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Regulatory Effect of Elastin Based Biomaterial on Cellular Behavior and Its Application on Wound Repair and Regeneration

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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DEDICATION

I would like to dedicate this work to my wonderful family, particularly to my wife, Lili Huang, who always believed in me and is there to support; and my baby daughter, Jiayin Yuan, who always encouraged me through the tough times with her laughter.

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ABSTRACT

Elastin-like peptides (ELPs) are stimulus-responsive protein-based polymers which are attractive material for biomedical research due to their biocompatibility and unique properties. The physical properties of ELPs are dependent on the chain length and the chosen amino acid at the guest residue position. This imparts unlimited flexibility in designing ELP based biomaterials with the desired physical properties.

We have shown that in addition to their physical properties, ELPs have biological activities that are conducive to tissue regeneration. Specifically, we found that ELPs induce fibroblast proliferation via cell surface heparan sulfate proteoglycans (HSPG). Furthermore, our data suggests that ELP based materials with differential proliferative potential can be designed by controlling the interaction of ELPs with HSPGs by incorporating either hydrophobic or positively charged residues within the ELP sequence. Fibroblast proliferation is important for granulation tissue formation which is important in chronic wounds as well as in healing of other tissues. The customizable biological activity of ELPs coupled with their unique physical properties will enable us to design novel, sustainable and cost effective therapies for different tissue regeneration applications.

ELPs can be genetically fused to biologically active peptides or proteins. These fusions can be expressed and readily purified since they maintain the phase transitioning property of the fused ELP domain. Moreover, depending on the ELP sequence chosen the chimeric fusion sequences can self-assemble into unique structures such as nanoparticles. These structures can then be applied to the injury site where they not only provide unique topographical cues or structural support but

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also act as delivery vehicles for the fused bioactive protein. We developed a multifunctional nanoparticle that is comprised of PMP-D2-ELP fusion protein and different functional peptide ELP fusion proteins to preserve the bioactivity of the functional group with the existence of elastase. These heterogeneous particles will be beneficial for the delivery of combination therapies to solve multiple problems that often existed in chronic wound healing or other tissue regeneration process.

In summary, this study adds to our understanding of the biological activity of ELP and the interaction mechanism that allow the regulation of cellular behavior. Furthermore this work also investigated the potential therapeutic application of ELP as a delivery platform for chronic wound healing.

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CHAPTER 1: INTRODUCTION

1.1 Skin

Skin is the largest organ and is the outer covering of the body. It is comprised of multiple layers including epidermis, dermis, and hypodermis (Wilkinson 2009) (Figure 1.1). Since skin interfaces with the environment, it is the first line of defense against pathogens and protects the body from excessive water loss (Madison 2003, Proksch, Brandner et al. 2008). Other functions include insulation, temperature regulation, sensation, synthesis of vitamin D, and the protection of vitamin B folates.

Epidermis is the outermost layer of the skin, which is about 0.2 mm. According to the different stages of cell development, it can be further divided to 5 layers: stratum corneum, which protects skin damage from friction and leakage of body fluid. Cells in this layer don't have nuclei; transparent layer, which is composed of 2 to 3 layer of cells with dead nuclei, also called barrier zone; granular layer, which is made of flat spindle cells; prickle cell layer, in which the intercellular bridge is forming; and basal layer, cells in this layer constantly proliferate and gradually move up, keratosis, deformation, forming the other layers epidermis, finally shedding keratosis. The cycle for basal cells from proliferation to shedding takes about 28 days (Ovaere, Lippens et al. 2009). Melanocytes mixed in with the basal cells can produce melanin which determines the color of skin.

Underneath the epidermis lies the dermis. It is mainly composed of collagen fibers, elastic fibers, reticular fibers which give skin certain degree of flexibility and roughness and the amorphous matrix and other connective tissue, of which there are nerves and nerve endings, blood

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vessels, lymphatic vessels, muscle and skin appendages(Marks 2006). Cellular components include fibroblasts, histiocytes and mast cells. The thickness of the dermis and the amount of fibrous tissue are closely related to the density of the skin, fullness, relaxation and the wrinkling phenomenon.

There is no clear boundary line between dermis and hypodermis. Hypodermis, also called subcutaneous adipose tissue is a layer of relatively loose organization, it is a natural cushion that can buffer external pressures, and it is also a heat insulator, and capable of storing energy. In addition to fat, subcutaneous adipose tissue is also rich in blood vessels, lymphatic vessels, nerves, sweat glands and hair follicles (O'Rahilly 2013).

1.2 Wound Healing and Chronic Wounds

Wound healing is subjected to the healing process of skin and other tissues after the break or defect caused by an external force. It is a complex combination of various processes including a variety of tissue regeneration, proliferation of granulation tissue, and scar tissue formation process which exhibit synergy (Stadelmann, Digenis et al. 1998, Midwood, Williams et al. 2004) (Figure 1.2).

In early wounds with varying degrees of tissue necrosis and vascular rupture bleeding, inflammation will occur within a few hours which manifests as hyperemia, serous exudate and leukocyte guerrilla, and local swelling. In the early inflammation phase, neutrophils are the dominant cell type, while macrophage starts to take over after 2-3 days (Martin and Leibovich 2005, de la Torre J. 2006).

The proliferation phase starts as early as 12 hours after the initial injury. In this phase, fibroblasts proliferate and secrete collagen and fibronectin to form granulation tissue to fill up the wound gap (Stadelmann, Digenis et al. 1998). Meanwhile, vascular endothelial cells form new

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blood vessels, a process called angiogenesis (R. 2007). Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and 'crawl' atop the wound bed, providing cover for the new tissue (Santoro and Gaudino 2005).

In the remodeling phase, the levels of collagen production and degradation equalize and start to realign. During maturation, collagen type III which is prevalent during proliferation, is replaced by type I collagen (C. 1999). Depending on different types of wounds, remodeling can last for a year or longer (Mercandetti M. 2005).

Chronic wounds are a major source of morbidity for our aging population, affecting 6.5 million patients in the United States annually (Daamen, Veerkamp et al. 2007). There are mainly three types of chronic wounds: venous stasis ulcers, diabetic foot ulcers and pressure sores. Many factors contribute to chronic wound formation, including ischemia, neuropathy, and repeated trauma (Kamoun, Landeau et al. 1995). Aging is another factor contributing to chronic wound formation (Mustoe 2004). Specifically, the proliferation rate of cells in the skin of old people is slower. Although the causes of chronic wounds vary, there are shared symptoms, such as being stuck in one or more phases of wound healing, such as the inflammatory stage (Snyder 2005).

Since poor granulation and dermal remodeling are often associated with chronic wounds, research on treatments focuses on fibroblast proliferation and migration, such as growth factor therapy (Chao, Yang et al. 2012) and the use of stem cells (Devy, Duca et al. 2010). Platelet-derived growth factor (PDGF) is the first FDA approved biotech product for treatment of diabetic foot ulcers, and has been widely used clinically. However repeated direct injection of the growth factor is needed, and requires large amounts due to diffusion and a hostile environment in the wound area (Schonfelder, Abel et al. 2005). Therefore an effective delivery method is critical for growth factor therapy for chronic wounds. Stem cells are undifferentiated cells that can self-

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replicate and differentiate into more specialized cells. Studies have shown that transplanted stem cells derived from bone marrow and adipose tissue are conducive to chronic wound healing through five major paths (Crisostomo, Markel et al. 2008)- (1) increased angiogenesis, (2) decreased local inflammation, (3) anti-apoptotic and chemotactic signaling, (4) normalization of the extracellular matrix, and (5) stimulation of nearby resident stem cells. Stem cell therapy as a potential treatment method for chronic wounds still needs extensive study, mainly focusing on the delivery mechanism, the amount of stem cells required for the treatment, and the length of time for the culture, expansion and characterization of the stem cells (Hu, Leavitt et al. 2015).

1.3 Growth Factors

Growth factors are naturally occurring substances that are capable of stimulating cell growth or differentiation. Wound healing is a complex biological process involving proliferation, differentiation and synthesis secretion of fibroblasts that enter the wound site, extracellular matrix fibrosis, angiogenesis, and re-epithelialization (Figure 1.3). Most growth factors that are produced in the process of skin wound healing have regulatory effect on one or more of processes mentioned above through: 1) chemotaxis, induce inflammatory cells and fibroblasts to migrate into wound site; 2) induce cell proliferation; 3) promote angiogenesis; 4) up-regulate the expression of cytokines (Bodnar 2015, Marti-Carvajal, Gluud et al. 2015).

Platelet derived growth factor (PDGF) can be divided into a 31 KDa 7% sugar containing PDGF I and 28 KDa 4% sugar containing PDGF II. Both are composed of two highly homologous A chain and B chains, which makes the structure of PDGF dimers have three forms, i.e., PDGF-AA, PDGF-BB and PDGF-AB. Experiments show that PDGF is an important mitogenic factor, which has the ability to stimulate proliferation of specific cell populations (Hannink and Donoghue 1989, Heldin 1992). When the skin tissue is injured, formation of a blood clot will stop the bleeding.

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At the same time, platelets release several growth factors - the most important one is the platelet-derived growth factor. It induces fibroblasts proliferation and collagen type I and III production to promote the formation of granulation tissue. PDGF also helps reduce the degradation of the extracellular matrix by up-regulation of tissue inhibitors of metalloproteinase (TIMP-1) to inhibit collagenase activity. The PDGF exerts its biological effects through binding with the corresponding receptors on the cell membrane. PDGF receptor consists of two subunits α and β, having a molecular weight of 170 ~ 180 KDa (Heldin and Lennartsson 2013). The binding affinity of the two subunits to the A chain and B chain vary greatly. The α subunit has a good binding affinity to both the A chain and B chain, while the β subunit only binds to the B chain. So the α subunit can bind PDGF-AA, PDGF-AB and PDGF-BB, the β subunit binds only PDGF-BB and PDGF-AB (Matsui, Heidaran et al. 1989).

Keratinocyte growth factor (KGF), also known as FGF-7, is a member of the fibroblast growth factor family. It is a basic growth factor secreted by skin tissue cells and can stimulate physiological metabolism processes of epithelial cells, including cell proliferation, differentiation and migration (Seeger and Paller 2015). Mature KGF has 163 amino acids, which have an N-terminal glycosylation site that binds to a specific receptor on the surface of epithelial cells through a complex signaling process , starting to participate in gene expression and the division of epithelial cells , thereby stimulating epithelial tissue metabolism (Rotolo, Ceccarelli et al. 2008).

1.4 Elastin-Like Peptides

Elastin-like peptides are biodegradable, non-immunogenic protein-based polymers composed of tandemly-repeated blocks of $(Val\text{-}Pro\text{-}Gly\text{-}X\text{-}Gly)_N$ where X can be any residue, except *Pro*. This sequence motif is derived from the hydrophobic domain of tropoelastin (Urry 1997), a soluble precursor form of elastin. An interesting property of ELPs is their ability to

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undergo a phase transition at physiological temperatures. When the temperature is below their inverse transition temperature, also known as the lower critical solution temperature (LCST), they assume a random coil structure and are soluble in aqueous solution. However, at temperatures higher than the LCST, ELPs undergo an entropy driven self-assembly rendering them insoluble (Koria, Yagi et al. 2011) **(**Figure 1.4**)**. This property enables recombinant ELPs to be expressed in bacteria and rapidly purified to high homogeneity using inverse temperature cycling (ITC) (Meyer and Chilkoti 1999).

Due to its attractive physical properties, ELPs have been genetically fused to functional peptides and used in many applications. These fusions can be expressed and readily purified since they maintain the phase transitioning property of the fused ELP domain (Meyer and Chilkoti 1999, Koria, Yagi et al. 2011). Moreover, depending on the ELP sequence chosen, the chimeric fusion sequences can self-assemble into unique nanostructures such as nanoparticles (Osborne, Farmer et al. 2008, Koria, Yagi et al. 2011) or gels (Wright and Conticello 2002, Martin, Alonso et al. 2009) or other cross-linked materials (Srokowski and Woodhouse 2008). These nanostructures can then be applied to the injury site where they not only provide unique topographical cues or structural support but also act as delivery vehicles for the fused bioactive protein. Due to these benefits, ELPs have been widely used for different applications (Chilkoti, Christensen et al. 2006), including protein purification and separation (Bellucci, Amiram et al. 2013), drug delivery (Walker, Perkins et al. 2012), and scaffolds for tissue engineering (Amruthwar and Janorkar 2013).

Even though ELPs' physical properties have been well studied over the past decade, there is only limited information about its biomedical applications. Examples of the later include the use of cross-linked ELP based hydrogels for repair of bone defects in goats (Nettles, Kitaoka et al. 2008) and rabbits (Hrabchak, Rouleau et al. 2010), repair of intervertebral discs in rabbits (Moss,

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Gordon et al. 2011), investigation of biocompatibility of elastin particles using subcutaneous injection in rabbits (Rincon, Molina-Martinez et al. 2006), and stability of ELP peptides in blood (Liu, Dreher et al. 2006).

To address this gap in our knowledge, in Chapter 2 and 3, I discussed the proliferative activity of ELP on fibroblasts and their interaction mechanisms. I further show that this interaction can be modulated by designing ELP sequences having different phase transition behavior or charge; thereby controlling the mitogenic activity of ELPs. Fibroblast proliferation and function is essential for granulation tissue formation not only in chronic wound healing but also in healing of injuries of other tissues such as bone or neural tissue.

Overall this thesis gives the reader a new perspective about ELP as a potential biomaterial for chronic wound healing. There are several novel observations that add to existing knowledge in ELP applications and wound healing. For example, ELP induces fibroblasts proliferation through interaction with heparan sulfate proteoglycans (chapter 2). In chapter 3 I described a method for controlling the mitogenic activity of ELPs. A novel chronic wound model that significantly delayed wound healing process on diabetic mice is detailed in chapter 4. I hope this thesis is a start for the development of a rational framework for designing ELP based materials with the desired proliferative activity. The customizable biological activity of ELPs coupled with their unique physical properties will enable us to design novel, sustainable and cost effective therapies for healing of different tissues.

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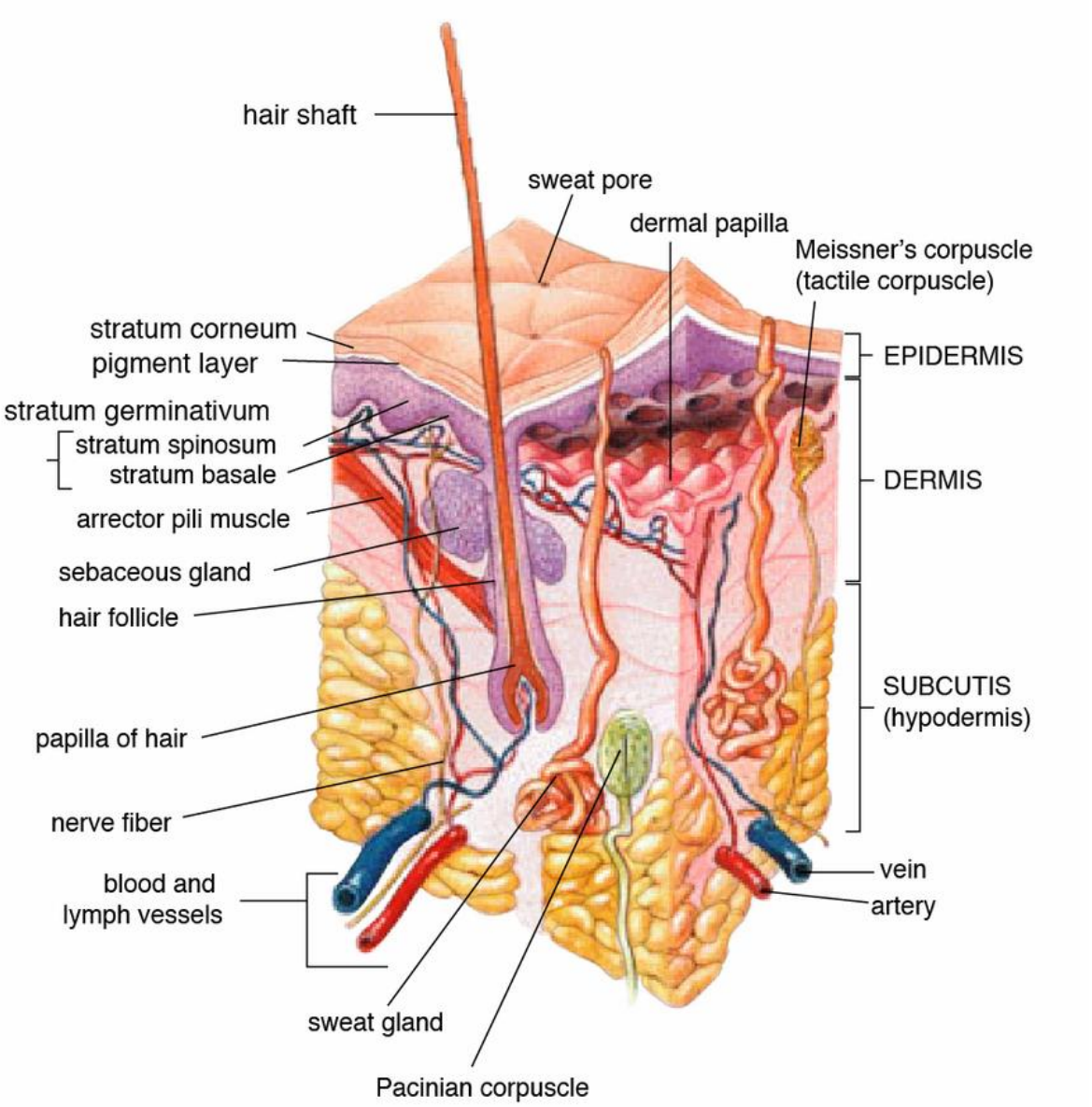

Figure 1.1 Schematic of skin. The skin consists of three major layers namely the epidermis, the dermis and the hypodermis. (US Gov, 2005)

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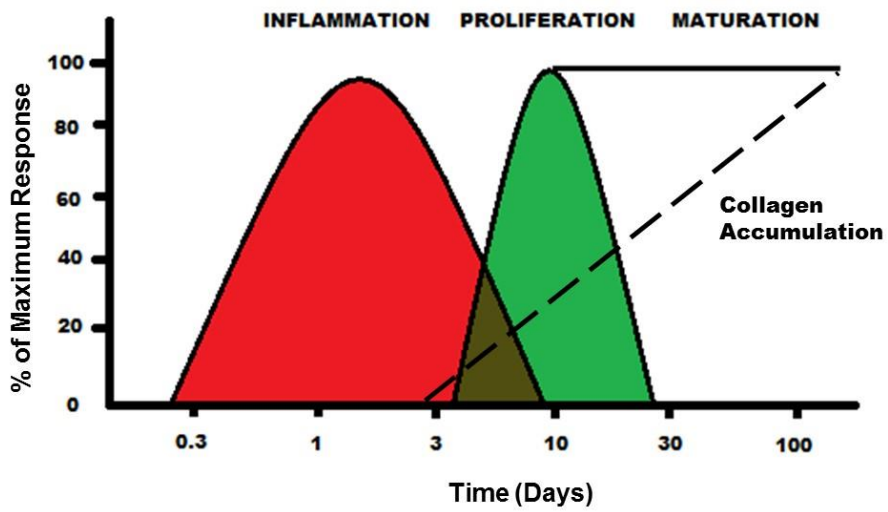

Figure 1.2 Schematic of wound healing process. Wound healing is a complex combination of various processes including inflammation, proliferation, and maturation.

