mg/mL EC growth supplement (Sigma) and 1% Hanks balance salt solution (Gibco).

B.2.3 Cell seeding

B.2.3.1 Scaffolds preparation prior to cell seeding

Prior to cell seeding, the D-PHI scaffolds were placed in 70% ethanol overnight. The ethanol was then removed and the scaffolds were dried in a sterile environment for 24 hours. Scaffolds were pre-wetted with sterile F-12 K medium overnight. The scaffolds were then partially dried in a sterile environment prior to seeding.

B.2.3.2 Estimating total cell number for cell seeding

Cell seeding was performed when the cells reached 80-90% confluence. Prior to seeding, the cells in the culture flask were trypsinized and total cell number was estimated. Trypan Blue (Gibco) was mixed with the cell suspension thoroughly and the mixture was transferred onto a hemocytometer for cell counting.

B.2.3.3 Static cell seeding

The scaffolds were placed into a 96-well plate. Cells were seeded onto D-PHI scaffolds at corresponding cell seeding density. The 96-well plate was then placed in the incubator for overnight incubation, after which media was changed every other day.

B.2.3.4 Vacuum cell seeding

Each scaffold was placed into a 5mL sterile disposable syringe and the plunger was then re-inserted to remove most of the air from the syringe. The cell suspension was drawn into the syringe by pulling the plunger backward to ensure that the scaffold was in the cell suspension.
The syringe was closed with a luer lock cap and the plunger was pulled back for 2mL for 5 seconds in order to assure that a vacuum was created inside the syringe (Fig. B.1). This procedure was repeated 3 times. The seeded scaffold was then transferred into a 96 well-plate and placed in the incubator for overnight incubation, after which media was changed every other day.

**Figure B.1.** a) Each scaffold was placed into a 10mL sterile disposable syringe. b) The plunger was then re-inserted to remove most of the air from the syringe. c) The cell suspension was drawn into the syringe by pulling the plunger backward to ensure that the scaffold was in the cell suspension. d) The syringe was closed with a luer lock cap. e) The plunger was pulled back for 2mL for 5 seconds in order to assure that a vacuum was created inside the syringe. This procedure was repeated 3 times (the scaffold is floating in the media).
B.2.4 Dynamic culture (medium perfusion)

The scaffolds were placed into a 96-well plate. Cells were seeded statically onto D-PHI scaffolds at a corresponding cell seeding density. The 96-well plate was then placed in the incubator for overnight incubation. After 24 hours, the scaffolds were transfer into a bioreactor for constant medium perfusion in an incubator (Cheung et al., 2013).

B.2.5 DNA assay

DNA assay was performed at days 0, 1, 7, and 14 of culture. The scaffolds were rinsed twice with PBS. The samples were minced and placed in a cell lysis buffer (100 mM NaCl, 100 mM Tris-HCl, 25 mM EDTA, 0.1% SDS) for 1h at 65°C for incubation. The mixture was then centrifuged at 14000 rpm for 15 minutes at 8°C to allow the scaffold pieces to settle. A Hoechst 33258 dye solution was added to each well of a black polypropylene, flat-bottomed 96-well plate (Thermo Scientific). The cell lysates was added to each well containing the dye solution. Fluorescence was measured using a Bio-Tek® FL600 microplate reader (Faculty of Dentistry, University of Toronto, Canada) with the excitation wavelength set at 360nm and the emission wavelength at 460nm. The fluorescence values were compared to a standard curve (calf thymus DNA, Sigma-Aldrich) to estimate DNA content. The background fluorescence emitted from the scaffolds (no cells) was subtracted from the reading to obtain the reported value.

B.2.6 Statistical analysis

Values were presented as mean ± standard error (SE). Statistical significance was determined by one-way ANOVA and student’s t-test (SPSS, version 19.0) with a significance level of p<0.05.

