1 INTRODUCTION

1.1 Wound healing

The wound healing process has been studied for decades. It involves a series of intricate cellular events involving cell migration, proliferation, and remodeling (*1-2*). Upon the onset of the inflammatory response, fibroblasts begin to proliferate and migrate into the wound area. Collagen and fibronectin are subsequently deposited in the wound bed (*3*), serving as a temporary matrix on which epithelial cells can migrate (*4-5*). Throughout the process, cells often migrate as groups (*6-7*). The mechanisms by which they do so are under active investigation (*8*).

While understanding how cells move together in tissue repair is important, this process is also fundamentally relevant to other complex events such as morphogenesis and tumor metastasis (*6, 9-10*). Two mechanisms of collective migration have been identified (*11*). Lamellipodial crawling involves active migration of cells at the wound edge, mediated primarily through cell-ECM interactions in the wound area (*12-14*). This process is commonly observed in adult wound healing and is well-studied in *in vitro* wound healing models (*15-17*). The second mechanism is known as the "purse string" model, and is the primary mode of cell movement in fetal wound healing (*18-20*). Upon wounding, an actomyosin cable assembles around the periphery of the wound, and contracts to close the wound by transmitting tension through intercellular junctions (*21*). While the lamellipodial crawling mechanism results in rapid wound closure, the purse string mechanism leads to scarless wound healing (*22*). Understanding the factors

involved in both wound healing mechanisms might enable us to achieve optimal wound healing rates and to minimize scarring.

1.2 Challenges in tissue regeneration

The role of ECM in wound healing has been traditionally thought of as a passive structural support for cells. It is now clear that cell-ECM interactions, in concert with growth factors, are necessary for rapid wound healing (*23-24*). Hence, the main challenge in wound therapeutics is to provide an ideal microenvironment for optimal cell migration and proliferation (*25*).

Many strategies have been adopted for accelerating tissue repair. Exogenous growth factors (*26-27*), ECM molecules (*28*), and short peptide sequences (*29*) targeting specific integrin receptors have been shown to accelerate wound healing both *in vitro* and *in vivo*. However, native ECM molecules or growth factors lack structural properties, and are expensive to produce in large quantities. On the other hand, synthetic materials offer excellent physical support, but do not possess any biological activity. To circumvent these disadvantages, synthetic materials have also been functionalized with bioactive peptide sequences (*30-32*) and growth factors (*33*). However, issues arising from steric hindrance and denaturation of these molecules have yet to be resolved (*34*).

1.3 Artificial proteins as biomaterials

The advent of DNA recombinant technology has brought about the discovery of a novel class of protein-based biomaterials (*35*). Using a series of genetic manipulations, DNA sequences encoding functional moieties can be readily integrated into bacterial hosts and artificial proteins containing user-specified functionalities can be easily generated.

The genetic approach to biomaterial design allows the synthesis of complex protein macromolecules, which are otherwise difficult to fabricate by chemical means. Specific mechanical and biological functionalities can also be expressed combinatorially to direct cell behavior. Biological domains in fibronectin, laminin, and other ECM molecules have been widely incorporated into artificial proteins (*35*). The short Arg-Gly-Asp (RGD) sequence found in fibronectin has been shown to promote cell spreading of a multitude of cell types (*36*). Likewise, recombinant proteins derived from spider silk (*37*), collagen (*38*), and elastin (*39*) have been shown to display mechanical properties resembling their native proteins. In particular, Urry and coworkers have synthesized and characterized a series of recombinant elastin-like materials bearing repetitive motifs Val-Pro-Gly-X-Gly (VPGXG), where X can be any amino acid. These materials can be designed to yield a range of viscoelastic properties, by substituting the appropriate amino acid in the position X (*40*).

The artificial proteins prepared in our laboratory consist of functional domains derived from fibronectin and elastin (*41-44*). The artificial extracellular matrix (aECM) proteins are modular in nature, allowing simple substitution of either the biological or the

structural domains. Non-canonical amino acids have also been incorporated to introduce alternative crosslinking chemistries (*45*) and to create novel materials with tunable moduli (*46*). Figure 1.1 shows the amino acid sequences of the aECM proteins discussed in this thesis. In all constructs **1** to **5**, lysine residues were interspersed within the elastin-like sequences, and subsequently crosslinked to form viscoelastic materials (*41, 47*). These aECM proteins also exhibit the characteristic inverse transition temperature of elastin (*48*), which allows simple and effective purification via thermal cycling (*49*).