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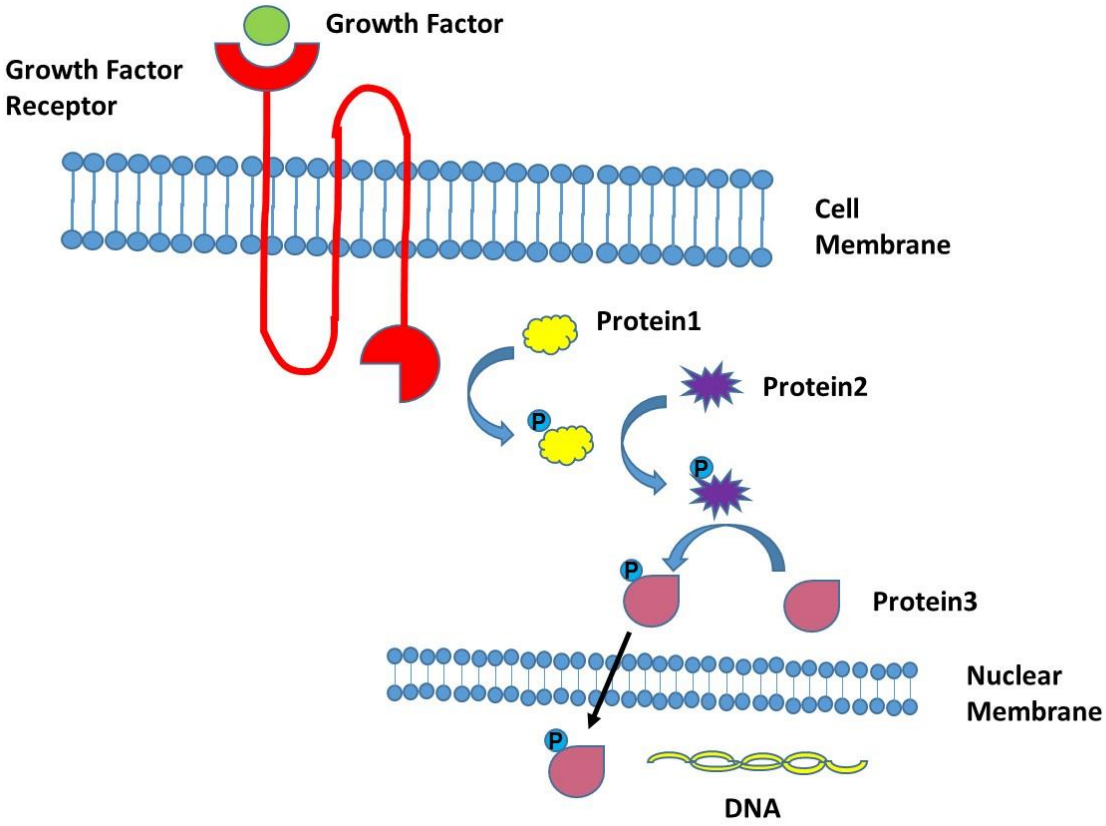

Figure 1.3 Schematic of growth factors interaction with cell. Growth factor binds to the specific receptor located on cell membrane and activates a series of signaling protein through phosphorylation.

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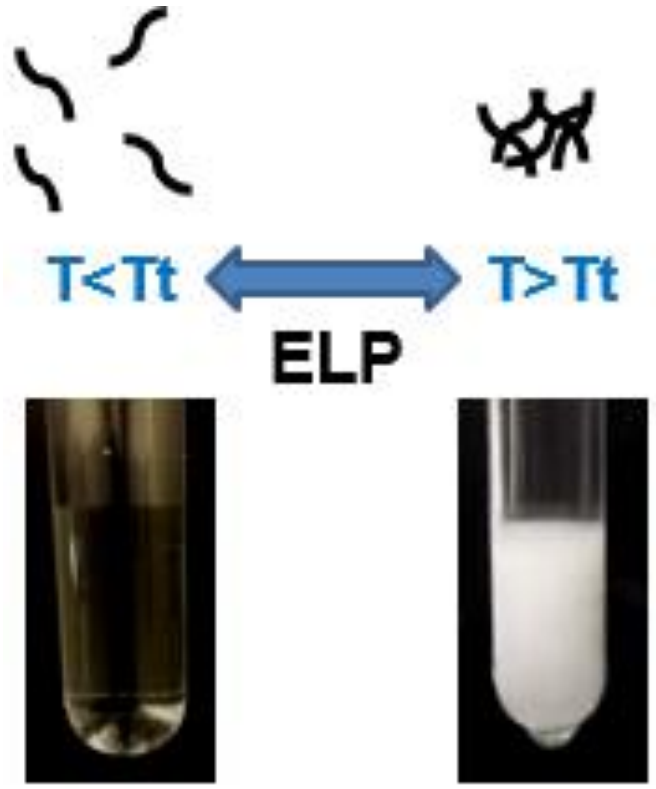

Figure 1.4 Phase transition behavior of ELP. An interesting property of ELPs is their ability to undergo phase transition at physiological temperatures. When the temperature is below their inverse transition temperature, also known as the lower critical solution temperature (LCST), they assume a random coil structure and are soluble in aqueous solution. However, at temperatures higher than the LCST, ELPs undergo an entropy driven self-assembly rendering them insoluble.

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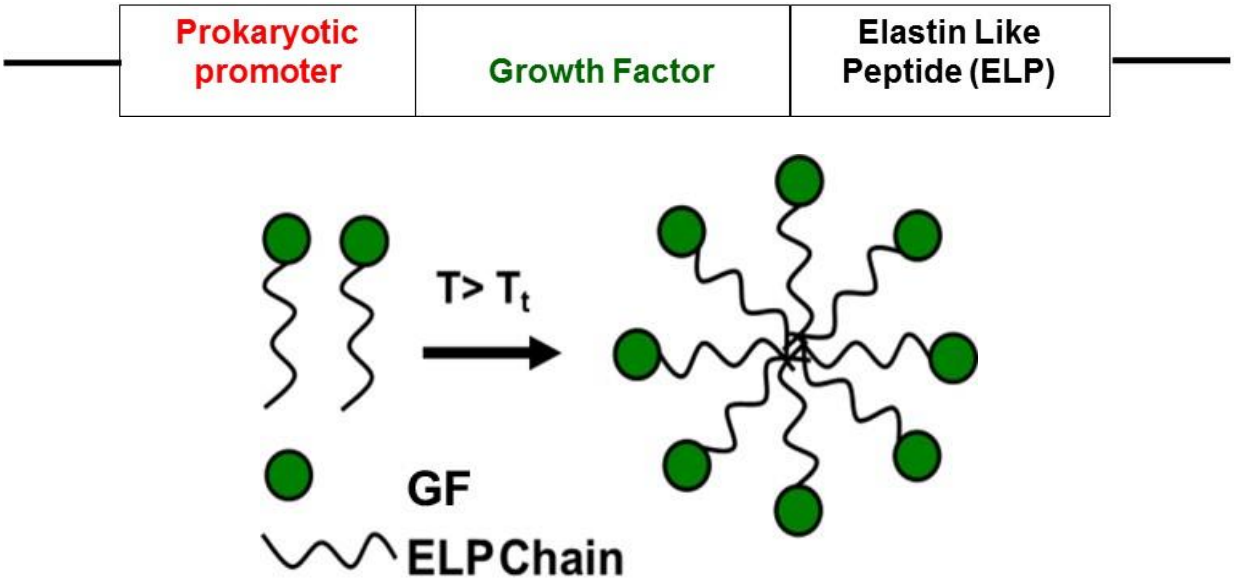

Figure 1.5 Schematic of growth factor ELP fusion protein.

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Figure 1.6 Cloning using recursive directional ligation.

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CHAPTER 2: BIOACTIVITY OF ELASTIN-LIKE PEPTIDES ON FIBROBLASTS: PROLIFERATION AND MATRIX METALLOPROTEINASE EXPRESSION

2.1 Note to Reader

Part of this chapter was published in Yuan, Y. and P. Koria (2015). "Proliferative activity of Elastin-like-Peptides Depends on Charge and Phase transition." J Biomed Mater Res A. Permission is included in appendix B.

2.2 Introduction

Protein-based polymers comprised of repeating peptide sequences, where the repeating unit can be as few as two residues or as many as hundred residues (Urry 1997) are attractive biomaterials for applications of tissue regeneration (Koria 2012). Elastin like peptides are biodegradable, non-immunogenic protein-based polymers composed of tandemly-repeated blocks of $(Val\text{-}Pro\text{-}Gly\text{-}X\text{-}Gly)_N$ where X can be any residue but *Pro*. This sequence motif is derived from the hydrophobic domain of tropoelastin (Urry 1997), a soluble precursor form of elastin. An interesting property of ELPs is their ability to undergo a phase transition. When the temperature is below their inverse transition temperature, also known as the lower critical solution temperature (LCST), they assume a random coil structure and are soluble in aqueous solution. However, at temperatures higher than the LCST, ELPs undergo an entropy driven self-assembly rendering them insoluble (Koria, Yagi et al. 2011). This property enables recombinant ELPs to be expressed in bacteria and rapidly purified using inverse temperature cycling (ITC) (Meyer and Chilkoti 1999).

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ELPs can be genetically fused to biologically active peptides or proteins. These fusions can be expressed and readily purified since they maintain the phase transitioning property of the fused ELP domain (Meyer and Chilkoti 1999, Koria, Yagi et al. 2011). Moreover, depending on the ELP sequence chosen the chimeric fusion sequences can self-assemble into unique structures such as nanoparticles (Osborne, Farmer et al. 2008, Koria, Yagi et al. 2011) or gels (Wright and Conticello 2002, Martin, Alonso et al. 2009) or other cross-linked materials (Srokowski and Woodhouse 2008). These structures can then be applied to the injury site where they not only provide unique topographical cues or structural support but also act as delivery vehicles for the fused bioactive protein. Due to these benefits, ELPs have been widely used for several biomedical applications (Chilkoti, Christensen et al. 2006), including protein purification and separation (Bellucci, Amiram et al. 2013) and drug delivery (Walker, Perkins et al. 2012).

The physical properties of ELPs are dependent on the chain length and the chosen amino acid at the guest residue position (Urry 1997). This imparts unlimited flexibility in designing ELP based biomaterials with the desired physical properties. Elegant work by several researchers has elucidated the role of sequential variations on the physical properties of ELPs (Meyer and Chilkoti 2004, Trabbic-Carlson, Meyer et al. 2004, Cho, Zhang et al. 2008, Ribeiro, Arias et al. 2009, Jeon 2011). This has provided further insights and design rules that have to be followed to develop ELP based biomaterials with the desired physical properties and molecular structure. For example, substitution of a more hydrophobic amino acid at the guest residue position or increase in the chain length yields a polymer with a reduced transition temperature (Meyer and Chilkoti 2004). Additionally, Chilkoti and co-workers have described the formation of micellar nanoparticles using a block co-polymer comprised of two ELPs with different transition temperatures (Dreher, Simnick et al. 2008). Moreover, the formation of ELP based hydrogels has also been described by

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selection of unique ELP sequences (Wright and Conticello 2002, Srokowski and Woodhouse 2008, Martin, Alonso et al. 2009).

However, the biological characterization of ELPs is missing in all of the aforementioned studies. Very few studies have reported the implantation and effects of ELP based biomaterials in vivo. These include the use of cross-linked ELP based hydrogels for repair of bone defects in goats (Nettles, Kitaoka et al. 2008) and rabbits (Hrabchak, Rouleau et al. 2010), repair of intervertebral disc in rabbits (Moss, Gordon et al. 2011), investigation of biocompatibility of elastin particles using subcutaneous injection in rabbits (Rincon, Molina-Martinez et al. 2006) and stability of ELP peptides in blood (Liu, Dreher et al. 2006). All of these have reported that ELPs are biocompatible, well tolerated and do not induce inflammation. Nevertheless, these studies mainly focused on the mechanical strength of the ELP hydrogels and their application was mainly limited as providers of mechanical support within the defect. While, some effects on chondrocytes proliferation were observed but there was no detailed mechanistic investigation or report of any possible biological activity of ELPs within the injury environment. Since, elastin is an integral part of the extracellular matrix elastin derived peptides (EDPs) or ELPs may affect certain cellular functions. Indeed, EDPs which are elastin breakdown peptide products have been shown to modulate cellular behavior of a variety of cell types such as fibroblasts and monocytes (Senior, Griffin et al. 1984), hepatocytes (Janorkar, Rajagopalan et al. 2008), smooth muscle cells (Mochizuki, Brassart et al. 2002), endothelial cells (Faury, Garnier et al. 1998) and keratinocytes (Fujimoto, Tajima et al. 2000). Specifically in fibroblasts that play a key role in regeneration, EDPs have been shown to induce proliferation and expression of several matrix remodeling proteins such as collagenase (Brassart, Fuchs et al. 2001, Duca, Debelle et al. 2002). However, in these experiments the precise molecular

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weights and sequences that induce effects on cells is not known since the degradation product is a complex mixture of several elastin peptides having different sequences and molecular weight.

Here, we created several ELPs with precise sequences and molecular weight and evaluated their biological activity. We found that ELPs induced proliferation and matrix metalloproteinase 1 (MMP1) expression in fibroblasts which both are crucial for tissue regeneration process. We further report that these two activities were a result of interaction with two different molecules present on the cell surface.

2.3 Materials and Methods

2.3.1 Materials

Human dermal fibroblasts (CRL-2522) were purchased from ATCC (Manassas, VA). PBS, FBS and DMEM were all purchased from life technologies. The restriction enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). The reagents for RT-PCR were purchased from Bio-Rad. BrdU cell proliferation assay kit was purchased from BD Pharmingen. Miniprep kit and gel extraction kit used for sub cloning process were purchased from Qiagen.

2.3.2 Generation of ELP Plasmids and Expression of the Protein

The PUC57 plasmids containing the elastin cassette **(**$(VPGVG)_5$ and $(VPGVG)_2VPGCG(VPGVG)_2$ were purchased from Genscript (Piscataway, NJ). V40C2 and V50 encoding genes were made using recursive direction ligation method as described previously (Meyer and Chilkoti 2002). PUC19-C plasmid was used as template in site-directed mutagenesis for the generation of $(VPGVG)_2$ VPGLG $(VPGVG)_2$, $(VPGVG)_2$ VPGKG $(VPGVG)_2$ and $(VPGVG)_2$ VPGDG $(VPGVG)_2$ cassette. L10 and K10 encoding gene were made using same method mentioned above.

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The expression vector pET25b+ was digested with SfiI and dephosphorylated. Ligation was performed using linear pET25b+ vector and respective ELP encoding gene cassettes. E.coil BLR cells were transformed with expression vector with ELP encoding genes. For protein production, a starter culture of 75ml terrific broth medium was inoculated overnight and added to 1L culture the next day. After 16hrs of induction, bacterial cells were harvested by centrifugation at 4^0C, re-suspended in ice cold phosphate buffered saline (PBS). Cells were lysed on ice using sonication. Cell debris was removed by centrifugation at 4^0C followed by polyethyleneimine treatment (final concentration: 0.5% w/v) for DNA precipitation. After another centrifugation at 4^0C, the clear supernatant was heated up to 40^0C in water bath for 15min to transition the ELPs followed by centrifugation at 40^0C to pellet the ELPs. The ELP pellet was then dissolved in ice cold PBS followed by centrifugation at 4^0C to remove the insoluble impurities. This hot and cold spin cycle was then repeated twice for a total of three cycles. The final protein pellet was dissolved in deionized water and dialyzed overnight. The protein was then lyophilized and stored in a desiccator.

2.3.3 BrdU Cell Proliferation Assay

Human skin fibroblasts were cultured in 10 cm cell culture plates with 10% fetal bovine serum (Invitrogen, Grand Island, NY). 100,000 cells were plated in 6-well plate and were allowed to attach for 24 hours. After 24 hours, the cells were serum starved for another 24 hours followed by treatment with different ELPs for 48 hours. New DNA synthesis was quantified using the BrdU cell proliferation kit (BD Pharmingen, San Jose, CA) according to the manufacturer's instruction. Briefly, cells were treated with BrdU for 24 hours. After 24 hours the cells were fixed and stained using a fluorescently tagged BrdU antibody. Then cells were stained with 7-AAD for cell cycle. BrdU incorporation in the cells was evaluated using flow cytometry.

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To explore the signaling pathways involved in ELP induce proliferation the plated cells were pre-incubated for 1hr with the following inhibitors: (i) 5mM of lactose to block elastin binding protein, (ii) 10 μM of PD98059 to inhibit MAPK, (iii) 100nM of wortmannin to inhibit PI3K, (iv) 20mIU/ml of Heprinase III or 10 μM of surfen to block hepran sulfate activity. Each concentration of inhibitor was chosen based on previous studies (Schuksz, Fuster et al. 2008, Rusciani, Duca et al. 2010, Nikmanesh, Shi et al. 2012, Huang, Sun et al. 2013). ELPs were then added to the cells in the presence of different inhibitors. After 48hrs, new DNA synthesis was quantified using the Brdu cell proliferation assay as described above.

2.3.4 Crosslinking of Surface Immobilized and Free ELP

Maleimide Activated Plates (Pierce, Waltham, MA) were used to immobilize the sulfhydryl-containing ELP (V40C2). Plate wells were washed three times with 200 μL wash buffer (0.1M sodium phosphate, 0.15M sodium chloride, 0.05% Tween®-20 Detergent; pH 7.2). V40C2 at 50 M in Binding Buffer (0.1M sodium phosphate, 0.15M sodium chloride, 10mM EDTA; pH 7.2) was prepared and 100-150 μL of the ELP solution was added to each well and incubated overnight at 4 ℃ with continuous mixing. Next day before use, wells were washed three times (200 μL of Wash Buffer/wash) followed by cell plating for the proliferation experiment described above. ELP with His tag was used to detect successful immobilization with anti-His antibody (Sigma, St. Louis, MO) using ELISA.

Free ELP was crosslinked by oxidation using 3% hydrogen peroxide. 3% hydrogen peroxide was added in 50 μM ELP solution and incubated for 1hr at room temperature with continuous shaking. The crosslinked ELP was purified from the peroxide by centrifugation at 400C for 10 minutes.

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2.3.5 Flow Cytometry for ELP Internalization

The cysteines in the ELP (V40C2) were labeled with fluorescein conjugated maleimide (Invitrogen, Grand Island, NY). ELPs were incubated with the maleimide overnight at 4 ^{0}C. The labeled ELPs were purified from the excess fluorescein tagged maleimide by 1 cycle of inverse temperature cycling. Fibroblasts were treated with the labeled ELPs for 24 hours. The cells were then washed 3 times with ice cold PBS. Cells were trypsinized from the wells pelleted and incubated with Trypan blue for 15 minutes to quench the fluorescence on the cell surface. The internalization of the labeled ELP was evaluated by quantifying the fluorescence per cell using flow cytometry.

2.3.6 Dynamic Light Scattering

Lyophilized protein samples were resuspended in PBS at desired concentration and then filtered through 0.22 µm syringe filter. 1ml filtered sample solution in cuvette was read in dynamic light scattering (DLS) (Zetasizer Nano S, Malvern, UK) using a temperature trend analysis from 30˚C to 42˚C at a step of 2˚C. An equilibrium time of 10 min and 3 readings were performed in each temperature point.

2.3.7 Real Time PCR for Gene Expression

Fibroblasts (80,000 cells/well) were plated in 6-well plate and then lysed for RNA isolation after 48hrs of ELP treatments. The RNA from these cells was isolate using the Total RNA isolation kit (Promega, Madison, WI) as per manufacture recommendations. cDNA was synthesized from total cellular RNA using the iScrpt cDNA synthesis kit (Biorad, Ipswich, MA). Briefly, 50 ng of total RNA was reverse transcribed in a 20µL volume containing 5µL 4× RT reaction mix (Biorad) and 1µL enzyme for 5 minutes at 25 ^{0}C, followed by 30 minutes at 42 ^{0}C. The cDNA was then incubated at 85 ^{0}C for 5 minutes. Quantitative real-time PCR was performed using the SsoFast

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EvaGreen supermix (Biorad). The primers used to detect MMP-1 were 5'-AGTGACTGGGAAACCAGATGCTGA-3' and 5'-GCTCTTGGCAAATCTGGCCTGTAA-3'. The primers used to detect GAPDH were 5'-CCTGCACCACCAACTGCTTA-3' and 5'-GCCTGCTTCACCACCTTCTT-3'. The quantitative analysis was done using Biorad CFX manger.