B.3 Results and discussion

B.3.1 Cell seeding density and cell seeding technique

The cell seeding experiment at 0-, 1- and 7-days post seeding was performed on D-PHI scaffolds to determine the optimal cell seeding density and cell seeding technique. Cells were seeded at two different densities: 40,000 cells/scaffold and 80,000 cells/scaffold with two different techniques: static and vacuum. The optimal seeding density and cell seeding technique were evaluated for each density and each technique by comparing DNA content.
In the static seeding technique, for both seeding densities, the growth of HUVEC was increased from day 0 to day 1 of culture but decreased after day 1 (Fig. B.2). This indicates that a significant number of HUVECs did not survive the long-term culture period of 7 days in a monoculture. In a study by Hofmann et al, HUVECs did not proliferate or form any apparent tube-like structures in monocultures and the cells began to die rapidly after one week without passage (Hofmann et al., 2008). Since HUVEC proliferation was similar between 40,000 and 80,000 cells/scaffold, based on the studies with human gingival fibroblast (HGF) monocultures (Cheung et al., 2013), 40,000 cells/scaffold was chosen as the cell seeding density for other experiments.

The DNA content of HUVECs seeded with the static and vacuum seeding techniques (Fig. B.2 and B.3) showed that the growth of HUVECs was enhanced from day 0 to day 1 with the static technique but not for the vacuum technique. This suggests that some HUVECs died in the process of vacuum seeding. During vacuum seeding, only a small volume of cell suspension (200 µL) was used and being transferred from the syringe to the scaffold. Since the scaffold did not absorb all the cell suspension, the remaining suspension was collected after vacuum seeding and was added directly onto the scaffold in a 96 well-plate. Since the vacuum seeding technique involved too much handling (possibly leading to a loss in cell population), the static seeding technique was chosen for the other studies.

![Bar graph showing DNA content of HUVECs](image)

**Figure B.2.** Average DNA content of HUVECs, seeded with the static seeding technique, at days 0, 1 and 7 of culture for 40,000 and 80,000 cells/scaffold. n = 9, ±SE.
Figure B.3. Average DNA content of HUVECs, seeded with the vacuum seeding technique, at days 0, 1 and 7 of culture for 40,000 and 80,000 cells/scaffold. n = 9, ±SE.

B.3.2 Dynamic vs. static culture

HUVECs were cultured on D-PHI scaffolds statically and dynamically (i.e. under medium perfusion) up to 7 days to compare the growth of HUVECs on D-PHI scaffolds in these two cell culturing conditions (Fig. B.4). In tissue engineering approaches, constant medium perfusion with the use of a bioreactor can improve cell distribution, cell activity and efficiency of seeding processes into 3D structures. From the DNA data, it was observed that the HUVEC survival was maintained in the dynamic culture over 7 days in the D-PHI scaffold, while it was significantly decreased from day 1 to day 7 in the static culture (p<0.001). At day 7 of culture, the HUVEC population in the dynamic culture was greater than that in the static culture. The reported data indicated that the combination of the D-PHI scaffold and medium perfusion improved HUVEC survival. In the study of Mathews et al., a dynamic culture of ECs in a tube structure stabilized the cells, and ECs were aligned along the direction of flow, with improved deposition of insoluble elastin and type IV collagen (Mathews et al., 2012). Moreover, in a previous study by Cheung et al., it was shown that medium perfusion promoted greater HGF proliferation, suggesting that HGFs are more metabolically active in the dynamic vs. static culture (Cheung et al., 2013).

The decreased HUVEC growth at day 7 suggests that HUVECs could not survive the long-term monoculture. In a study by Kyriakidou et al., it was shown that, as opposed to the use of
monocultures, co-cultures encouraged cell-cell interaction between ECs and osteoblasts, which supported EC survival and blood vessel formation (Kyriakidou et al., 2008). Similarly, a study by Hofmann et al. demonstrated the need for osteoblasts to support cell proliferation and EC tubulogenesis in long-term culture in polyurethane scaffolds (Hofmann et al., 2008). Therefore, HUVECs may need to be co-cultured with other cell types (e.g. HGFs) in future studies in order to maintain long-term survival and promote tubulogenesis in the D-PHI scaffolds.

Figure B.4. DNA content of HUVECs cultured in D-PHI scaffolds in static or dynamic cultures over 7 days. All samples (40,000 cell/scaffold) were cultured statically at day 0, followed by separation into static or dynamic cultures at day 1. n = 9, ±SE. ǂSignificantly different from each other (p<0.001).