1 M-MASMTGGQQMG-HHHHHHH-DDDDK (LD-YAVTGRGDSPASSKIA ((VPGIG)$_2$VPG*K*G(VPGIG)$_2$)$_4$VP)$_3$-LE

T7 tag; His tag; cleavage site; RGD cell-binding domain; Elastin-like repeats

2 M-MASMTGGQQMG-HHHHHHH-DDDDK (LD-YAVTGRDGSPASSKIA((VPGIG)$_2$VPG*K*G(VPGIG)$_2$)$_4$VP)$_3$-LE

Scrambled RGD domain

3 M-MASMTGGQQMG-HHHHHHH-DDDDK-[(VPGIG)$_2$VPG*K*G(VPGIG)$_2$]$_6$ FN910m [(VPGIG)$_2$VPG*K*G(VPGIG)$_2$]$_6$-LE

4 M-MASMTGGQQMG-HHHHHHH-DDDDK-[(VPGIG)$_2$VPG*K*G(VPGIG)$_2$]$_6$ FN10m [(VPGIG)$_2$VPG*K*G(VPGIG)$_2$]$_6$-LE

5 M-MASMTGGQQMG-HHHHHHH-DDDDK-[(VPGIG)$_2$VPG*K*G(VPGIG)$_2$]$_6$ RGDm [(VPGIG)$_2$VPG*K*G(VPGIG)$_2$]$_6$-LE

Figure 1.1 Amino acid sequences of aECM proteins. Each aECM protein contained a T7 tag, a hexahistidine tag, an enterokinase cleavage site, and elastin-like domains containing lysine residues (italicized) for crosslinking. Constructs **1** and **2** containing the short RGD sequence were developed by Julie Liu in our laboratory. Constructs **3** to **5** were designed and cloned in this work and will be discussed in greater detail in Chapter 3. The full amino acid sequences of the underlined cell binding domains in constructs **3** to **5** can be found in Figure 3.1.

In our work and that of others, cell responses on RGD surfaces were never identical to those observed on native fibronectin (*50*). An obvious strategy to improve biological activity of the existing aECM proteins is hence to expand the cell binding region to include full-length fibronectin domains. Efforts to expand on the biological activity of the aECM proteins are described in Chapter 3.

1.4 Methods for studying wound healing *in vitro*

In vitro wound healing assays have been used for decades to study the major signaling transduction pathways in wound healing (*51-54*). These assays have also been used to examine various mechanisms responsible for cell sheet movement (*12-13, 15*). The most commonly used setup is the "scratch" wound assay, which is performed by denuding an area of a confluent cell sheet using a small tool (e.g., pipette tip). The method is simple but often results in wounds that vary significantly between experiments. More importantly, the method is unsuitable for studying cell-material interactions. A major challenge in designing wound healing assays for studying cell-material interaction is to allow cells to form a confluent monolayer without modifying the underlying surface. A "barrier" wound healing assay not only allow precise control the surface chemistry of the wound area (*54*), it has also been shown to trigger wound responses similar to those observed in the scratch wound assays (*16, 55*). Recently, microfabrication has provided new tools for fabricating barriers (*55*), micropatterns (*56*), and microfluidic systems (*57*) for wound healing studies.

The *in vitro* "barrier" wound healing assay discussed in Chapter 2 was adapted from the work of Nikolić et al. (*55*). We used a polydimethylsiloxane (PDMS) block to protect the underlying aECM protein surface, while allowing cells to grow around it. Fibronectin was also added to allow cells to spread on surfaces that do not support cell attachment (i.e., BSA and aECM proteins containing a scrambled "RDG" sequence). When cells grow to confluence, the PDMS slab is removed, exposing the wounded cell sheet to the initial protein surface. The same assay was used again in Chapter 3 to compare different protein surfaces. The "barrier" assay provided a convenient way to examine cell-aECM interactions *in vitro*, but the wound areas were too large to allow the visualization of the entire wound periphery.

Attempts to study cell decisions along the periphery of the wound prompted further development of the wound healing assay. Using standard lithography methods, we prepared PDMS blocks bearing micron-sized barriers to replace the previous PDMS blocks. These patterns were made to create wounds of precise wound size and shape, allowing a systematic study of the role of wound geometry in wound healing.