2.3.8 Animals

The mouse strain that we have chosen is B6.BKS-Leprdb from Jackson Laboratories. These mice are homozygous for the diabetes spontaneous mutation (Leprdb) and become identifiably obese around 3 to 4 weeks of age. They exhibit elevations of plasma insulin at 10 to 14 days and of blood sugar at four to eight weeks. Moreover, wound healing in these mice is delayed. Hence, it is a good and well accepted model to study chronic diabetic wounds in the research community.

2.3.9 Excisional Wounding of Skin

Mice will be anesthetized using Isoflurane inhalation (3-4% induction, 1-2% maintenance) on a heating pad at 37˚C and anesthesia deemed sufficient when the animal lacks a contracting reflex in response to interdigital pinch and palpebral/eyeblink reflex. The eyes of the animal will be covered with medical ointment to prevent dryness. Hair will be removed from the dorsal area using electric clippers in an area away from the surgical site. The anesthetized animal would then be brought to the surgical site and the dorsum will be prepared by a three-fold alternating application of Betadine SCRUB and 70% alcohol solution. A 1cm x 1cm square area will be drawn with a black marker on the back of the animal using a template. The skin will be gently lifted up and cut along the marked boundary to generate a full-thickness skin defect on the dorsum. 100µl of fibrin gel containing different treatments will be administered into the wounds. The mice will

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then be dressed with a 2.5 cm x 2.5 cm piece of adherent silicone dressing (Tegaderm™) encompassing an area extending at least 1 cm beyond the wound boundaries. Animals will be single caged after the procedures and standard bedding will be used.

2.3.10 Post Procedure Observations

Animals will be under continuous examination until they recover from the anesthesia as evidenced by the ability to maintain themselves in an upright position, and by assessment of the respiratory rate and response to visual, acoustic, and touch stimuli. Then, they will be returned to the animal housing area. After housing, animals will be observed every 12 hours for 72 hours then daily till 28 days. Animals with be euthanized at 14 days post wounding and tissue surrounding the wound area (including the wound) will be excised and collected for histological analysis.

2.3.11 Statistical Analysis

Cell proliferation assay results were expressed as normalized mean ± SEM to control. Statistical significance of the observed differences was evaluated by ANOVA: single factor whereas p value < 0.05 were considered significant (n=3).

2.4 Results

2.4.1 ELPs Induce Proliferation of Human Skin Fibroblasts

We have recently shown that a fusion protein comprising of ELP and growth factor retains the bioactivity of the growth factor and physical phase transition of the ELP (Koria, Yagi et al. 2011). This fusion protein was successful in healing of diabetic wounds. Interestingly, ELPs by themselves induced significant granulation (Koria, Yagi et al. 2011) (Figure 2.2). This was a surprising result that warranted further attention as several studies involving ELPs in animal models have reported that ELPs are biologically inert and exhibit no significant biological activity (Nettles, Kitaoka et al. 2008, Hrabchak, Rouleau et al. 2010). Since fibroblasts are the main cells

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responsible for granulation we reasoned that ELPs may affect fibroblast functions particularly proliferation. Therefore, we used human dermal fibroblasts to evaluate whether ELPs induced proliferation in these cells. Indeed, ELPs increased proliferation of dermal fibroblasts as high as 4.7 folds at a treatment of 50 μM of ELP (Figure 2.3). Since, increasing of ELPs concentration beyond 50 μM did not increase fibroblast proliferation significantly; we decided to use a concentration of 50 μM for subsequent experiments.

2.4.2 Immobilization or Crosslinking Leads to Loss of Proliferative Potential of ELPs

Previous experiments investigating the ELP bioactivity used either crosslinked ELPs or ELP immobilized on a solid surface (Nettles, Kitaoka et al. 2008). We reasoned that this could be a possible reason why previous studies did not report any proliferative activity of ELPs. To test this hypothesis we immobilized ELP on a cell culture plate using maleimide chemistry. Immobilized ELPs did not induce cell proliferation (Figure 2.4a) thereby confirming the hypothesis that immobilization of ELPs leads to loss in their proliferative activity. However, the loss in proliferative activity could also be attributed to the inability of cells to uptake the immobilized ELPs. To eliminate this possibility we cross-linked free ELP particles using hydrogen peroxide. The cross-linked ELP particles were free to move in the solution but were unable to induce proliferation in fibroblasts (Figure 2.5a). Finally, we further show that ELPs that induce proliferation in fibroblasts are not uptaken by them (Figure 2.6). Thus, these data show that internalization of ELPs is not necessary for their proliferative activity.

2.4.3 ELP Induced Matrix Metalloproteinase Induction via Elastin Receptor

Previous research has demonstrated that the GXXPG motif contained in tropoelastin induces pro-MMP1 expression (Moroy, Alix et al. 2005). Since ELP has the similar sequence motif VPGXG, we tested the induction of MMP1 gene by ELPs in fibroblasts using quantitative

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real time polymerase chain reaction (qRT-PCR). Indeed similar to elastin derived monomeric peptides, ELP significantly induced MMP1 expression in fibroblasts (Figure 2.7a). Interestingly, lactose blocked the ELP induced MMP1 expression suggesting the involvement of the elastin receptor in MMP1 induction unlike proliferation. Furthermore, we investigated the effect of phase transition or charges in the induction of MMP-1 by ELPs. We found that the monomer VPGVG induced MMP1 induction suggesting that it is independent of the phase transition property of ELPs (Figure 2.7b).

2.4.4 ELP Accelerated the Wound Closure and Re-epithelialization *in vivo*

Fibroblasts play a crucial role in chronic wound healing. Since ELP induced fibroblast proliferation may be beneficial for wound healing, we also tested how ELP perform as a potential therapeutic for wound healing in diabetic mice wound model. Interestingly, we found that indeed ELP promoted wound healing process, within 14 days complete wound closure was observed with significant faster re-epithelialization comparing to control. Mice treated with PDGF-ELP fusion protein as a positive control were also observed complete wound closure in 14 days **(**Figure 2.8**)**.

2.5 Discussion

Current research on biomaterials mostly describes inert biomaterials that provide mechanical support or act as drug delivery vehicles carrying an active drug for tissue regeneration. The bioactivity of the biomaterials is typically achieved by the inclusion of an active drug or an active sequence that induces cellular response. However, our work suggests that the biomaterial backbone (VPGXG) is also capable of inducing fibroblast proliferation with no active biological signal embedded within. Thus, ELP based biomaterials are not only capable of providing mechanical support but also have biological activities conducive to fibroblast proliferation which is crucial for healing of different tissues.

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Elastin derived peptides that are prepared by proteolytic degradation of κ-elastin or elastin based tissues have been shown to increase proliferation of arterial smooth muscle cells (Mochizuki, Brassart et al. 2002) and fibroblasts (Shiratsuchi, Ura et al. 2010) respectively. Since, these studies were done using a complex mixture of degradation products of elastin, the exact sequence or molecular weight of the species that induced proliferation could not be identified. On the other hand our work reports the induction of proliferation by a single elastin sequence with precise molecular weight, thus allowing us to identify the exact sequence responsible for proliferation. Our work indicates that the model sequence $(VPGVG)_{50}$ induced fibroblast proliferation. However, previous work has reported that the monomeric or polymeric form of this model sequence VPGVG created by solid peptide synthesis did not demonstrate any significant proliferative activity on fibroblasts (Tajima, Wachi et al. 1997). This could be attributed to the very low concentrations of ELP used in that study compared to our experiments. The concentration of ELP is directly related to their transition behavior, which seems to be critical for inducing fibroblast proliferation as demonstrated by our experiments.

The observed mitogenic activity of ELPs is lost due to crosslinking or when they are immobilized on a solid substrate. This result explains why previous work performed with ELP hydrogels report no significant biological activity; since it involved chemically cross-linked ELP hydrogels leading to the loss of the mitogenic activity of ELPs (Nettles, Kitaoka et al. 2008, Hrabchak, Rouleau et al. 2010). Interestingly, the inability of the cells to internalize the cross-linked ELP will not explain the loss in bioactivity since the induced proliferation was independent of ELP internalization. Crosslinking results in loss of ability of ELPs to toggle between aggregates or single monomeric forms, which may lead to the loss of the biological activity. Further studies

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are needed to investigate the exact role of cross-linking and its effect on the biological activity of ELPs.

In summary, our work demonstrates that the biological activity of ELPs can be modulated by simply tweaking their physical properties. Specifically, we show for the first time that ELPs induce fibroblast proliferation which is dependent on cell surface HSPGs. Fibroblast proliferation and function is essential for granulation tissue formation in not only chronic wound healing but in healing of injuries of other tissues such as bone or neural tissue. Thus, ELP based materials with differential proliferative activity on fibroblasts can be designed and will have broad application in the development of therapeutics for tissue regeneration.

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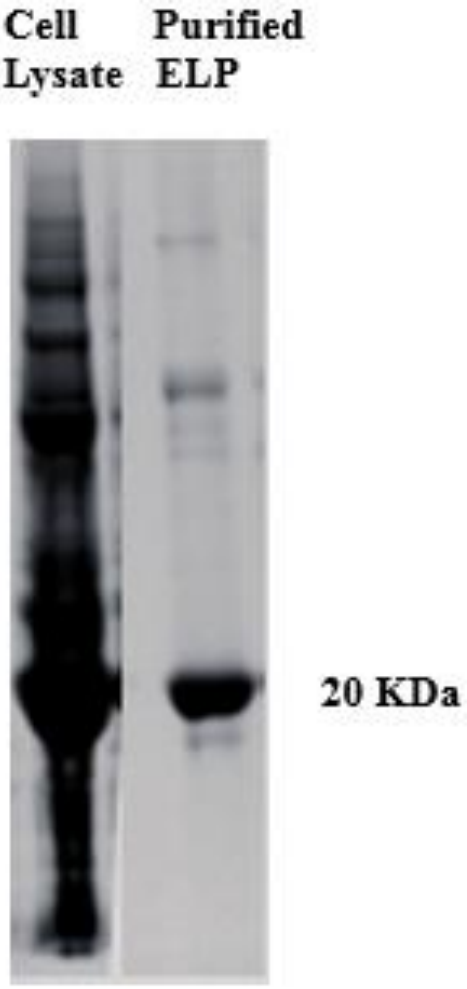

Figure 2.1(a) ELP purification and characterization: protein stain. ELPs were purified using inverse temperature cycling. The bacterial lysate and purified ELP was run on a SDS-PAGE gel and stained with simply safe blue stain for total protein. Lane 1, bacteria lysate; lane 2, purified ELP (V40C2).

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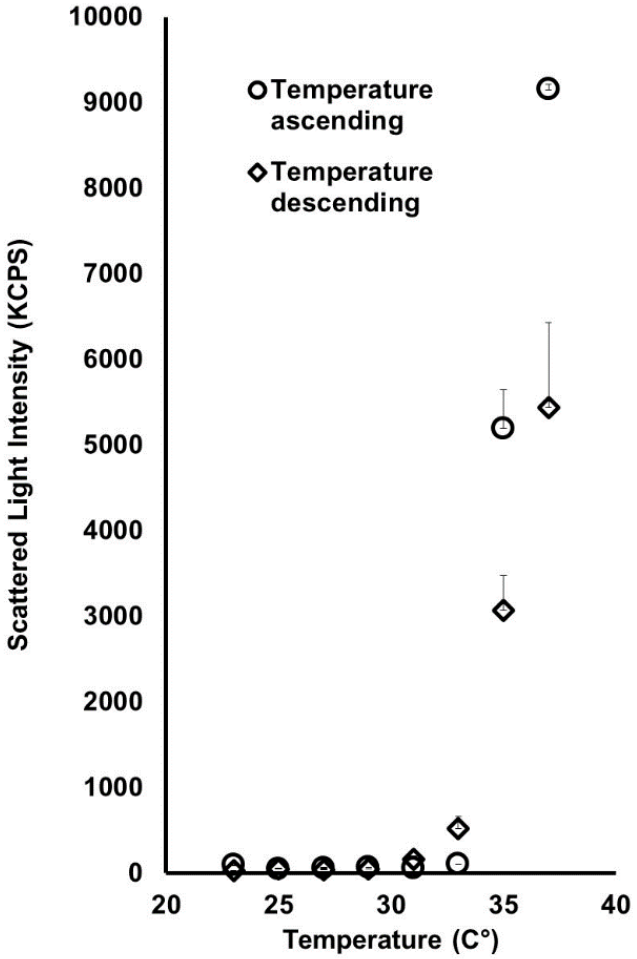

Figure 2.1(b) ELP purification and characterization: dynamic light scattering. Scattered light intensity as a function of temperature.

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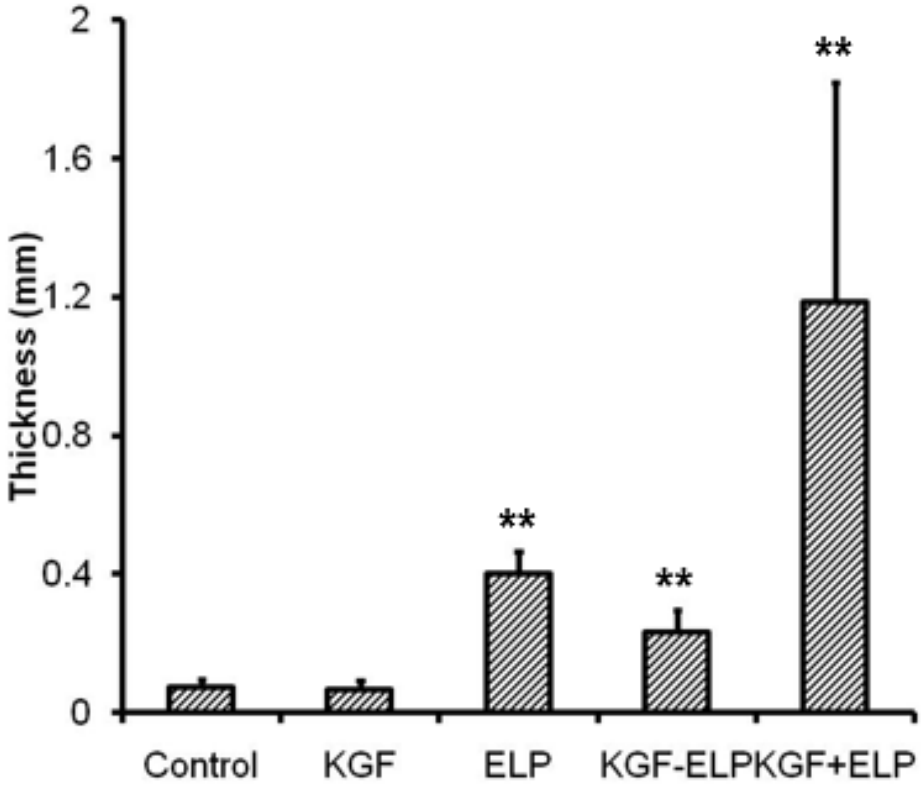

Figure 2.2 ELP enhances granulation tissue formation in diabetic mice wound.(Koria, 2010)

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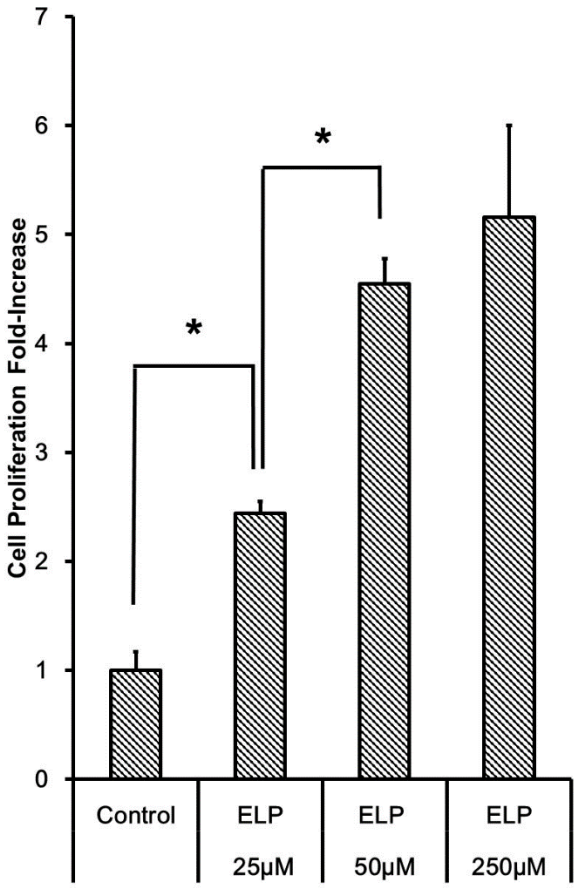

Figure 2.3 ELPs induce fibroblast proliferation. Cells were serum starved for 24hrs then treated for 48 hrs with the indicated concentrations of ELPs. BrdU assay was performed for cell proliferation as described in materials and methods. All the values are obtained from triplicate experiments and normalized to control. * = $p < 0.05$.

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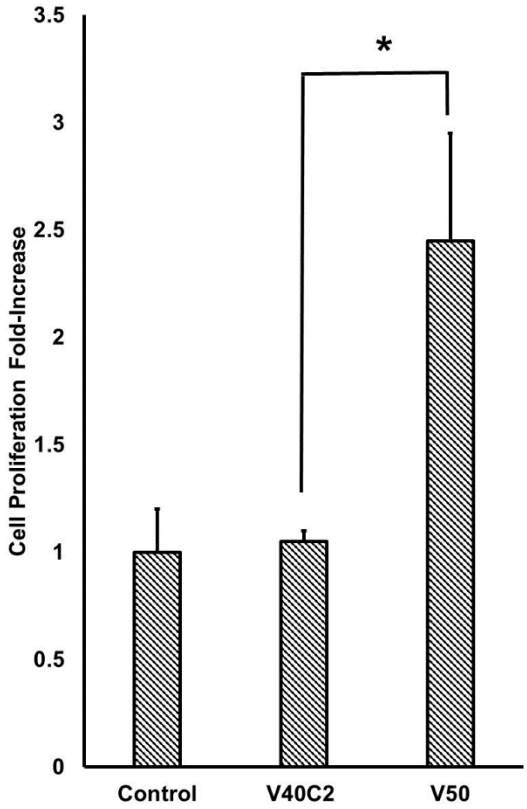

Figure 2.4(a) Immobilized ELP didn't induce fibroblast proliferation. Cell proliferation assay was performed in the plates containing cysteine immobilized ELPs (V40C2) by maleimide chemistry as described in the text. Non cysteine containing ELP (V50) was used as a control. Cells were cultured on ELP containing plates for 48 hours and proliferation was evaluated using the BrdU assay. *=$p<0.05$.

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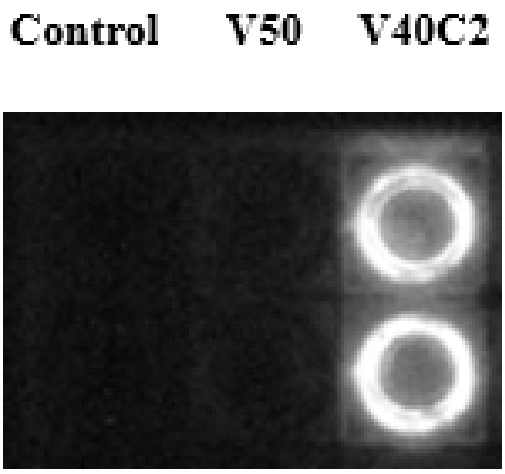

Figure 2.4(b) Immobilization of ELP on cell culture plate. Elisa shows His-tag V40C2 was successful immobilized in maleimide plate.