B.4 Conclusion

In summary, a seeding density of 40,000 cells/scaffold and the static cell seeding technique may be more favourable for HUVEC culture. HUVECs did not survive the long-term static culture of 7 days in a monoculture. Dynamic culture was shown to increase HUVEC survival. For future studies, HUVECs may need to be co-cultured with other cell types in order to support cell proliferation and EC tubulogenesis.
B.5 References


Appendix C
Supplementary data

Figure C.1. (a) Amount of vimentin (Vim) produced by HGFs in static vs. dynamic monocultures throughout 28-day period. (b) Direct relationship between the amount of Vim and DNA content in HGFs in static and dynamic monocultures. n = 9, ±SE. *Significantly different from each other (p<0.05).

Figure C.2. (Left) Immunofluorescence image of HGFs in a D-PHI scaffold section (5x magnification). Cell nuclei were stained with Hoechst, which also stained the scaffold. (Right)
A magnified view (15x) of the framed area in the image on the left. Oval-shaped nuclei can be distinguished from the scaffold (arrows). Scale bar = 60 µm.

**Figure C.3.** Immunofluorescence images of HUVEC-HGF co-culture in a D-PHI scaffold section (15x magnification). (Left) Cell nuclei were stained with Hoechst, which also stained the scaffold. (Right) Cells were stained for von Willebrand factor (vWF, red) and cell nuclei were stained with Hoechst (blue). The nuclei can be seen localized with the vWF stain (arrows), which can be distinguished from the scaffold. These images were used for the ease of counting. Scale bar = 60 µm.

**Figure C.4.** Immunofluorescence images of HUVEC-HGF co-culture (in 50/50 mix medium, at 1:2 (HUVEC: HGF)) in a D-PHI scaffold section (15x magnification). (Left) Cell nuclei were stained with Hoechst, which also stained the scaffold. (Right) HUVECs were stained for vWF
(red) and cell nuclei were stained with Hoechst (blue). It was observed that HUVECs (arrows) formed large clusters at a ratio of 1:2 (HUVEC:HGF). Scale bar = 60 µm.

Figure C.5. Amount of ED-A Fn produced by HGFs in static vs. dynamic monocultures throughout 28-day period. n = 9, ±SE. *Significantly different from each other (p<0.05).

Figure C.6. HGFs were cultured on TCPS for 24 hrs, after which 3µM SIS3 (a Smad inhibitor) was added to selected wells to test the functionality of SIS3. After 1 hr of incubation, α-SMA production was significantly reduced in the treated vs. non-treated condition, proving that SIS3 was effective in blocking the Smad pathway. The results also indicate that Smad was activated when HGFs were cultured on TCPS. n = 9, ±SE. *Significantly different from each other (p<0.05).
Appendix D
Mechanical drawings of the perfusion bioreactor

Figure D.1. Plan views of the top and the platform of the bioreactor chamber.
Figure D.2. Side views of cross-sections A and B indicated in Fig. D.1 at the platform and at the top of the bioreactor’s chamber.
Figure D.3. Side view of the bioreactor’s chamber with the parts mounted together. Cross-sections A and B correspond to those indicated in Fig. D.1.
Appendix E
Calculations of Reynolds number and shear stress in the perfusion bioreactor

E.1 Calculations

Reynolds Number:

To verify if the flow in the channel is laminar, the Reynolds number was calculated. Since the length of the channel is much greater than the height and width of the channel, the channel is simplified as two infinitely long parallel plates. The equations used to calculate the Reynolds number (Re) in the bioreactor channel include:

\[ V = \frac{Q}{A} \]

\[ Re = \frac{\rho V L}{\mu} \]

\textbf{Equation E.1.} Speed of fluid flow.

where \( V \) is the speed, \( Q \) is the flow rate, and \( A \) is the cross-sectional area of the channel, and

\[ Re = \frac{\rho V L}{\mu} \]

\textbf{Equation E.2.} Reynolds number (Re) calculation.

where \( \rho \) is the density of the fluid, \( L \) is the height of the channel, and \( \mu \) is the viscosity of the fluid.

Assume \( \rho_{\text{media}} \approx \rho_{\text{water}} \) (1 kg/L) and \( \mu_{\text{media}} \approx \mu_{\text{water}} \) (801.5 Pa·s).