1.5 Thesis organization and description of contributions

The thesis reports efforts towards understanding the fundamentals of cell-ECM interactions in wound healing. We studied extensively epithelial and fibroblast wound healing; both play distinct roles in the process of wound healing. The aECM proteins were used to understand and engineer specific cell-ECM interactions to accelerate wound

healing. More specifically, Chapter 2 examines corneal epithelial wound healing on crosslinked aECM protein with varying RGD densities. Crosslinked aECM films were produced by mixing aECM proteins with the RGD cell-binding domain and aECM proteins containing the RDG scrambled control (Figure 1.1; constructs **1** and **2**). I developed and performed the wound healing experiments, and analyzed the experimental data. The theoretical portion of this chapter was performed by Dr. Shelly Tzlil. Details of the simulation and data analysis were discussed jointly with Dr. Tzlil. I designed and performed all the experiments. We collaborated on writing the manuscript.

Another aspect of this work was focused on improving the design of the artificial extracellular matrix protein (aECM) materials to accelerate wound healing. In Chapter 3, I re-engineered the existing aECM protein constructs to incorporate full-length fibronectin domains 9 and 10 (Figure 1.1, constructs **3** to **5**). Rat-1 fibroblasts spread rapidly on these aECM protein surfaces. More importantly, aECM proteins containing full-length fibronectin domains 9 and 10 promoted rapid wound healing by supporting cell migration and proliferation, comparable to native fibronectin. I designed, cloned, and expressed these aECM proteins. I also performed all experiments and wrote the chapter.

Using the aECM protein containing fibronectin domain 10 (Figure 1.1; construct **4**) as described previously, we look to further understand how cells select the wound healing mechanism along the periphery of the wound. Chapters 4 and 5 resulted from collaborations with Dr. Chin-lin Guo. We used standard photolithography methods to create wound patterns with controlled wound size and geometry. I fabricated the master molds and made the PDMS micropatterned blocks. The MATLAB program used in

Chapter 4 was co-written by Jiang Bor-yuan and Dr. Guo. I acquired and analyzed all experimental data. I performed all other experiments. Dr. Guo wrote the description of the model while I wrote the experimental section. We co-wrote the rest of the chapter.

Chapter 5 describes how pre-disposing the cell sheets in a zigzag configuration allows cells to exploit the contraction of actomyosin cables and accelerate wound healing. I performed all the experiments and analyzed all the data with useful advice from Dr. Guo. I wrote the chapter.

1.6 References

1. A. J. Singer, R. A. F. Clark, *New Eng. J Med.* **341**, 738 (1999).
2. C. Steele, *Optometry Today*, 28 (1999).
3. F. Grinnell, *J. Cell Biochem.* **26**, 107 (1984).
4. R. A. F. Clark et al., *J. Invest. Dermatol.* **79**, 264 (1982).
5. R. A. F. Clark et al., *J. Invest. Dermatol.* **94**, 128S (1990).
6. P. Friedl, Y. Hegerfeldt, M. Tusch, *Int. J. Dev. Biol.* **48**, 441 (2004).
7. P. Rorth, *Trends in Cell Biol.* **17**, 575 (2007).
8. R. B. Vaughan, J. P. Trinkaus, *J. Cell Sci.* **1**, 407 (1966).
9. P. Martin, S. M. Parkhurst, *Development* **131**, 3021 (2004).
10. M. J. Redd, L. Cooper, W. Wood, B. Stramer, P. Martin, *Phil. Trans. R. Soc. Lond. B.* **359**, 777 (2004).
11. P. Martin, J. Lewis, *Science* **360**, 179 (1992).
12. R. Farooqui, G. Fenteany, *J. Cell. Sci.* **118**, 51 (2004).
13. X. Trepat et al., *Nat. Phys. Lett.* **5**, 426 (2009).
14. M. Zhao, B. Song, J. Pu, J. V. Forrester, C. D. McCaig, *FASEB J.* **17**, 397 (2003).
15. M. Bindschadler, J. L. McGrath, *J. Cell Sci.* **120**, 876 (2006).
16. E. R. Block et al., *J. Biol. Chem.* **279**, 24307 (2004).
17. K. Y. Chan, D. L. Patton, Y. T. Cosgrove, *Invest. Opthalmol. Vis. Sci.* **30**, 2488 (1989).
18. W. M. Bement, P. Forscher, M. S. Mooseker, *J. Cell. Biol.* **121**, 565 (1993).
19. K. M. Bullard, M. T. Longaker, H. P. Lorenz, *World J. Surg.* **27**, 54 (2003).
20. Y. Danjo, I. K. Gipson, *J. Cell Sci.* **111**, 3323 (1998).
21. M. Tamada, T. D. Perez, W. J. Nelson, M. P. Sheetz, *J. Cell. Biol.* **176**, 27 (2007).
22. D. P. Kiehart, *Curr. Biol.* **9**, R602 (1999).