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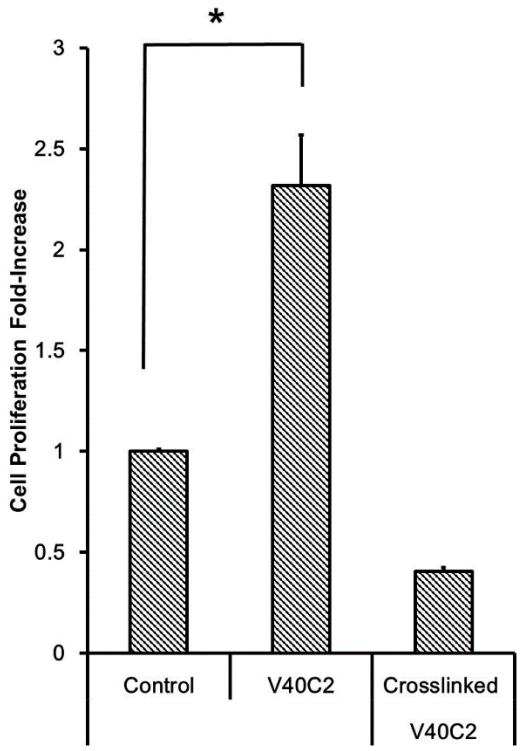

Figure 2.5(a) Crosslinked ELP didn't induce fibroblast proliferation. Cells were starved for 24hrs and then treated with 50 μM of crosslinked V40C2 for 48hrs. BrdU assay was performed for cell proliferation. All the values are obtained from triplicate experiments and normalized to control.

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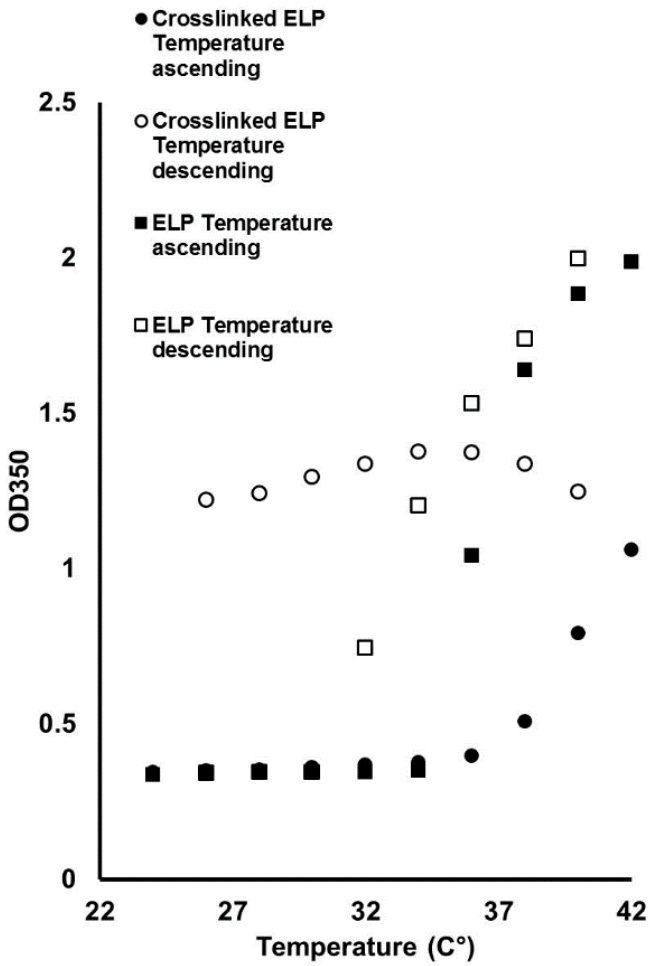

Figure 2.5(b) Successful crosslinking of ELP was detected by monitoring the absorbance in PBS at 350 nm as a function of temperature.

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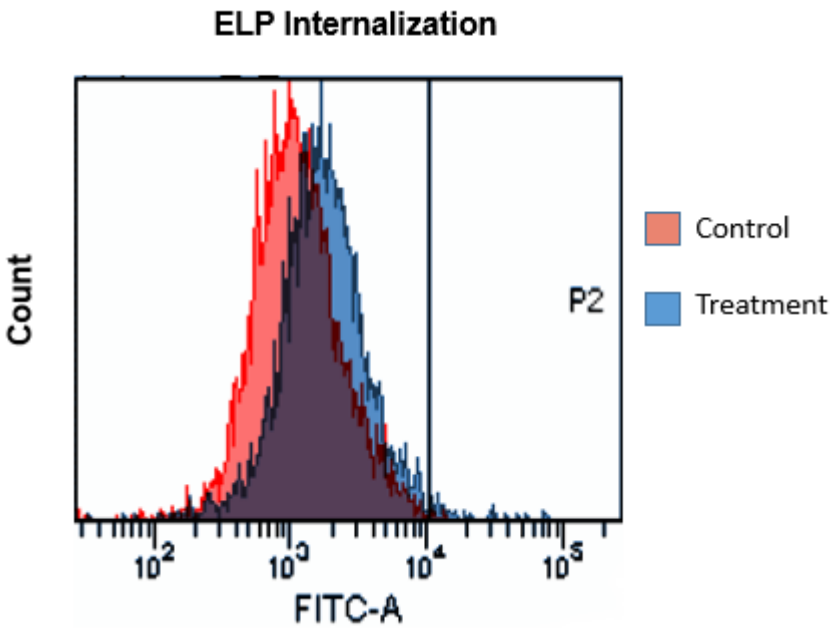

Figure 2.6 ELP was not internalized by fibroblasts. Cells were treated with FITC labeled ELP for 24hrs and ELP internalization was evaluated by flow cytometry.

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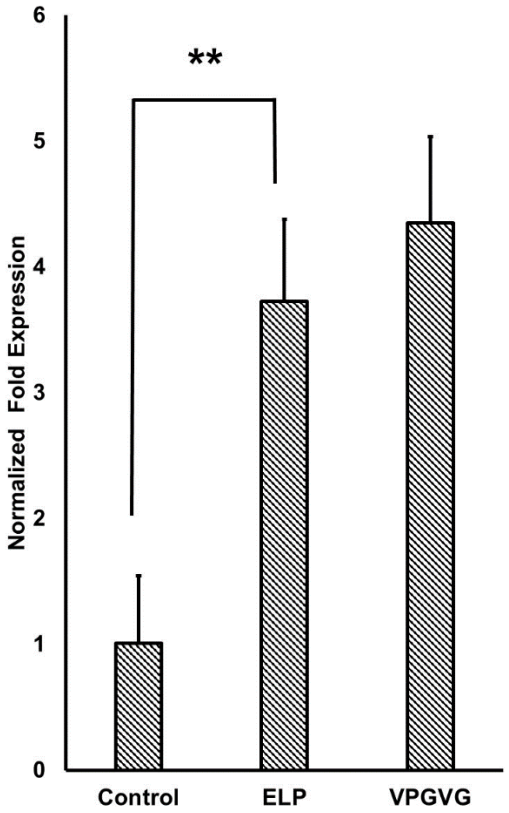

Figure 2.7(a) ELPs induce MMP1 expression is independent of phase transition property of ELP. Cells were serum starved for 24hrs and then treated with 50 μM of ELPs and penta-peptide VPGVG.

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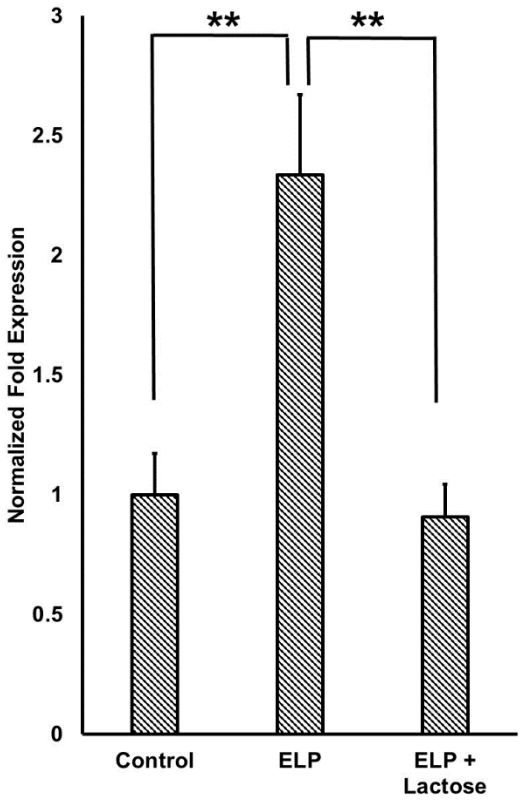

Figure 2.7(b) ELPs induce MMP1 expression in fibroblasts through elastin receptor. Cells were serum starved for 24hrs and then treated with 50 μM of ELPs and pentapeptide VPGVG with or without lactose.

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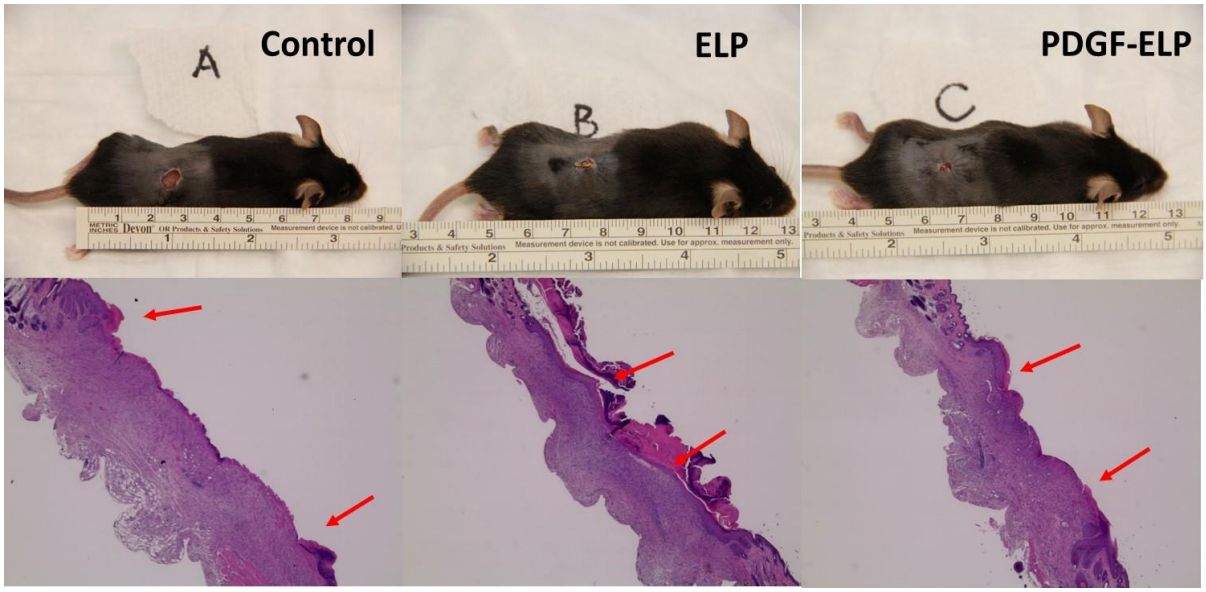

Figure 2.8 ELP accelerated the wound closure and re-epithelialization *in vivo*. 1 cm ×1 cm full thickness wound was created on the dorsal area of the mouse. Either ELP or PDGF-ELP was treated on the wound. Animals with be euthanized at 14 days post wounding and tissue surrounding the wound area (including the wound) will be excised and collected for histological analysis.

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CHAPTER 3: PROLIFERATIVE ACTIVITY OF ELASTIN-LIKE PEPTIDES DEPENDS ON CHARGE AND PHASE TRANSITION

3.1 Note to Reader

Part of this chapter was published in Yuan, Y. and P. Koria (2015). "Proliferative activity of Elastin-like-Peptides Depends on Charge and Phase transition." J Biomed Mater Res A. Permission is included in appendix B.

3.2 Introduction

Heparan sulfate proteoglycan (HSPG) is widely distributed in the cell membrane and extracellular matrix of animal tissues. It plays a crucial role in body development and physiological balance maintenance (Iozzo 1998, Gallagher 2000). Polysaccharide heparan sulfate chains unique molecular structure makes this kind of macromolecular complexes with a variety of biological functions which is achieved mainly through the implementation of the ligand and protein (Williams and Fuki 1997). HSPG on cell surface mediate a variety of cellular activity factors binding with their receptors, participating in signal transduction processes (Guimond, Maccarana et al. 1993, Feyzi, Lustig et al. 1997, Mitsi, Forsten-Williams et al. 2008, Gutierrez and Brandan 2010). It is also an important part of intercellular substance, and maintain the stability of interstitial structure along with collagen.

HSPG can be divided to four different categories, including glypican, syndecan, perlecan and agrin. Glypican and syndecan are located on the cell membrane, while perlecan and agrin are secretive HSPG that found in intercellular substance and basement membrane (Sarrazin, Lamanna et al. 2011). Syndencan family consists of four members (Sdc-1 ~ Sdc-4), although the molecular

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weight of each member is different (22 ~ 45 kD), but they all have an intracellular portion (C -terminus), a transmembrane region and extracellular domain (N -terminal) as structural components (Bernfield, Kokenyesi et al. 1992, Carey 1997). Extracellular domain sequence of four members vary greatly, but commonly they all have heparan sulfate and chondroitin sulfate modification sites. At present, for syndecan associated expression and physiological function are not completely understood, but existing research results suggest syndecan plays an important role in the regulation of body development process. Sdn-1 is dominant in endothelial cells, participating in angiogenesis, wound healing and the interactions between leukocytes and endothelial cells (Abu El-Asrar, Nawaz et al. 2014, Andersen, Kristensen et al. 2015). Sdn-2 expresses in the kidney, lung and gastric stromal cells, cartilage and osteoblasts (Munesue, Kusano et al. 2002, Vicente, Ricci et al. 2013, Mansouri, Hay et al. 2015). Sdn-3 mainly exists in the nerve cells and is the co-receptor of Agouti related peptide (AGRP) which regulate feeding behavior by binding to melatonin receptors (Reizes, Benoit et al. 2003, Creemers, Pritchard et al. 2006). Sdn-4 is widely present in the embryonic development process, and also in most mature body tissue, but in low expression (Suga, Sugimura et al. 2012). The main function of sdn-4 is to adjust the structure of matrix and cell adhesion (Carey 1997, De Luca, Klimentidis et al. 2010).

Elastin is a major component in skin dermis which gives the skin tissue the elasticity and tensile capacity. Elastin derived peptide (EDP), peptide products resulting from degradation of κ-elastin has been shown to modulate cellular behavior of a variety of cell types such as fibroblasts and monocytes (Senior, Griffin et al. 1984), hepatocytes (Janorkar, Rajagopalan et al. 2008), smooth muscle cells (Mochizuki, Brassart et al. 2002), endothelial cells (Faury, Garnier et al. 1998) and keratinocytes (Fujimoto, Tajima et al. 2000). Specifically in fibroblasts that play a key role in regeneration, EDPs have been shown to induce proliferation and expression of several matrix

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remodeling proteins such as collagenase (Mochizuki, Brassart et al. 2002, Dorecka, Francuz et al. 2014). Study haven shown that the activity of EDP on cells are achieved through interaction with elastin receptor complex, which is composed of three subunits, elastin binding protein, neuraminidase, and cathepsin A (Hance, Tataria et al. 2002). Two downstream signaling pathways are associated with the activation of elastin receptor, Erk1/2 and PI3K (Duca, Lambert et al. 2005, Chao, Yang et al. 2012, Shi, Wang et al. 2012). MAPK/Erk is a Ser/Thr protein kinases. When MEK is activated, it connects directly to Erks through its N-terminus. MEK is also the anchor for Erk in cytoplasm. When there is no active signal, MEK fixes Erk in the cytoplasm, once Erk is phosphorylated and dimerized, MEK will transfer it into nucleus or other activation site for the further phosphorylation of downstream substrates (Avruch, Khokhlatchev et al. 2001). Wilsbacher et al. studied the translocation process of phosphorylated Erk2 into nucleus, and found that the translocation of Erk2 is dependent on the formation of homodimers between a phosphorylated Erk2 and another Erk2, either phosphorylated or un-phosphorylated, which suggested that the formation of homodimer is critical for Erk2 translocation into nucleus (Wilsbacher, Juang et al. 2006). PI3K pathway is another intracellular signaling pathway that is important in regulating the cell cycle. Phosphorylation of PI3K activates AKT which locates in the plasma membrane. Many factors are known for enhancing PI3K/AKT pathway including EGF, shh, IGF-1, insulin, and CaM(Ju, Liao et al. 2015, Li, Hu et al. 2016, Zheng, Ye et al. 2016).

3.3 Materials and Methods

3.3.1 Materials

Human dermal fibroblasts (CRL-2522) were purchased from ATCC (Manassas, VA). PBS, FBS and DMEM were all purchased from life technologies. The restriction enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). The reagents for RT-PCR

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were purchased from Bio-Rad. BrdU cell proliferation assay kit was purchased from BD Pharmingen.

3.3.2 Generation of ELP Plasmids and Expression of the Protein

The PUC57 plasmids containing the elastin cassette **(**VPGVG)$_5$ and (VPGVG)$_2$VPGCG(VPGVG)$_2$ were purchased from Genscript (Piscataway, NJ). V40C2 and V50 encoding genes were made using recursive direction ligation method as described previously (Meyer and Chilkoti 2002). PUC19-C plasmid was used as template in site-directed mutagenesis for the generation of (VPGVG)$_2$ VPGLG (VPGVG)$_2$, (VPGVG)$_2$ VPGKG (VPGVG)$_2$ and (VPGVG)$_2$ VPGDG (VPGVG)$_2$ cassette. L10 and K10 encoding gene were made using same method mentioned above.

The expression vector pET25b+ was digested with SfiI and dephosphorylated. Ligation was performed using linear pET25b+ vector and respective ELP encoding gene cassettes. E.coil BLR cells were transformed with expression vector with ELP encoding genes. For protein production, a starter culture of 75ml terrific broth medium was inoculated overnight and added to 1L culture the next day. After 16hrs of induction, bacterial cells were harvested by centrifugation at 4^0C, re-suspended in ice cold phosphate buffered saline (PBS). Cells were lysed on ice using sonication. Cell debris was removed by centrifugation at 4^0C followed by polyethyleneimine treatment (final concentration: 0.5% w/v) for DNA precipitation. After another centrifugation at 4^0C, the clear supernatant was heated up to 40^0C in water bath for 15min to transition the ELPs followed by centrifugation at 40^0C to pellet the ELPs. The ELP pellet was then dissolved in ice cold PBS followed by centrifugation at 4^0C to remove the insoluble impurities. This hot and cold spin cycle was then repeated twice for a total of three cycles. The final protein pellet was dissolved

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in deionized water and dialyzed overnight. The protein was then lyophilized and stored in a desiccator.

3.3.3 BrdU Cell Proliferation Assay

Human skin fibroblasts were cultured in 10 cm cell culture plates with 10% fetal bovine serum (Invitrogen, Grand Island, NY). 100,000 cells were plated in 6-well plate and were allowed to attach for 24 hours. After 24 hours, the cells were serum starved for another 24 hours followed by treatment with different ELPs for 48 hours. New DNA synthesis was quantified using the BrdU cell proliferation kit (BD Pharmingen, San Jose, CA) according to the manufacturer's instruction. Briefly, cells were treated with BrdU for 24 hours. After 24 hours the cells were fixed and stained using a fluorescently tagged BrdU antibody. BrdU incorporation in the cells was evaluated using flow cytometry.

To explore the signaling pathways involved in ELP induce proliferation the plated cells were pre-incubated for 1hr with the following inhibitors: (i) 5mM of lactose to block elastin binding protein, (ii) 10 μM of PD98059 to inhibit MAPK, (iii) 100nM of wortmannin to inhibit PI3K, (iv) 20mIU/ml of Heprinase III or 10 μM of surfen to block heparan sulfate activity. Each concentration of inhibitor was chosen based on previous studies (Schuksz, Fuster et al. 2008, Rusciani, Duca et al. 2010, Nikmanesh, Shi et al. 2012, Huang, Sun et al. 2013). ELPs were then added to the cells in the presence of different inhibitors. After 48hrs, new DNA synthesis was quantified using the BrdU cell proliferation assay as described above.