Using \textbf{Equation E.1},

\[ V = \frac{Q}{A} = \frac{1.0 \text{ mL/min}}{0.3 \text{ cm} \times 0.6 \text{ cm}} = \frac{1.0 \text{ cm}^3/\text{min}}{0.18 \text{ cm}^2} = 5.556 \text{ cm/min} \]
Using **Equation E.2**, 

\[
Re = \frac{\rho VL}{\mu}
\]

\[
= \left( \frac{1 \text{ kg}}{L} \right) \left( \frac{5.56 \text{ cm}}{\text{min}} \right) (0.3 \text{ cm})
\]

\[
= \frac{801.5 \text{ Pa} \cdot \text{s}}{801.5 \text{ Pa} \cdot \text{s}}
\]

\[
= \left( \frac{0.001 \text{ kg}}{\text{cm}^3} \right) \left( \frac{0.0926 \text{ cm}}{\text{s}} \right) (0.3 \text{ cm})
\]

\[
= \frac{2.778 \times 10^{-5} \text{ kg}}{\text{cm} \cdot \text{s}}
\]

\[
= \frac{801.5 \text{ kg}}{\text{m} \cdot \text{s}}
\]

\[
= \frac{0.002778 \text{ kg}}{\text{m} \cdot \text{s}}
\]

\[
= \frac{801.5 \text{ kg}}{\text{m} \cdot \text{s}}
\]

\[
= 3.466 \times 10^{-6}
\]

Therefore, the Reynolds number is \(3.466 \times 10^{-6}\), which is less than 1000, indicating that flow in the bioreactor chamber is laminar.

Re can be affected by a scaffold’s pore size and porosity. To estimate the Re in the 3D porous D-PHI scaffold, an analytical model was adopted from Jungreuthmayer C et al. and Boschetti et al. (Boschetti et al., 2006; Jungreuthmayer et al., 2009). Assuming the scaffold can be represented as a bundle of parallel circular pipes, the diameter of each pipe is the pore diameter \(d_{\text{pore}}\). The average speed of fluid flow in the scaffold can be calculated as follows:

\[
V_{\text{scaf}} = \frac{Q}{(A_{\text{chamber}} \Phi)}
\]

**Equation E.3.** Average speed of fluid flow in a 3D porous scaffold.
where $Q$ is the flow rate, $A_{\text{chamber}}$ is the cross-sectional area of the bioreactor chamber (0.18 cm$^2$) and $\Phi$ is the porosity of the scaffold (80%). $Re$ for flow in a 3D porous scaffold can be estimated by:

$$Re = \frac{d_{\text{pore}} \rho V_{\text{sc}}}{\mu}$$

**Equation E.4.** Estimation of $Re$ for flow in a 3D porous scaffold.

where $\rho$ is the density of the fluid and $\mu$ is the dynamic viscosity of the fluid.

Using **Equation E.3**, 

$$V_{\text{sc}} = 0.5 \text{ cm}^3/\text{min}/(0.18 \text{ cm}^2 \times 0.8) = 3.472 \text{ cm/min} = 0.05787 \text{ cm/s}$$

Using **Equation E.4**, $d_{\text{pore}} \approx 0.003 \text{ cm} – 0.025 \text{ cm}$, $\mu = \text{dynamic viscosity of culture medium} = 0.001 \text{ Pa} \cdot \text{s}$,

$$Re = \frac{d_{\text{pore}} \rho u_{\text{sc}}}{\mu} \approx 0.01736–0.1447$$

Therefore the Reynolds number for flow in a 3D porous D-PHI scaffold is laminar ($Re < 1000$). 

**Wall Shear Stress:**

Similar to the calculations for the Reynolds number, a parallel plate model is used for calculating the wall shear stress in the bioreactor chamber. The equation for a parallel plate model is:

$$\tau_w = \frac{6 \mu Q}{h^2 w} \left(1 - \frac{z}{L}\right)$$

**Equation E.5.** Wall shear stress in a parallel plate model.

where $\tau_w$ is the wall shear stress, $\mu$ is the fluid viscosity, $Q$ is the flow rate, $h$ is the gap width between the parallel plates (i.e. height of the channel), $w$ is the width of the channel, $z$ is the length along the $z$-axis (i.e. height of the channel/2, as the zero of $z$-axis is at the centre of the channel), and $L$ is the length of the channel.
Assume $\mu_{\text{media}} \approx \mu_{\text{water}} (8.9 \times 10^{-3} \text{ dyn} \cdot \text{s/cm}^2)$.