23. K. Watanabe, S. Nakagawa, T. Nishida, *Invest. Opthalmol. Vis. Sci.* **28**, 205 (1987).

24. H. Brotchie, D. Wakefield, *Australas J. Dermatol.* **31**, 47 (1990).

25. R. Langer, D. A. Tirrell, *Nature* **428**, 487 (2004).

26. K. Hori et al., *J. Control. Res.* **118**, 169 (2007).

27. J. A. Hubbell, *J. Control. Res.* **39**, 305 (1996).

28. G. Maheshwari, A. Wells, L. G. Griffith, D. A. Lauffenburger, *Biophys. J* **76**, 2814 (1999).

29. D. L. Livant et al., *J. Clin. Invest.* **105**, 1537 (2000).

30. L. Aucoin, C. M. Griffith, G. Pleizier, Y. Deslandes, H. Sheardown, *J. Biomater. Sci. Polymer Edn.* **13**, 447 (2002).

31. A. Mardilovich, E. Kokkoli, *Biomacromolecules* **5**, 950 (2004).

32. U. Hersel, C. Dahman, H. Kessler, *Biomaterials* **24**, 4385 (2003).

33. J. A. Hubbell, *Swiss Med. Wkly* **136**, 387 (2006).

34. J. A. Neff, P. A. Tresco, K. D. Caldwell, *Biomaterials* **20**, 2377 (1999).

35. M. P. Lutolf, J. A. Hubbell, *Nat. Biotechnol.* **23**, 47 (2005).

36. M. D. Pierschbacher, E. Ruoslahti, *Nature* **309**, 30 (1984).

37. G. H. Altman et al., *Biomaterials* **24**, 401 (2003).

38. H. Kurihara, T. Morita, M. Shinkai, T. Nagamune, *Biotechnol. Lett.* **27**, 665 (2005).

39. S. Zhang, *Nat. Biotechnol.* **21**, 1171 (2003).

40. D. W. Urry, *J. Phys. Chem. B* **101**, 11007 (1997).

41. K. Di Zio, D. A. Tirrell, *Macromolecules* **36**, 1553 (2003).

42. S. C. Heilshorn, K. A. Di Zio, E. R. Welsh, D. A. Tirrell, *Biomaterials* **24**, 4245 (2003).

43. J. C. Liu, S. C. Heilshorn, D. A. Tirrell, *Biomacromolecules* **5**, 497 (2003).

44. J. C. Liu, D. A. Tirrell, *Biomacromolecules* **9**, 2984 (2008).

45. I. S. Carrico et al., *J. Am. Chem. Soc.* **129**, 4874 (2007).

46. P. J. Nowatzki, C. Franck, S. A. Maskarinec, G. Ravichandran, D. A. Tirrell, *Macromolecules* **41**, 1839 (2008).

47. P. J. Nowatzki, D. A. Tirrell, *Biomaterials*, 1261 (2003).

48. D. W. Urry, *Angewandte Chemie-International Edition in English* **32**, 819 (1993).

49. A. Panitch, T. Yamaoka, M. J. Fournier, T. L. Mason, D. A. Tirrell, *Macromolecules* **32**, 1701 (1999).

50. H. B. Streeter, D. A. Rees, *J Cell Biol* **105**, 507 (1987).

51. G. Fenteany, P. J. T. Stossel *Curr. Biol.* **10**, 831 (2000).

52. Y. Matsubayashi et al., *Curr. Biol.* **14**, 731 (2004).

53. P. J. Sammak, L. E. Hinman, P. O. T. Tran, M. D. Sjaastad, T. E. Machen, *J. Cell Sci.* **110**, 465 (1997).

54. R. van Horssen, N. Galjart, J. A. P. Rens, A. M. M. Eggermont, T. L. M. ten Hagen, *J. Cell. Biochem.* **99**, 1536 (2006).

55. D. L. Nikolić, A. N. Boettiger, D. Bar-Sagi,J. D. Carbeck, S. Y. Shvartsman, *Am J Physiol Cell Physiol* **291**, 68 (2006).

56. M. Poujade et al., *Proc. Natl. Am. Soc. USA* **104**, 15988 (2007).

57. A. D. van der Meer, K. Vermeul, A. A. Poot, J. Feijen, I. Vermes, *Am. J. Physio. Heart Circ. Physiol.* **298**, H719 (2010).