3.3.4 Dynamic Light Scattering

Lyophilized protein samples were resuspended in PBS at desired concentration and then filtered through 0.22 μm syringe filter. 1ml filtered sample solution in cuvette was read in DLS

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(Zetasizer Nano S, Malvern, UK) using a temperature trend analysis from 30˚C to 42˚C at a step of 2˚C. An equilibrium time of 10 min and 3 readings were performed in each temperature point.

3.3.5 Real Time PCR for Gene Expression

Fibroblasts (80,000 cells/well) were plated in 6-well plate and then lysed for RNA isolation after 48hrs of ELP treatments. The RNA from these cells was isolate using the Total RNA isolation kit (Promega, Madison, WI) as per manufactures' recommendations. cDNA was synthesized from total cellular RNA using the iScrpt cDNA synthesis kit (Biorad, Ipswich, MA). Briefly, 50 ng of total RNA was reverse transcribed in a 20-µL volume containing 5µL 4× RT reaction mix (Biorad) and 1µL enzyme for 5 minutes at 25 ^{0}C, followed by 30 minutes at 42 ^{0}C. The cDNA was then incubated at 85 ^{0}C for 5 minutes. Quantitative real-time PCR was performed using the SsoFast EvaGreen supermix (Biorad). The primers used to detect MMP-1 were 5'-AGTGACTGGGAAACCAGATGCTGA-3' and 5'-GCTCTTGGCAAATCTGGCCTGTAA-3'. The primers used to detect GAPDH were 5'-CCTGCACCACCAACTGCTTA-3' and 5'-GCCTGCTTCACCACCTTCTT-3'. The quantitative analysis was done using Biorad CFX manager.

3.3.6 Statistical Analysis

Cell proliferation assay results were expressed as normalized mean ± SEM to control. Statistical significance of the observed differences was evaluated by ANOVA: single factor whereas p value < 0.05 were considered significant (n=3).

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3.4 Results

3.4.1 ELPs Induced Proliferation of Fibroblasts Is Mediated through Cell Surface Heparan Sulfate Proteoglycans

ELPs are attractive biomaterials as their physical properties are tunable by sequence modification. We hypothesized that similar to the physical properties of ELPs the proliferative potential of ELPs can also be controlled via sequence modification. This can be achieved by modulating the ELP-cell interactions that lead to ELP mediated cell proliferation. Hence, we need to identify the interaction partners of ELPs present on the cell surface that lead to ELP mediated proliferation. Since, previous studies have shown that elastin derived peptides modulate cellular functions via the elastin receptor (Duca, Blanchevoye et al. 2007), we first targeted this receptor. Lactose sugar binds to the elastin receptor thereby shedding it from the cell surface. Thus, we carried out the proliferation experiments in the presence of lactose. The increase of fibroblast proliferation mediated by ELPs remained unaffected by the presence of lactose (Figure 3.1). Moreover, it has been reported that ELPs activate the ERK ½ MAP Kinase and the PI3Kinase pathway via the elastin receptor (Duca, Lambert et al. 2005). Blocking of either pathway with pharmacological inhibitors PD98059 (Erk/MAPK) or Wortmannin (PI3Kinase) had no effect on ELP induced proliferation (Figure 3.1). Hence, these experiments demonstrate that the interaction of ELPs with the elastin receptor is not responsible for ELP induced proliferation.

After ruling out the elastin receptor we focused on the possible role of cell surface heparan sulfate proteoglycans since several studies have shown that ELPs interact with them (Broekelmann, Kozel et al. 2005, Gheduzzi, Guerra et al. 2005, Tu and Weiss 2008). We pretreated the fibroblasts with Heparinase III, an enzyme that specifically cleaves cell surface heparan sulfates and Surfen a small molecule antagonist of heparan sulfate (Schuksz, Fuster et al. 2008). Indeed, Heparinase III

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and Surfen showed 95% and 85% blockage of ELP induced fibroblast proliferation respectively (Figure 3.2) suggesting that the interaction of ELPs with cell surface heparan sulfates is responsible for ELP mediated proliferation.

Further downstream signaling pathway was also investigated, we used go6983, a pan protein kinase C (PKC) inhibitor to pretreat the fibroblasts. Interestingly, at lower concentration (500nM) of go6983, ELP was still able to induce proliferation, while at a higher concentration (1μM), this proliferative effect was reversed, suggesting that PKC δ and PKC ζ may be involved in this interaction. Further study is needed to confirm the responsible signaling pathway of ELP-HSPG interaction.

3.4.2 Mitogenic Potential of ELPs Can Be Controlled by Modulating ELP-HSPG Interactions through Sequence Modification

Since, we discovered that HSPG interaction with ELP's is responsible for ELP mediated proliferation we hypothesized that the mitogenic potential of ELPs can be controlled by modulating their interaction with HSPG. Previous work has suggested the presence of β-strand is important for the interaction of proteins with heparan sulfates (Margalit, Fischer et al. 1993). Since during phase transition, ELP structure changes from random coils to β-turn (Hong, Isailovic et al. 2003), we investigated the effect of phase transition on ELP induced cell proliferation. Monomer peptide VPGVG which has same sequence motif as ELP, doesn't transition at physiological temperature. Indeed, without phase transition, the monomer failed to induce cell proliferation **(**Figure 3.5**)**.

Our data demonstrate clearly that the proliferative potential of ELPs is dependent on their phase transitioning properties. We further reasoned that ELP sequences having different transition properties will have varying proliferative potential. To prove this, we designed an ELP sequence

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--- L10 containing 10 leucine having the same molecular weight as V40C2 (Table 1). Since leucine is more hydrophobic than valine or cysteine, L10 has a lower transition temperature than V40C2 at the same concentration. Dynamic light scattering (DLS) showed that at 5 μM concentration, V40C2 didn't transition while L10 transitioned at about 35^0C (Figure 3.6b). At this concentration, V40C2 failed to induce cell proliferation; while L10 increased cell proliferation by 2.5 folds (Figure 3.6a). These data indicate that the proliferative potential of ELPs is directly dependent on their ability to transition.

Heparan sulfate proteoglycans are highly negatively charged. So we reasoned that besides transition, ELP-HSPG interaction can be modulated by creating positively charged ELPs; thereby resulting in ELPs having differential proliferative potential. ELPs containing positively charged lysine (K10, Table 1) at the guest position were created. Indeed, K10 induced proliferation of fibroblasts at higher folds than V40C2 or L10 suggesting that the presence of charges induce a stronger proliferation response than the ability to transition (Figure 3.7a). Interestingly, at physiological temperature K10 doesn't transition due to the presence of hydrophilic lysine residues (Figure 3.7b) suggesting that phase transition is not the only dominant mechanism of ELP mediated fibroblast proliferation.

3.4.3 ELP Induced Matrix Metalloproteinase Induction via Elastin Receptor and Is Independent of Phase Transition or Charge

Previous research has demonstrated that the GXXPG motif contained in tropoelastin induces pro-MMP1 expression (Moroy, Alix et al. 2005). Since ELP has the similar sequence motif VPGXG, we tested the induction of MMP1 gene by ELPs in fibroblasts using quantitative real time polymerase chain reaction (qRT-PCR). Indeed similar to elastin derived monomeric peptides, ELP significantly induced MMP1 expression in fibroblasts. Interestingly, lactose

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blocked the ELP induced MMP1 expression suggesting the involvement of the elastin receptor in MMP1 induction unlike proliferation. Furthermore, we investigated the effect of phase transition or charges in the induction of MMP-1 by ELPs. We found that the monomer VPGVG induced MMP1 induction suggesting that it is independent of the phase transition property of ELPs. Furthermore, while high concentrations of L10 and V40C2 (50 μm) induced MMP-1 induction, lower concentrations of L10 (5 μM) did not result in MMP-1 induction, unlike proliferation (Figure 3.8).

3.5 Discussion

Current research on biomaterials mostly describes inert biomaterials that provide mechanical support or act as drug delivery vehicles carrying an active drug for tissue regeneration. The bioactivity of the biomaterials is typically achieved by the inclusion of an active drug or an active sequence that induces cellular response. However, our work suggests that the biomaterial backbone (VPGXG) is also capable of inducing fibroblast proliferation with no active biological signal embedded within. Thus, ELP based biomaterials are not only capable of providing mechanical support but also have biological activities conducive to fibroblast proliferation which is crucial for healing of different tissues.

Elastin derived peptides that are prepared by proteolytic degradation of κ-elastin or elastin based tissues have been shown to increase proliferation of arterial smooth muscle cells (Mochizuki, Brassart et al. 2002) and fibroblasts (Shiratsuchi, Ura et al. 2010) respectively. Since, these studies were done using a complex mixture of degradation products of elastin, the exact sequence or molecular weight of the species that induced proliferation could not be identified. On the other hand our work reports the induction of proliferation by a single elastin sequence with precise molecular weight, thus allowing us to identify the exact sequence responsible for proliferation.

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Our work indicates that the model sequence $(VPGVG)_{50}$ induced fibroblast proliferation. However, previous work has reported that the monomeric or polymeric form of this model sequence VPGVG created by solid peptide synthesis did not demonstrate any significant proliferative activity on fibroblasts (Tajima, Wachi et al. 1997). This could be attributed to the very low concentrations of ELP used in that study compared to our experiments. The concentration of ELP is directly related to their transition behavior, which seems to be critical for inducing fibroblast proliferation as demonstrated by our experiments.

The observed mitogenic activity of ELPs is lost due to crosslinking or when they are immobilized on a solid substrate. This result explains why previous work performed with ELP hydrogels report no significant biological activity; since it involved chemically cross-linked ELP hydrogels leading to the loss of the mitogenic activity of ELPs (Nettles, Kitaoka et al. 2008, Hrabchak, Rouleau et al. 2010). Interestingly, the inability of the cells to internalize the cross-linked ELP will not explain the loss in bioactivity since the induced proliferation was independent of ELP internalization. Crosslinking results in loss of ability of ELPs to toggle between aggregates or single monomeric forms, which may lead to the loss of the biological activity. Further studies are needed to investigate the exact role of cross-linking and its effect on the biological activity of ELPs.

Our studies indicate that the interaction of ELPs with cell surface heparan sulfate proteoglycans (HSPGs) is responsible for the proliferation induced by ELPs. Although the interaction of the elastin sequence VPGXG with heparin and HSPGs has been demonstrated (Gheduzzi, Guerra et al. 2005, Tu and Weiss 2008) in several in vitro binding assays, no studies have reported that the interaction of ELPs with cell surface HSPG leads to cell proliferation or any biological activity by ELPs. Also, this finding is truly novel as previous studies involving the

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mechanism of ELP-cell interactions leading to its biological activity primarily focused on the elastin receptor complex (ERC) (Duca, Blanchevoye et al. 2007, Antonicelli, Bellon et al. 2009). For example, elastin derived peptides have been shown to interact with the elastin receptor leading to MMP-1 induction in fibroblasts. Similar, to this study our work also shows induction of MMP-1 activity by ELPs through the elastin receptor suggesting that ELPs also interact with the receptor. However, the observed mitogenic effect of ELPs is independent of this interaction and is dependent on HSPGs. Thus, these results indicate that ELPs affect several important processes for tissue regeneration namely inflammation, matrix remodeling (MMP-1 activity) and proliferation, via distinct receptors present on the cells. Furthermore, MMP-1 induction by ELPs was independent of the phase transition property unlike proliferation. Further studies are underway to elucidate the differences in ELP binding to these two receptors leading to activation of different pathways resulting in different bioactivity.

The mitogenic activity of ELPs is closely related to their ability of phase transition. One possible explanation for this is that during phase transition, ELPs form β-strand structure, which has been reported as a common secondary structure for variety of proteins that interact with cell membrane bound HSPG (Hileman, Fromm et al. 1998). Thus, the phase transition of ELPs enables the interaction of ELPs with HSPG leading to fibroblast proliferation. Since, immobilization prevents the ELP chains to come together affecting their structural transition this may also explain the loss of bioactivity of ELPs due to crosslinking. We further demonstrate that it is possible to modulate the proliferative activity of ELPs by designing ELP sequences having different transition behavior. ELP sequences that transition easily by incorporation of hydrophobic leucine were more mitogenic. We further show modulation of this ELP interaction with HSPGs by inclusion of charged amino acids within the ELP sequence, since HSPGs are negatively charged. We show that

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ELPs containing positively charged lysine residues were highly mitogenic compared ELPs containing no charged residues. These studies demonstrate a simple way of controlling the mitogenic activity of ELPs by simply including either hydrophobic amino acids affecting phase transition or charged amino acids. Controlling the mitogenic activity of ELPs in context to tissue regeneration is extremely crucial. Take chronic wound healing for example, little or no fibroblast proliferation leads to chronic wounds; while excessive fibroblast proliferation is also not desired as it leads to fibrosis (Rieder, Brenmoehl et al. 2007).

In summary, our work demonstrates that the biological activity of ELPs can be modulated by simply tweaking their physical properties. Specifically, we show for the first time that ELPs induce fibroblast proliferation which is dependent on cell surface HSPGs. We further show that this interaction can be modulated by designing ELP sequences having different phase transition behavior or charge; thereby controlling the mitogenic activity of ELPs. Fibroblast proliferation and function is essential for granulation tissue formation in not only chronic wound healing but in healing of injuries of other tissues such as bone or neural tissue. Thus, ELP based materials with differential proliferative activity on fibroblasts can be designed and will have broad application in the development of therapeutics for tissue regeneration.

Table 1 ELPs sequences and characterization

ELP	Sequence	Charge
V50	$(VPGVG)_{50}$	Neutral
V40C2	$(VPGVG)_{40}$(VPGVGVPGVGVPG**C**GVPGVGVPGVG)$_2$	Neutral
L10	(VPGVGVPGVGVPG**L**GVPGVGVPGVG)$_{10}$	Neutral
K10	(VPGVGVPGVGVPG**K**GVPGVGVPGVG)$_{10}$	Positive

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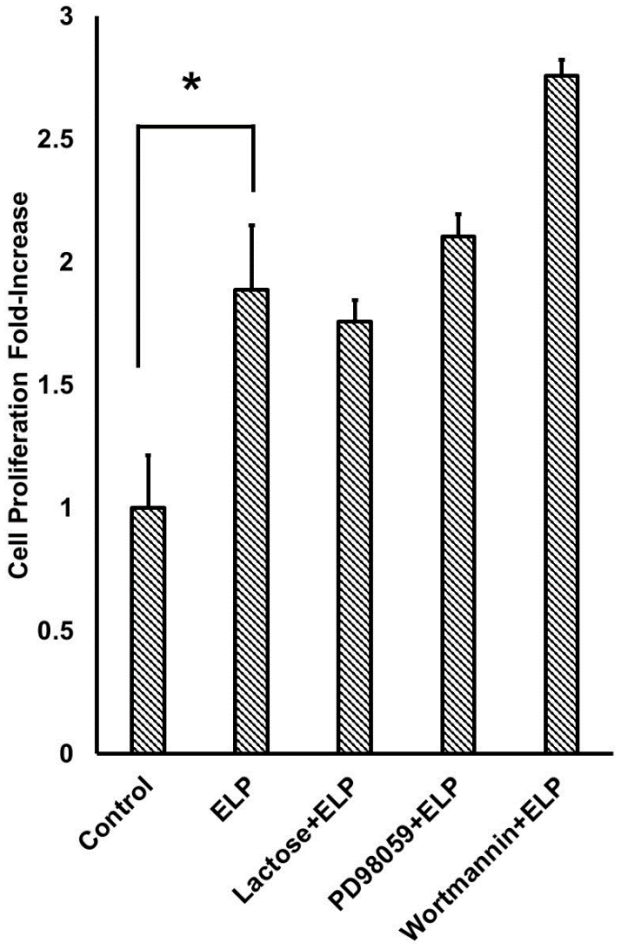

Figure 3.1 ELP induced cell proliferation is not dependent on elastin receptor complex. Cells were starved for 24hrs and pre-blocked with different inhibitors lactose 5mM, PD98059 10 μM and Wortmannin 100 nM. They were then treated with 50 μM ELP for 48hrs in the presence of the inhibitors. Cell proliferation was evaluated using the BrdU assay. All the values are obtained from triplicate experiments and normalized to control. *= $p < 0.05$ when the indicated samples were compared.

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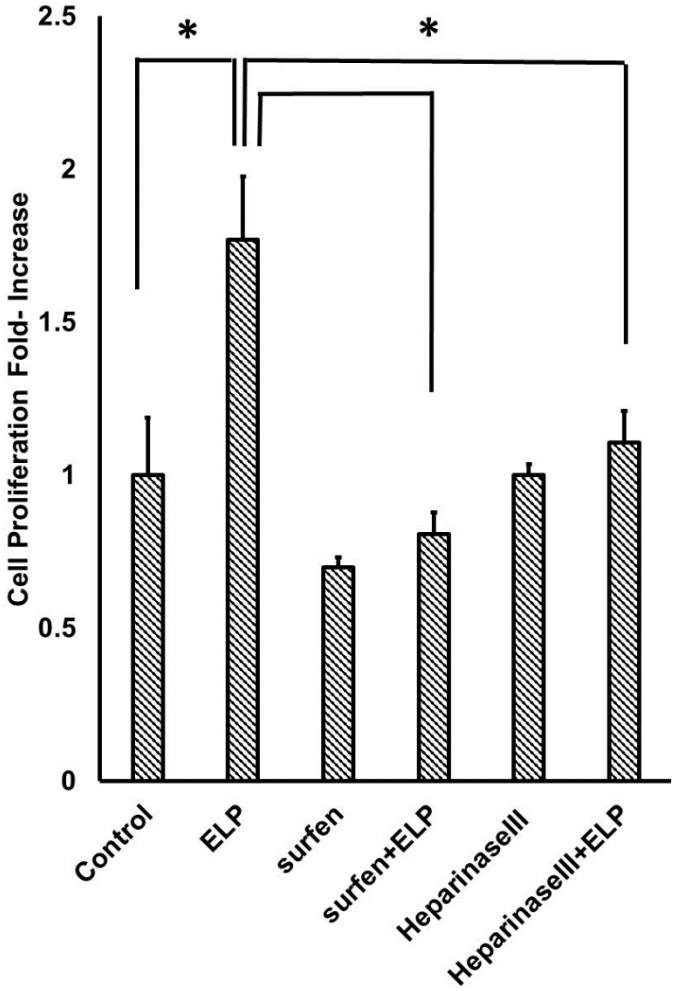

Figure 3.2 ELP induced cell proliferation is dependent on HSPG. Cells were starved for 24hrs and pre-blocked with different inhibitors Heparinase III 20mIU or surfen 10μM. They were then treated with 50 μM ELP for 48hrs in the presence of the inhibitors. Cell proliferation was evaluated using the BrdU assay. All the values are obtained from triplicate experiments and normalized to control. *= $p < 0.05$ when the indicated samples were compared.