Using Equation E.5,

$$T_w = 6 \left( 8.9 \times 10^{-3} \frac{\text{dyn} \cdot \text{s}}{\text{cm}^2} \right) \left( 0.5 \frac{\text{mL}}{\text{min}} \right) \left( 1 - \frac{0.3 \text{ cm}}{2 \text{ cm}} \right) \left( 0.3 \text{ cm} \right)^2 \left( 0.6 \text{ cm} \right)$$

$$= \left( 0.0543 \frac{\text{dyn} \cdot \text{s}}{\text{cm}^2} \right) \left( 0.00833 \frac{\text{mL}}{\text{s}} \right)$$

$$= 0.000422 \frac{\text{dyn} \cdot \text{mL}}{0.054 \text{ mL} \cdot \text{s}}$$

$$= 0.00781 \frac{\text{dyn}}{\text{cm}^2}$$

Therefore, the estimated wall shear stress in the bioreactor chamber is 0.00781 dyn/cm$^2$.

Using the analytical model adopted from Jungreuthmayer C et al. and Boschetti et al. (Boschetti et al., 2006; Jungreuthmayer et al., 2009), the wall shear stress in the 3D porous D-PHI scaffold can be estimated by:

$$\tau_w = 8\mu V_{\text{scaf}}/d_{\text{pore}}$$


where $\tau_w$ is the wall shear stress and $\mu$ is the fluid viscosity.

Using Equation E.6, $d_{\text{pore}} \approx 0.003\text{cm} - 0.025\text{cm}$, $\mu = 0.001 \text{ Pa} \cdot \text{s}$,

$$\tau_w = 8\mu V_{\text{scaf}}/d_{\text{pore}} \approx 0.185 - 1.543 \text{ dyn/cm}^2$$

Therefore, the estimated wall shear stress in the 3D porous D-PHI scaffold is in the range of 0.185–1.543 dyn/cm$^2$. 

226
E.2 References


Appendix F
Biomaterials in co-culture systems: Towards optimizing tissue integration and cell signaling within scaffolds

Abstract

Most natural tissues consist of multi-cellular systems made up of two or more cell types. However, some of these tissues may not regenerate themselves following tissue injury or disease without some form of intervention, such as the use of tissue engineered constructs. Recent studies have increasingly used co-cultures in tissue engineering applications as these systems better model the natural tissues, both physically and biologically. This review aims to identify the challenges of using co-culture systems and to highlight different approaches with respect to the use of biomaterials in the use of such systems. The application of co-culture systems to stimulate a desired biological response and examples of studies within particular tissue engineering disciplines are summarized. A description of different analytical co-culture systems is also discussed and the role of biomaterials in the future of co-culture research are elaborated on. Understanding the complex cell-cell and cell-biomaterial interactions involved in co-culture systems will ultimately lead the field towards biomaterial concepts and designs with specific biochemical, electrical, and mechanical characteristics that are tailored towards the needs of distinct co-culture systems.

Appendix G

New degradable polyurethanes for use in tissue engineering: Inspired by mechanisms of biodegradation and wound healing

Abstract

The Biomedical Polymers Laboratory at the Institute of Biomaterials and Biomedical Engineering in Toronto in the Faculties of Applied Science and Engineering, Dentistry and Medicine is coordinated by Professor J Paul Santerre in the Institute’s area of Biomaterials, Tissue engineering and Regenerative Medicine. Research in the Santerre group includes polyurethane synthesis for biomedical implants and their biostability, conceiving new biomaterials for tissue engineering applications in the area of vascular and connective tissues, and utilizing co-culture techniques as a means of reducing the need for complicated biochemical modifications of the materials. Upon implantation of a biomaterial, monocytes play a critical role in directing subsequent cellular and wound healing response. The evaluation of a degradable polar hydrophobic ionic polyurethane (D-PHI) for vascular tissue engineering applications indicated the material’s ability to support an anti-inflammatory monocyte state while also supporting growth and a contractile vascular smooth muscle cell (VSMC) phenotype. These materials have also been applied toward the development of gingival tissue regeneration. Studies by the group on protein interactions with polymer surfaces and mechanisms of enzyme-catalyzed degradation have led to the development of novel nano structured fibres from linear degradable polyurethanes to form the annulus fibrosus tissues of spinal discs. This review highlights selective unique attributes of the group’s original research.