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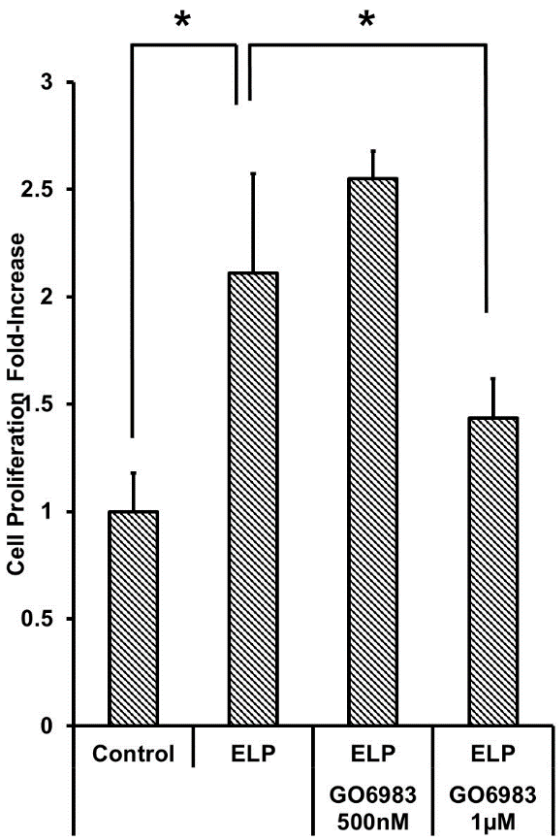

Figure 3.3 PKC signaling pathway may be involved in the ELP induced proliferation. Cells were starved for 24hrs and pre-blocked with Go6983 500nM or 1 μM. They were then treated with 50 μM ELP for 48hrs in the presence of the inhibitors. Cell proliferation was evaluated using the BrdU assay. All the values are obtained from triplicate experiments and normalized to control. *= $p < 0.05$ when the indicated samples were compared.

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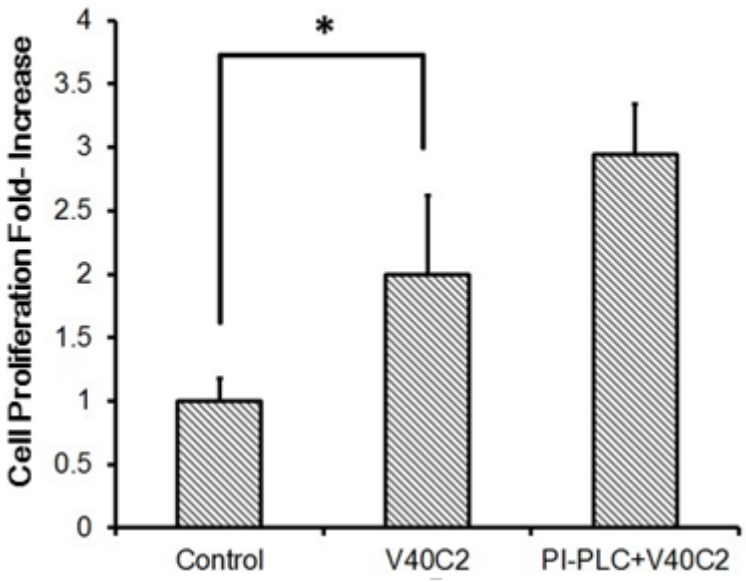

Figure 3.4(a) Syndecan 1 may be involved in ELP-cell interaction downstream signaling pathway.

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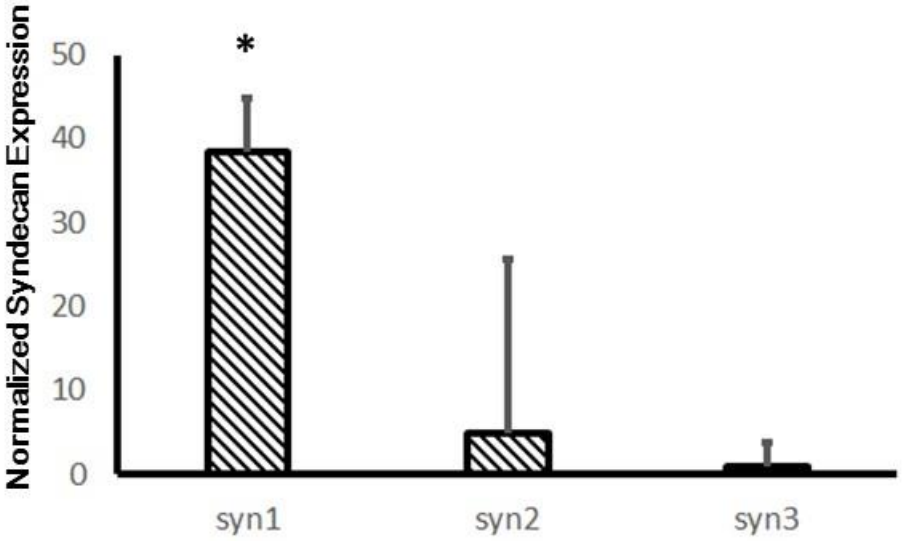

Figure 3.4(b) Syndecan 1 is dominantly expressed in skin fibroblasts.

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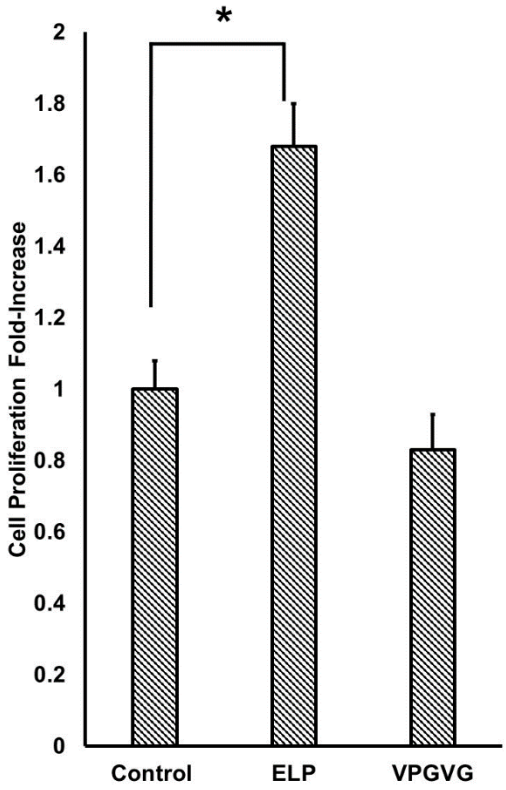

Figure 3.5 VPGVG monomer didn't induce the proliferation. ELPs were starved for 24hrs and then treated for 48 hrs with 50 μM of ELP (V40C2) or the peptide VPGVG. Cell proliferation was evaluated using the BrdU assay. All the values are obtained from triplicate experiments and normalized to control.

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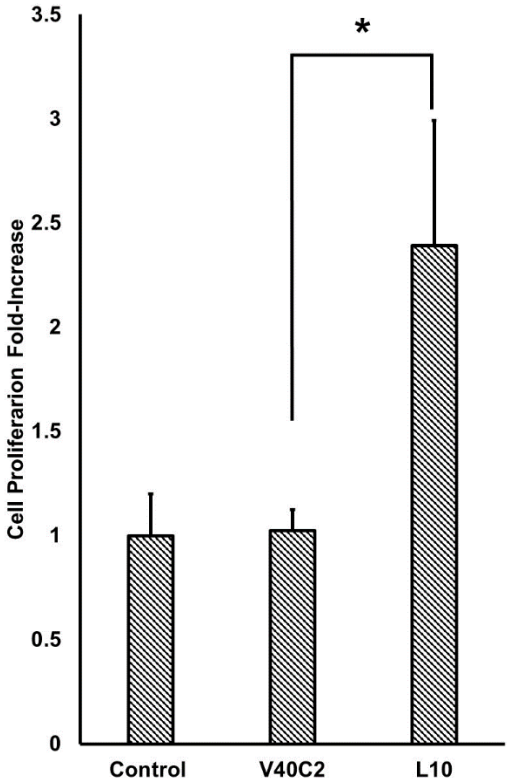

Figure 3.6(a) Controlling ELP induced cell proliferation through sequence modification. Cells were starved for 24hrs then treated for 48 hrs. with 5μM of ELPs. BrdU assay was performed for cell proliferation.

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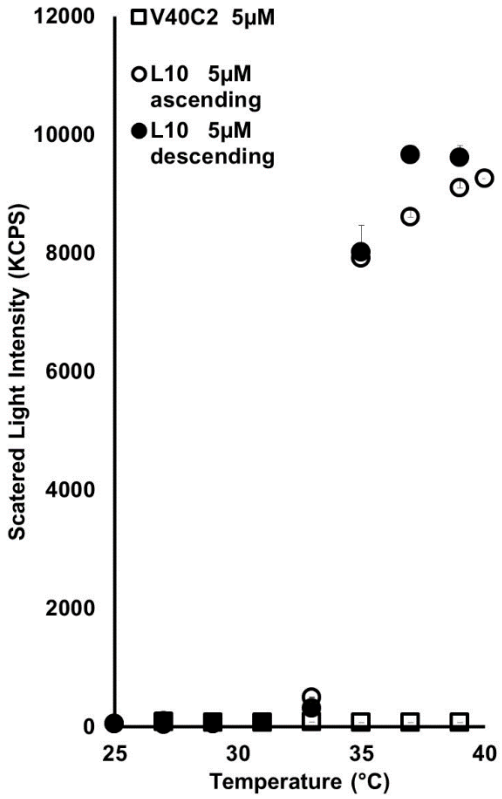

Figure 3.6(b) L10 transition before physiological temperature at 5μM. ELPs were dissolved in PBS to 5μM and phase transition was quantified using dynamic light scattering (DLS)All the values are obtained from triplicate experiments and normalized to control. *=$p<0.05$.

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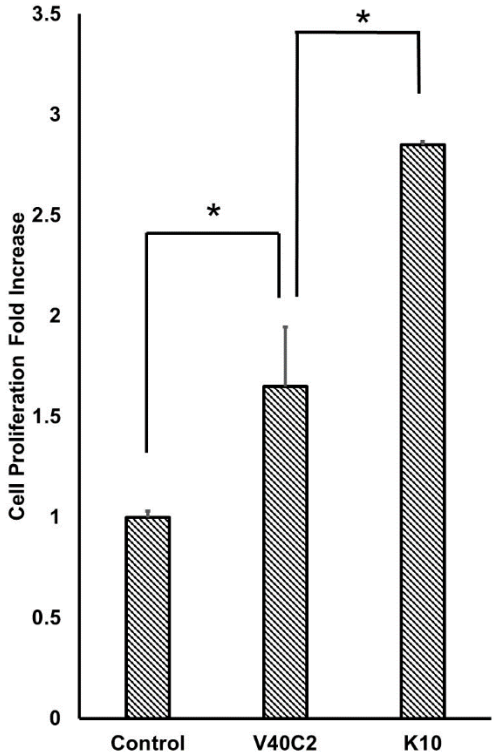

Figure 3.7(a) ELPs containing positively charged amino acids induce cell proliferation independent of phase transition. Cells were starved for 24hrs then treated for 48 hrs with either V40C2 or K10. BrdU assay was performed for cell proliferation. All the values are obtained from triplicate experiments and normalized to control *= P<0.05.

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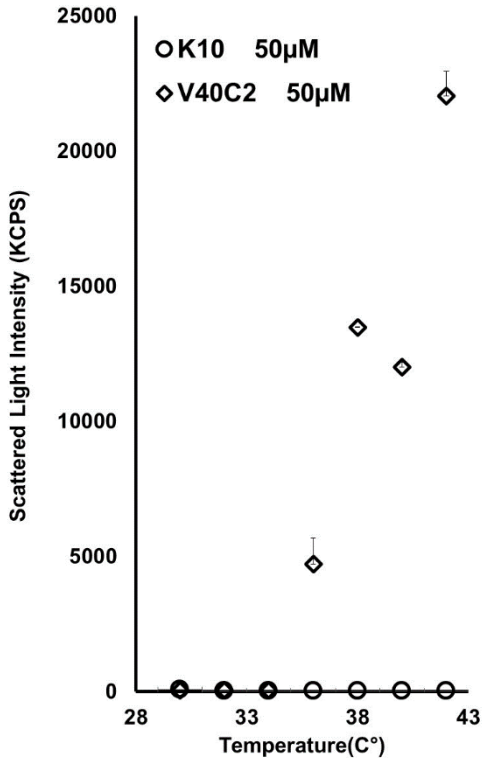

Figure 3.7(b) K10 didn't transition before physiological temperature at 50µM. V40C2 and K10 were dissolved in PBS and phase transition was evaluated using dynamic light scattering.

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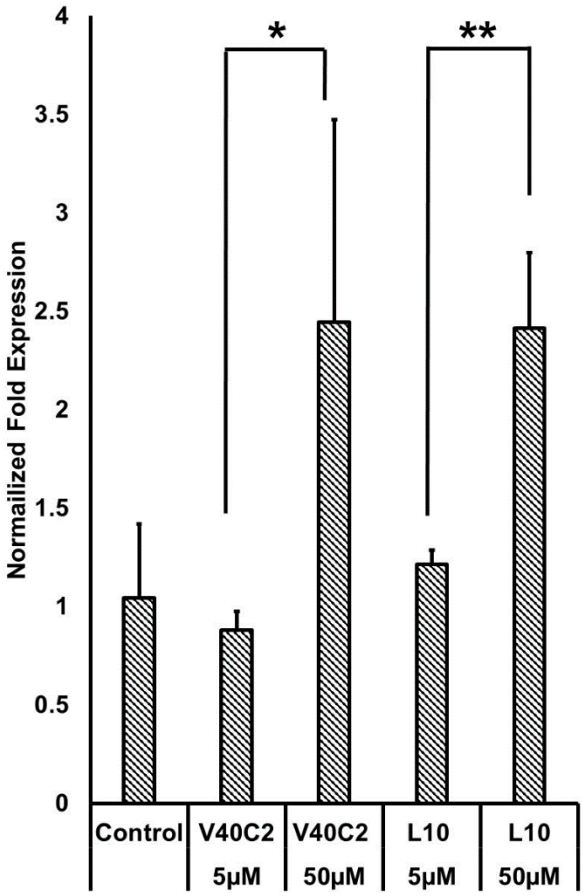

Figure 3.8 ELPs induced MMP1 expression in fibroblasts is independent of phase transition. Cells were serum starved for 24hrs and then treated with either 5 or 50 μM concentrations of the indicated ELPs. Total RNA was collected for qRT-PCR to quantify MMP-1 gene expression. All the values are obtained from triplicate experiments and normalized to control. *= indicates $p<0.1$, **= indicates $p<0.05$.

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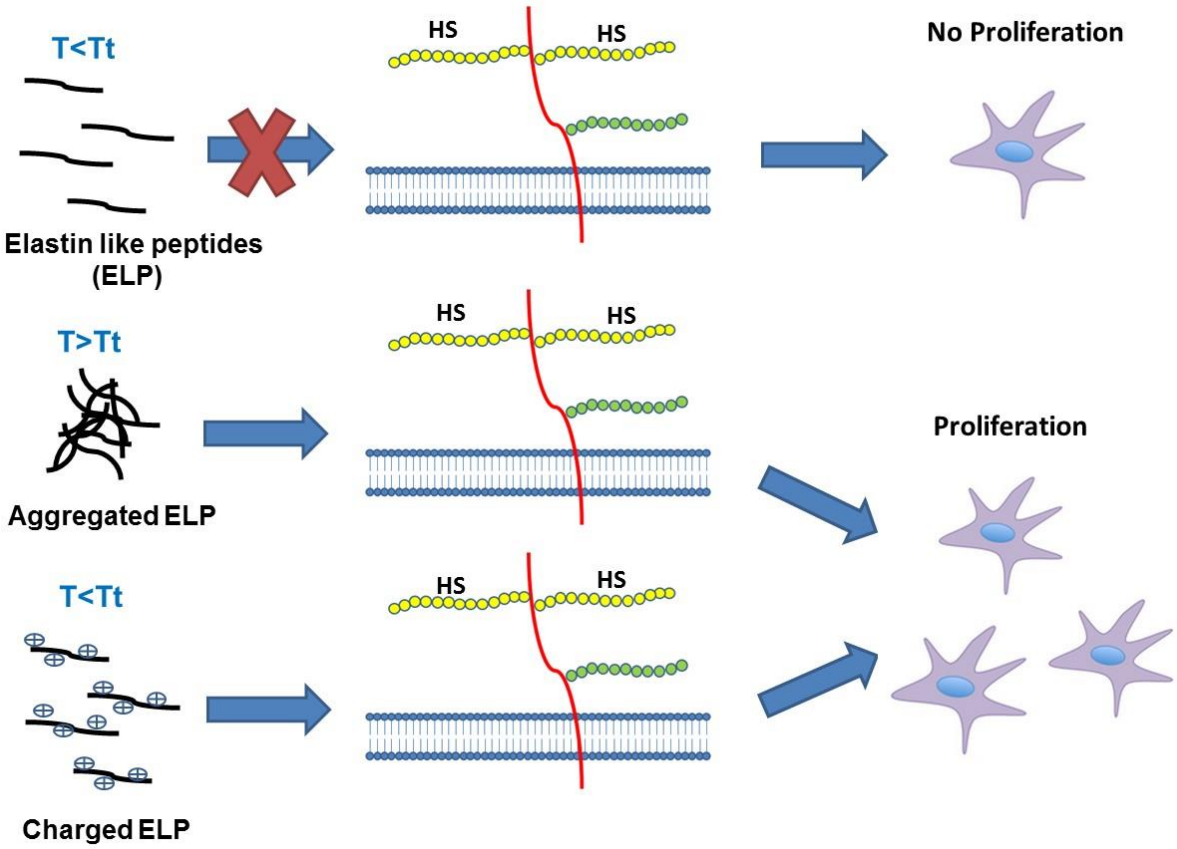

Figure 3.9 Schematic of interaction mechanism of ELP induced cell proliferation.

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CHAPTER 4: A PROTEASE RESISTANT FORMULATION FOR DELIVERY OF BIOACTIVE MOLECULES FOR TISSUE REGENERATION

4.1 Introduction

Protein therapeutics have obtained numerous attention for past decade and already have a significant role in almost every field of medicine. Compare to small molecule drugs, protein therapeutics have several advantages such as high specificity, less likely to elicit immune response and shorter clinical development and FDA approval time (Leader, Baca et al. 2008). The development of recombinant DNA technique greatly helped the widespread of protein therapeutics through the availability of modification of the protein and potentially limitless quantity (Barton, Brady et al. 1991, Grabowski, Barton et al. 1995).

In the field of regenerative medicine, proteins like growth factors and cytokines are often used to stimulate the proliferation, migration and differentiation of different cell type to promote the healing of skin tissue, bones and etc (Moura, da Silva et al. 2013, Liu, Ou et al. 2014, Valente and Ciavarella 2016). Platelet derived growth factor (PDGF) which stimulates fibroblasts proliferation has been approved by FDA for the treatment of diabetic ulcers (Smiell, Wieman et al. 1999). Other growth factors like epidermal growth factor (EGF), keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF) also have shown promising results in both *in vitro* and *in vivo* studies (Koria, Yagi et al. 2011, Barrientos, Brem et al. 2014, Johnson and Wang 2015). However, there are still some problems hindering the translation of these growth factors from clinical research to FDA approved medicines. Tissue regeneration is a complex process that

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depends on tightly regulated signaling molecules at exact times, locations, and concentrations (Clause and Barker 2013). Growth factors usually experience a short half-life after been delivered to the injury site due to excessive protease existed in the wounded area (Dinh, Braunagel et al. 2015). Engineered delivery system for growth factor therapy is a very attractive way to modulate cell behavior. Encapsulation of growth factors in hydro gel is a common way of achieving controlled delivery. Rybalko and co-worker have done a delicate work to deliver SDF-1α and IGF-I within a novel poly-ethylene glycol PEGylated fibrin gel matrix (Rybalko, Pham et al. 2015). Conjugation of growth factors to a stabilizing molecule is another way of extending half-life. Thi H. Nguyen and co-worker used a heparin-mimicking polymer conjugate to stabilize basic fibroblast growth factor (bFGF) (Nguyen, Kim et al. 2013). Poly(ethylene glycol) (PEG) is another stabilizing molecule which has been widely explored as a means to improve the half-lives of proteins (Wang, Cooke et al. 2013, Miao, Rao et al. 2014). PMP-D2 is a naturally occurring peptide derived from the brain and fat body of the insect *Locusta Migratoria (Kellenberger, Ferrat et al. 2003)*. It has 35 residues which cross-linked by three disulfide bonds. PMP-D2 variant (R29L/K30M) has a strong inhibitory activity towards serine protease through increased binding affinity of P1 residue within the reactive site of the inhibitor (Kellenberger, Boudier et al. 1995).

Elastin is a major component in connective tissue and may be beneficial for tissue regeneration. Elastin derived peptides which are short peptide pieces digested from tropoelastin have been showed to modulate cellular behavior of a variety of cell types such as fibroblasts and monocytes (Senior, Griffin et al. 1984), hepatocytes (Janorkar, Rajagopalan et al. 2008), smooth muscle cells (Mochizuki, Brassart et al. 2002), endothelial cells (Faury, Garnier et al. 1998) and keratinocytes (Fujimoto, Tajima et al. 2000). Specifically in fibroblasts that play a key role in regeneration, EDPs have been shown to induce proliferation and expression of several matrix

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remodeling proteins such as collagenase (Brassart, Fuchs et al. 2001, Duca, Debelle et al. 2002). Elastin like peptides are synthetic peptide that are composed of repeated blocks of (Val-Pro-Gly-X-Gly)n, a sequence motif that derived from hydrophobic domain of tropoelastin. An interesting property of ELPs is their ability to undergo phase transition at transition temperatures. When the temperature is below their inverse transition temperature, also known as the lower critical solution temperature (LCST), they assume a random coil structure and are soluble in aqueous solution. However, at temperatures higher than the LCST, ELPs undergo an entropy driven self-assembly rendering them insoluble (Koria, Yagi et al. 2011). This property enables recombinant ELPs to be expressed in bacteria and rapidly purified using inverse temperature cycling (ITC). Genetic fusion of ELP with protein or peptide has been studied and proved to be a promising delivery method since it forms certain nanostructure and can be easily purified.

Previously in our lab, we have created several recombinant growth factors fusion proteins with elastin like peptide including KGF-ELP and BMP-2-ELP which not only maintained ELP phase transition property but also retained growth factor bioactivity **(**Table 1**)**. In this study, we have fabricated a fusion protein comprising of recombinant PMP-D2 variant and elastin like peptide (PMP-D2-ELP), mixed it with the growth factor fusions and allow them transition at physiological temperature to form multifunctional nanoparticles which may address the stability issue and improve delivery of different growth factors for tissue regeneration application.

Currently, the golden standard animal model for studying of chronic wound healing is diabetic mice or rats (Ansell, Holden et al. 2012). They are the most widely used model, including genetically deficient type I diabetic mice, also known as non-obese diabetic (NOD) mice and type II diabetic mice which are leptin or leptin receptor knockout mice (Islam 2013). Although NOD mice do show a delayed wound healing process, they may not be a suitable model for diabetic foot

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ulcers which are prevalent in type II diabetes (Keswani, Katz et al. 2004, Boulton 2008). Leptin receptor deficient mice become obese from around 6 weeks of age, and consequently develop type II diabetes. Research have shown that the wound repair has been markedly delayed (Tsuboi and Rifkin 1990, Ring, Scully et al. 2000). Many growth factors have been identified their therapeutic potential using this model (Greenhalgh, Sprugel et al. 1990, Kitano, Yoshimura et al. 2001, Bevan, Gherardi et al. 2004, Hardwicke, Hart et al. 2011), however, only PDGF-BB has successfully gone through the human trials and approved by FDA for treatment of diabetic foot ulcers suggesting the poor predictivity of the diabetic mice model. In human chronic wounds, excessive protease level is found in the area which break the balance between itself and growth factors and other signaling molecules, thus cause a delay of the formation of granulation tissue. Currently animal chronic wound models including diabetes model, ageing model, skin flap model, and pressure model all lack of the important property of excessive protease level in human chronic wounds. In this study, we created a chronic wound model using leptin receptor deficient mice incorporating with elastase treatment in hydrogel which significantly delayed the wound healing process.

4.2 Materials and Methods

4.2.1 Materials

Human dermal fibroblasts (CRL-2522) were purchased from ATCC (Manassas, 4.2VA). PBS, FBS and DMEM were all purchased from life technologies. The restriction enzymes used for cloning were purchased from New England Biolabs (Ipswich, MA). The reagents for RT-PCR were purchased from Bio-Rad.

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4.2.2 Generation of PMP-D2-ELP Plasmids and Expression of the Protein

The PUC57 plasmids containing the PMP-D2 gene cassette were purchased from Genscript (Piscataway, NJ). V40C2 encoding genes were made using recursive direction ligation method as described previously (Meyer and Chilkoti 2002).

The expression vector pET25b+ was digested with SfiI and dephosphorylated. Ligation was performed using linear pET25b+ vector and respective PMP-D2-ELP encoding gene cassettes. E.coil BLR cells were transformed with expression vector with PMP-D2-ELP encoding genes. For protein production, a starter culture of 75ml terrific broth medium was inoculated overnight and added to 1L culture the next day. After 16hrs of induction, bacterial cells were harvested by centrifugation at 4^0C, re-suspended in ice cold phosphate buffered saline (PBS). Cells were lysed on ice using sonication. Cell debris was removed by centrifugation at 4^0C followed by polyethyleneimine treatment (final concentration: 0.5% w/v) for DNA precipitation. After another centrifugation at 4^0C, the clear supernatant was heated up to 40^0C in water bath for 15min to transition the ELPs followed by centrifugation at 40^0C to pellet the ELPs. The ELP pellet was then dissolved in ice cold PBS followed by centrifugation at 4^0C to remove the insoluble impurities. This hot and cold spin cycle was then repeated twice for a total of three cycles. The final protein pellet was dissolved in deionized water and dialyzed overnight. The protein was then lyophilized and stored in a desiccator.

4.2.3 Elastase Activity Assay

Neutrophil elastase colorimetric drug discovery kit (ENZO) was used to test the inhibition activity of PMP-D2-ELP fusion protein against elastase. Lyophilized PMP-D2-ELP and ELP control were dissolved separately in PBS to make 10mg/ml stock concentration and 0.2mg/ml final

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concentration was used in the assay. Elastase (2mU/μl) was diluted 1/90 in assay buffer for the experiment. Absorbance at 405 was read every minute from 0 to 20 minutes for each sample.

4.2.4 Western Blot

Growth factor ELP fusion proteins (PDGF-ELP and BMP2-ELP) were dissolved in PBS to reach a concentration of 1mg/ml. They either mix with 0.2mg/ml of PMP-D2-ELP to form multifunctional nanoparticles then treated with 20mU/ml elastase or directly treated with 20mU/ml elastase for up to 2 hours. At time point 15 minutes, 30 minutes, 1 hour, and 2 hours, we collected the sample for each treatment and prepare for western blot analysis.

4.2.5 BrdU Cell Proliferation Assay

Human skin fibroblasts and A431 cells were cultured in 10 cm cell culture plates with 10% fetal bovine serum (Invitrogen, Grand Island, NY). 20,000 cells were plated in 48-well plate and were allowed to attach for 24 hours. After 24 hours, the cells were serum starved for another 24 hours followed by treatment with different growth factor ELP fusion proteins (KGF-ELP and PDGF-ELP) for 48 hours. New DNA synthesis was quantified using the BrdU cell proliferation kit (BD Pharmingen, San Jose, CA) according to the manufacturer's instruction. Briefly, cells were treated with BrdU for 24 hours. After 24 hours the cells were fixed and stained using a fluorescently tagged BrdU antibody. BrdU incorporation in the cells was evaluated using flow cytometry.

To explore the inhibition activity of PMP-D2-ELP against elastase and the multifunctional nanoparticles performance in elastase, we treated the cells with growth factor fusion proteins alone (1mg/ml) as positive control, growth factor fusion proteins (1mg/ml) mixing with elastase, and multifunctional nanoparticles (1mg/ml growth factor ELP fusion and 0.2 mg/ml PMP-D2-ELP)

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with elastase. After 48 hours of treatments, cells were trypsinized and fixed, then treated with anti-BrdU antibody. 7-AAD was used to stain the cell nucleus for cell cycle.

4.2.6 Animals

The mouse strain that we have chosen is B6.BKS-Leprdb from Jackson Laboratories. These mice are homozygous for the diabetes spontaneous mutation (Leprdb) and become identifiably obese around 3 to 4 weeks of age. They exhibit elevations of plasma insulin at 10 to 14 days and of blood sugar at four to eight weeks. Moreover, wound healing in these mice is delayed. Hence, it is a good and well accepted model to study chronic diabetic wounds in the research community.

4.2.7 Excisional Wounding of Skin

Mice will be anesthetized using Isoflurane inhalation (3-4 % induction, 1-2 % maintenance) on a heating pad at 37C and anesthesia deemed sufficient when the animal lacks a contracting reflex in response to interdigital pinch and palpebral/eyeblink reflex. The eyes of the animal will be covered with medical ointment to prevent dryness. Hair will be removed from the dorsal area using electric clippers in an area away from the surgical site. The anesthetized animal would then be brought to the surgical site and the dorsum will be prepared by a three-fold alternating application of Betadine SCRUB and 70% alcohol solution. A 1cm x 1cm square area will be drawn with a black marker on the back of the animal using a template. The skin will be gently lifted up and cut along the marked boundary to generate a full-thickness skin defect on the dorsum. 100µl of fibrin gel containing 25mU/ml elastase will be administered into the wounds. The mice will then be dressed with a 2.5 cm x 2.5 cm piece of adherent silicone dressing (Tegaderm™) encompassing an area extending at least 1 cm beyond the wound boundaries. Animals will be single caged after the procedures and standard bedding will be used.

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4.2.8 Post Procedure Observations

Animals will be under continuous examination until they recover from the anesthesia as evidenced by the ability to maintain themselves in an upright position, and by assessment of the respiratory rate and response to visual, acoustic, and touch stimuli. Then, they will be returned to the animal housing area. After housing, animals will be observed every 12 hours for 72 hours then daily till 28 days. Animals with be euthanized at 21 days post wounding and tissue surrounding the wound area (including the wound) will be excised and collected for histological analysis.

4.2.9 Statistical Analysis

Cell proliferation assay results were expressed as normalized mean ± SEM to control. Statistical significance of the observed differences was evaluated by ANOVA: single factor whereas p value < 0.05 were considered significant (n=3).

4.3 Results

4.3.1 PMP-D2-ELP Inhibits Elastase Activity

After successfully purified PMP-D2-ELP using ITC, we tested the protease inhibition activity of the fusion protein using elastase activity assay. Human leukocyte elastase (HLE) is selected because it is the protease that found most abundant in skin chronic wounds. Indeed, PMP-D2-ELP inhibited HLE activity by 76% (Figure 4.3), suggested that the fusion protein retained the protease inhibition activity of PMP-D2. Interestingly, the data showed that ELP by itself also inhibited HLE activity by 33%. Since elastin is a known substrate of elastase, competitive inhibition could be an explanation of this finding.

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4.3.2 Multifunctional Nanoparticles Prevented Degradation of Growth Factors in HLE Environment

Next we the examined the stability of mNPs in HLE environment. Two growth factor fusion protein, BMP-2-ELP and PDGF-ELP were mixed at 5:1 ratio separately with PMP-D2-ELP to make mNPs. Then mNPs were incubated with HLE at 37 ˚C for different time points from 15 minutes to 2 hours. The western blot data showed that for BMP-2-ELP, with or without PMP-D2-ELP, there was an initial degradation, however with the help of PMP-D2-ELP, the mNPs were able the maintain stable after 2 hours; while the control BMP-2-ELP kept degrading. For PDGF-ELP, the mNPs kept almost 100% activity of PDGF after 2 hours; while the control was fully degrade by elastase after 30 minutes (Figure 4.4). This result clearly suggested that PMP-D2-ELP was able to prevent growth factor degradation from HLE.

4.3.3 mNPs Retained Proliferative Activity of Growth Factors *in vitro*

To compare the bioactivity of the recombinant growth factors in mNPs and growth factor fusion protein alone with the existence of HLE, we performed cell proliferation assay. KGF-ELP and PDGF-ELP were used in this experiment since they are both crucial growth factors for skin wound healing. The responsive cell lines are A431 and fibroblasts respectively. To test KGF mNPs, we serum starved the cells for 24 hours and then added KGF-ELP, KGF-ELP with HLE, mNPs with HLE as treatment. Indeed KGF-ELP alone induced cell proliferation by 2 folds, with HLE the proliferative effect has been reversed due to degradation; while mNPs restored KGF activity completely. PMP-D2-ELP had no effect on A431 cell as same as HLE (Figure 4.5a).

Similarly, for PDGF mNPs, we serum starved the fibroblasts for 24 hours and then added PDGF-ELP, PDGF-ELP with HLE, mNPs with HLE as treatment. Same pattern was found in proliferation data, PDGF mNPs was able to prevent degradation and induce fibroblast proliferation

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by around 1.5 folds (Figure 4.5b). Both cell proliferation assays suggested that comparing to growth factor alone, mNPs was able to improve the stability and retain the bioactivity of growth factor with the existence of HLE.

4.3.4 Excessive HLE Delayed the Healing of Full-Thickness Wound in Genetically Diabetic Mice

To evaluate the effect of excessive elastase on wound healing of genetically diabetic mice, we created a 1 cm ×1 cm full thickness wound on the dorsal area of the mouse. Elastase was treated on the wound with fibrin gel for either 14 days or 21 days. 15 days after the treatment, control wound closed about 80%; while for both 14-day and 21-day treatment wound closed about 50% (Figure 4.7c). Significant delay of wound healing was observed when wound treated with HLE. After 21 days, 14-day treatment wound and control both healed completely, while 21-day treatment wound had 30% wound area remain open showing that HLE didn't cause permanent damage to the wound, the delay of the wound healing is reversible by removing elastase.

4.4 Discussion

In this work we demonstrated the development of a formulation that preserving the bioactivity of different functional peptides and growth factors in excessive protease environment by incorporation of an elastase resistant peptide. Growth factors are crucial signaling molecules for tissue regeneration. The delivery of growth factors has been always the key to the successful clinical application. Currently the delivery methods of growth factors involve chemical conjugation and physical encapsulation (Koria 2012). However, for these methods, issues like growth factor release speed, the toxicity of the crosslinking agent and the correct balance between the factor dose and physical and chemical properties of the scaffold, which can regulate cell behavior are still require further investigation.

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The fusion PMP-D2-ELP inhibited elastase activity significantly which is in agreement with previous studies that ELP fusion protein not only maintain the ELP transition property but also retain the bioactivity of the functional group. One interesting finding of our experiment is that ELP by itself inhibited elastase activity by 33%. ELP is a synthetic peptide that has a repeated sequence motif derived from the hydrophobic domain of tropoelastin which is a known substrate of elastase. This data suggested that ELP part of the fusion protein also has the potential to preserve the growth factor activity by attenuating elastase activity through competitive inhibition.

Our fusion multifunctional nanoparticles clearly showed inhibition of elastase activity and prevention of degradation of growth factors. Western blot data suggested that with the existence of high concentration of elastase, most growth factors go through a complete degradation within 2 hours. This data explains why in chronic wound treatment, a daily dose of PDGF BB, the only FDA approved growth factor, is required. Interestingly, even though we mentioned that ELP has an inhibition activity towards elastase, the growth factor ELP fusion proteins were almost degraded completely within 2 hours, suggesting that an elastase resistant peptide is needed in the mNPs.

In wound healing process, fibroblasts and epithelial cells are two important cell types involved, growth factors like KGF and PDGF are often used to induce the proliferation of these cells. From our data, growth factors were able to induce cell proliferation with the existence of elastase with the help of PMP-D2-ELP. Interestingly, here ELP fusion protein maintained phase transition property which allowed rapid expression and purification of large quantity of these proteins using inverse transition cycling (ITC). Moreover, this controllable transition behavior allowed ELP fusion proteins with different functional group self-assemble and form nanoparticles. These particles not only serves as drug delivery vehicle but also the drug itself which targets different cell types and signaling molecules. Several advantages of our multifunctional

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nanoparticles comparing to other growth factor delivery methods include first of all, it doesn't require extra engineering procedures like physical encapsulation, release and chemical crosslinking; second, instead just induce the proliferation of certain cell type, the mNP could target multiple cell types using different functional peptides, this is especially important to chronic wound healing which is considered a complex process involved sequential phases that promoted by many cell types and ECM proteins.

Currently diabetic mouse model for studying chronic wound healing has several limitations such as the protease level is significant lower comparing to human chronic wound environment, and the healing process is also much faster, usually takes about 21 days due to contraction **(**Figure 4.6**).** Here we tried to develop a new chronic wound model by treating elastase onto the wound with fibrin gel. The wound closure was significantly delayed with the treatment of elastase. Interestingly, after treated the wound for 14 days with elastase, we took out the treatment, the wound closed completely by day 21, and histology showed full re-epithelialization. This suggested that the delay of wound closure effect is reversible, elastase didn't alter any cellular behavior permanently. This has been also proved in the cell proliferation assay experiment. Also, an interesting observation during the experiment is that the delay of the wound healing process is related to the yellow secretion which only found in the treatment wounds. One possible explanation is that the inflammatory phase had been prolonged by elastase treatment. Further investigation is needed to elucidate the mechanism of this delayed healing process.

In summary, we developed a multifunctional nanoparticle that comprised of PMP-D2-ELP fusion protein and different functional peptide ELP fusion proteins to preserve the bioactivity of the functional group with the existence of elastase. These heterogeneous particles will be beneficial for the delivery of combination therapy to solve multiple problems that often existed in chronic

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wound healing or other tissue regeneration process. We also tested a new animal chronic wound model by increasing the local elastase concentration to mimic human chronic wound. The healing process has been delayed significantly. The development of this chronic wound model may help the translation of therapeutics that tested in animal research to clinical approved drug.

Table 2 ELP fusion proteins sequences and molecular weight.

ELP fusion protein	**Sequence**	**Molecular Weight**
PMP-D2-ELP	EEKCTPGQVKQQDCNTCTCTPTGVWGCTLMGCQPA $(VPGVG)_{40}(VPGVGVPGVGVPGCGVPGVGVPGVG)_2$	24 KDa
PDGF-ELP	SLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFLVWPPCVEVQRCSGCCNNRNVQCRPTQVQLRPVQVRKIEIVRKKPIFKKATVTLEDHLACKCETVAAARPVT $(VPGVG)_{40}(VPGVGVPGVGVPGCGVPGVGVPGVG)_2$	32 KDa
BMP2-ELP	HGVGQAKHKQRKRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCRVP $(VPGVG)_{40}(VPGVGVPGVGVPGCGVPGVGVPGVG)_2$	33.5 KDa
KGF-ELP	CNDMTPEQMATNVNCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNNYNIMEIRTVAVGIVAIKGVESEFYLAMNKEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHNGGEMFVALNQKGIPVRGKKTKKEQKTAHFLPMAIT $(VPGVG)_{40}(VPGVGVPGVGVPGCGVPGVGVPGVG)_2$	39 KDa

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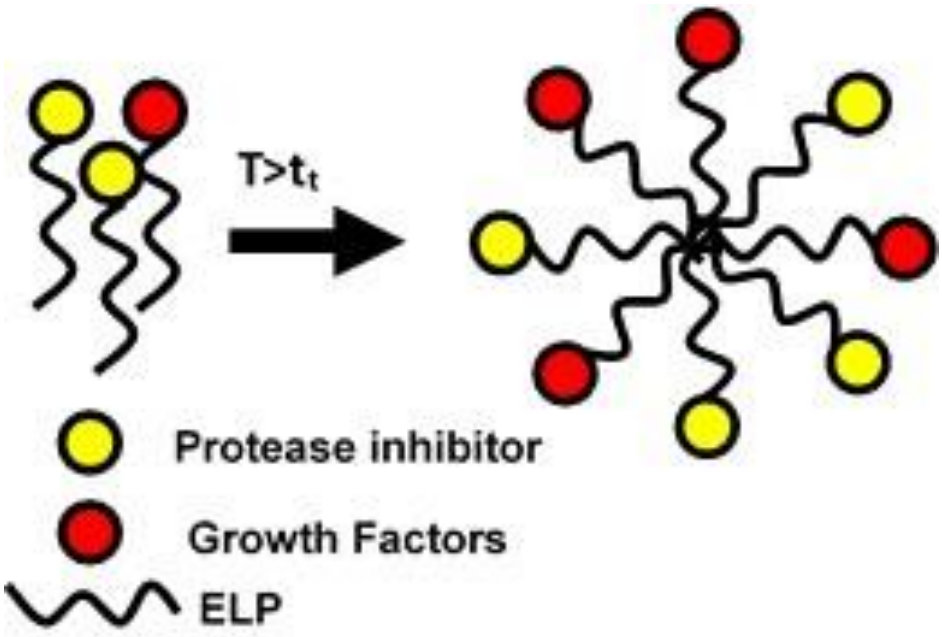

Figure 4.1 Schematic of a multifunctional nanoparticle. Fusion proteins comprising of different functional domains (yellow or red) self-assemble into a multifunctional nanoparticle.

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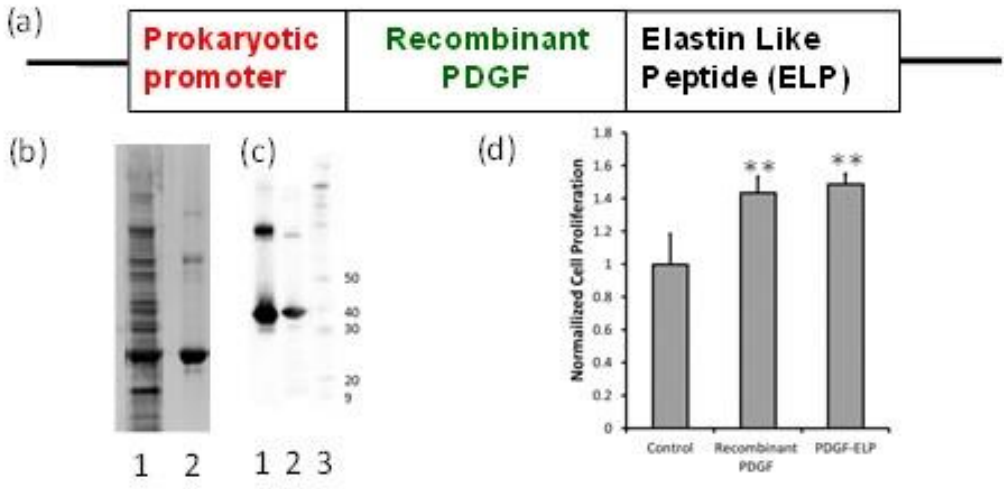

Figure 4.2 Generation of PDGF-ELP fusion peptides. (a) Gene encoding PDGF was cloned in frame with the gene encoding for the elastin cassette. (b) The indicated samples were run on a SDS PAGE gel and stained with simply safe blue stain. Lane 1– Bacterial Lysate, Lane 2 – Purified PDGF-ELP. (c) The corresponding gel was then subjected to western blot with a monoclonal PDGF antibody. Lane 1 – Bacterial Lysate, Lane 2 – Purified KGF-ELP, Lane 3 – Ladder. (d) PDGF-ELP induced proliferation of human fibroblasts similar to recombinant PDGF. ** = $P<0.05$ when compared to control (n=5).

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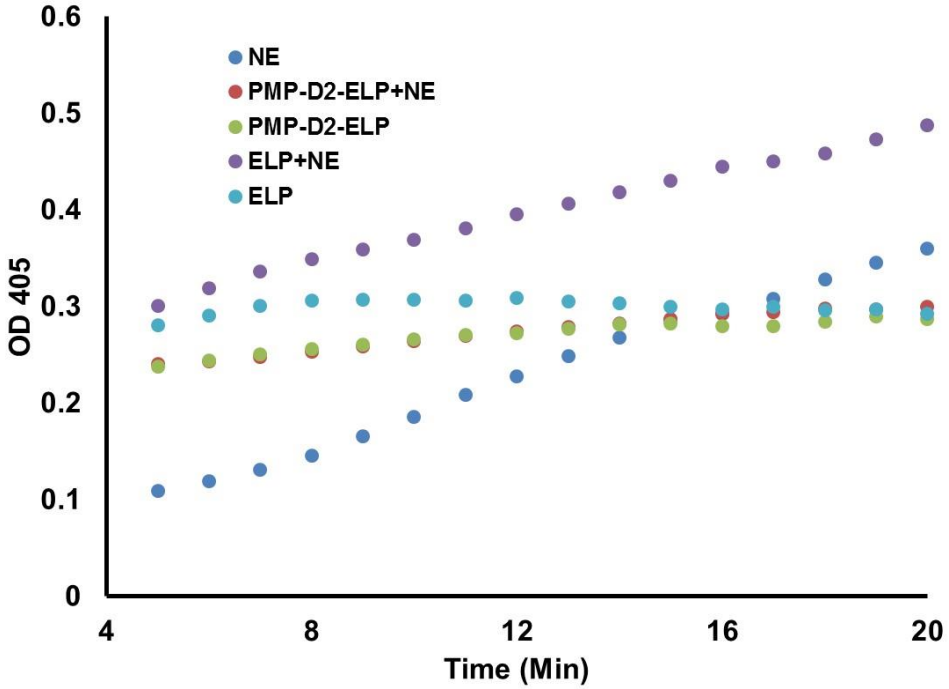

Figure 4.3 PMP-D2-ELP fusion protein retained PMP-D2 variant bioactivity. Elastase (2mU/µl) was diluted 1/90 in assay buffer for the experiment. Absorbance at 405 was read every minute from 0 to 20 minutes for each sample.

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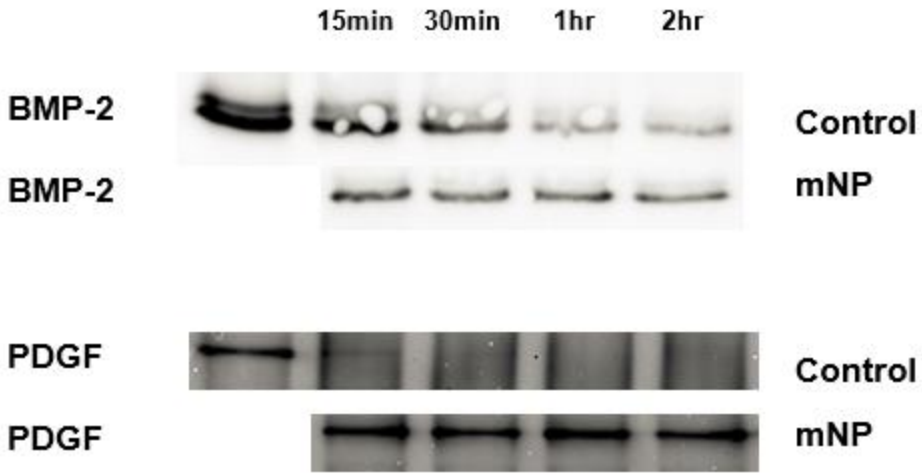

Figure 4.4 Western blot shows mNPs preserved growth factors from degradation. Growth factor ELP fusion proteins (PDGF-ELP and BMP2-ELP) were dissolved in PBS to reach a concentration of 1mg/ml. They either mix with 0.2mg/ml of PMP-D2-ELP to form multifunctional nanoparticles then treated with 20mU/ml elastase or directly treated with 20mU/ml elastase for up to 2 hours. At time point 15 minutes, 30 minutes, 1 hour, and 2 hours, we collected the sample for each treatment and prepare for western blot analysis.

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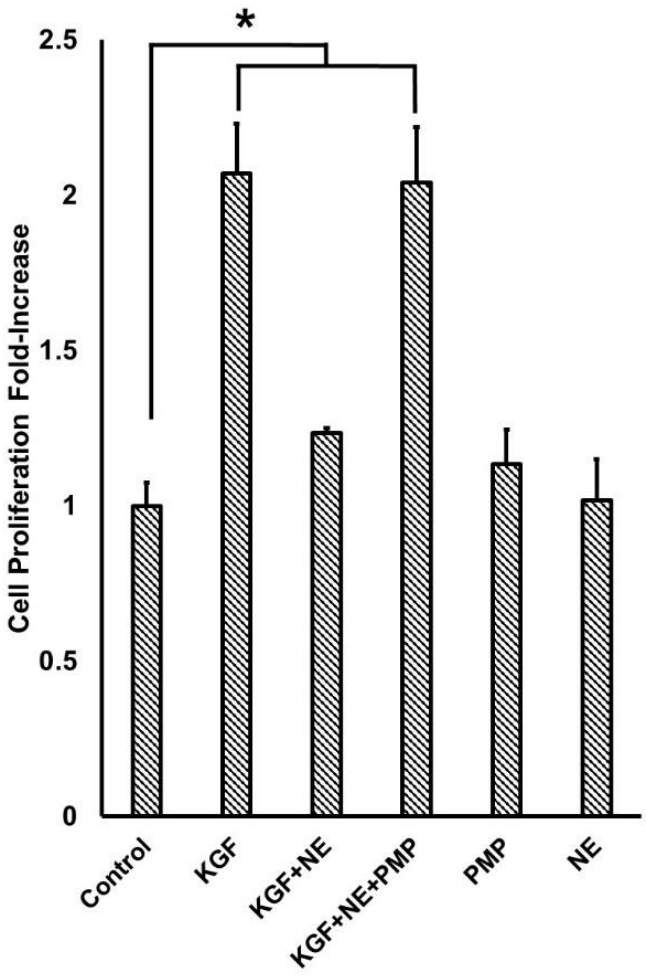

Figure 4.5(a) mNPs retained KGF proliferative activity. 20,000 cells were plated in 48-well plate and were allowed to attach for 24 hours. After 24 hours, the cells were serum starved for another 24 hours followed by treatment with PDGF-ELP fusion proteins for 48 hours. New DNA synthesis was quantified using the BrdU cell proliferation kit.

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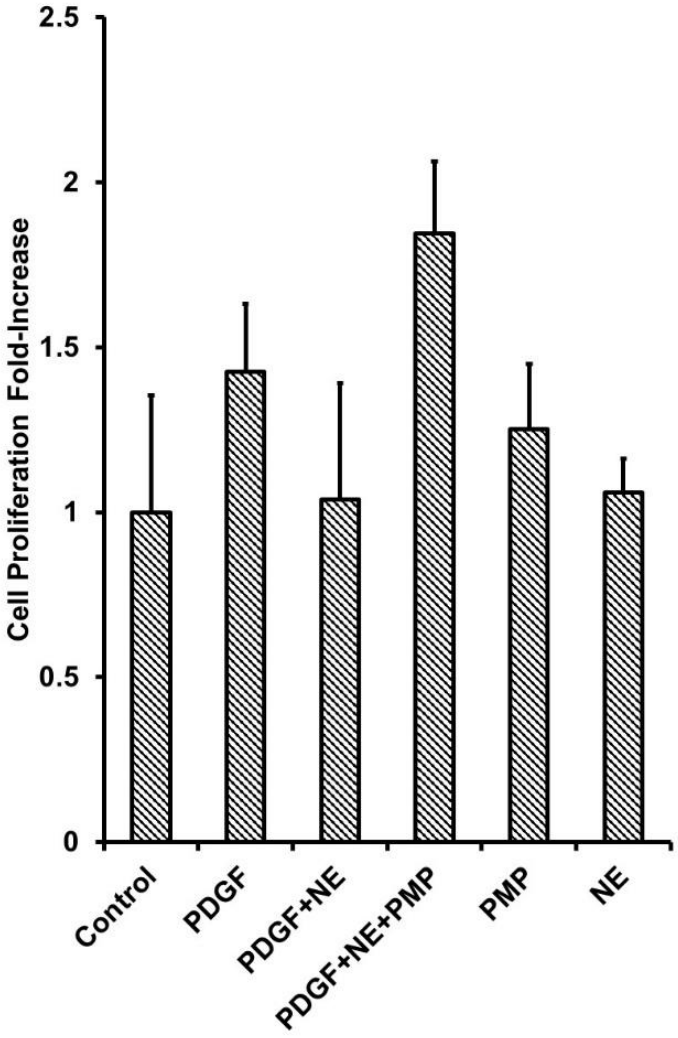

Figure 4.5(b) mNPs retained PDGF proliferative activity. 20,000 cells were plated in 48-well plate and were allowed to attach for 24 hours. After 24 hours, the cells were serum starved for another 24 hours followed by treatment with PDGF-ELP fusion proteins for 48 hours. New DNA synthesis was quantified using the BrdU cell proliferation kit.

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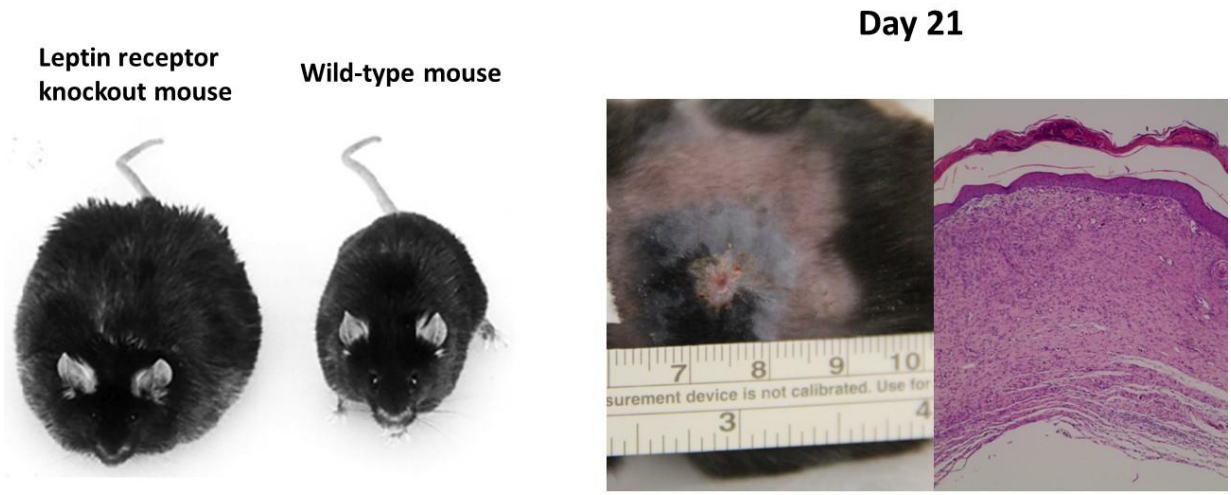

Figure 4.6 Type II diabetes mice model (Leptin knockout).

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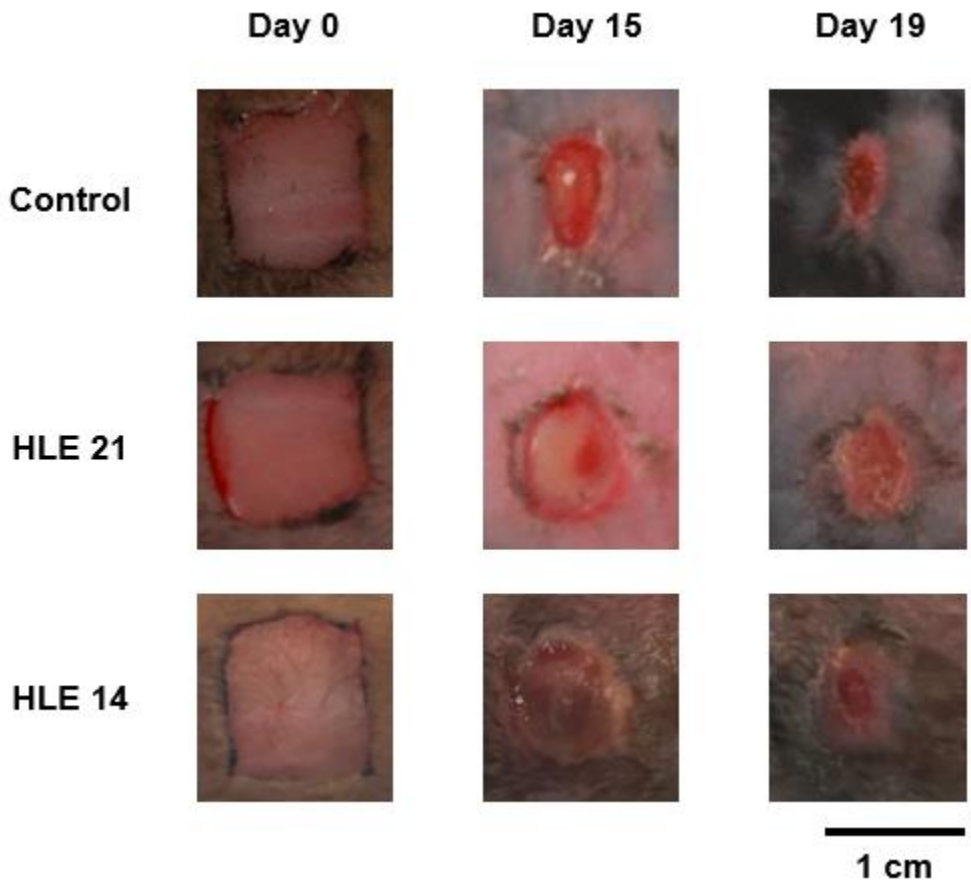

Figure 4.7(a) Elastase treatment delayed the healing process in diabetic mice wounds. 1 cm ×1 cm full thickness wound was created on the dorsal area of the mouse. Elastase was treated on the wound with fibrin gel for either 14 days or 21 days.

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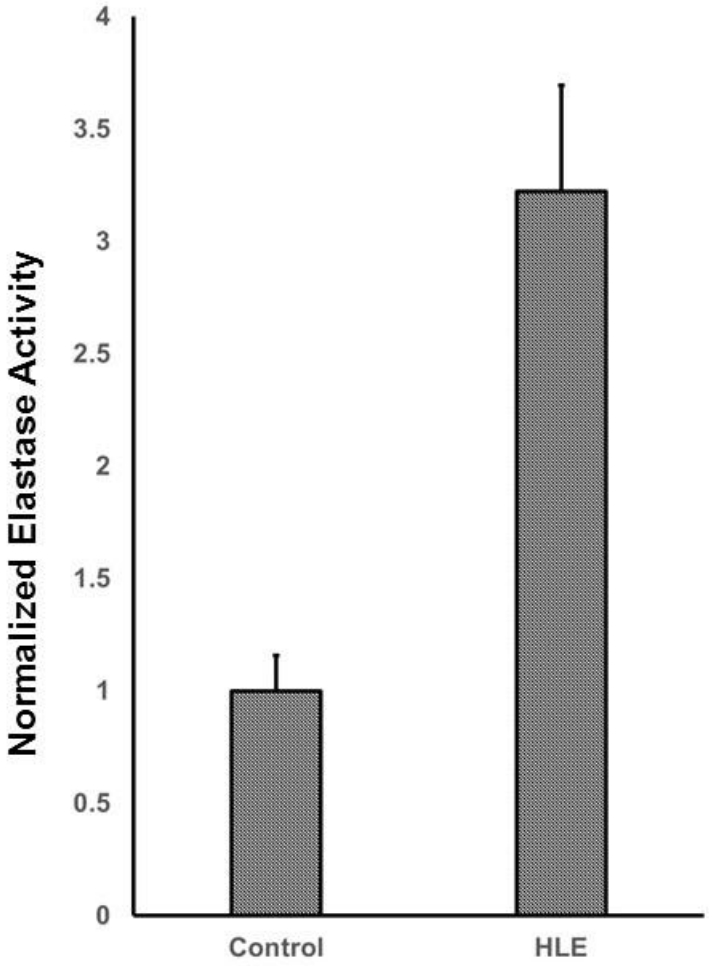

Figure 4.7(b) Elevated HNE levels block healing of diabetic wounds. Full thickness wounds were treated either with vehicle (control) or with HNE (0.5 U/ml) every day. The HNE activity was measured in the wound fluid using HNE substrate MeOSuc-AAPV-pNA. The HNE activity was normalized to control. Two individual measurements of protease activity are shown.

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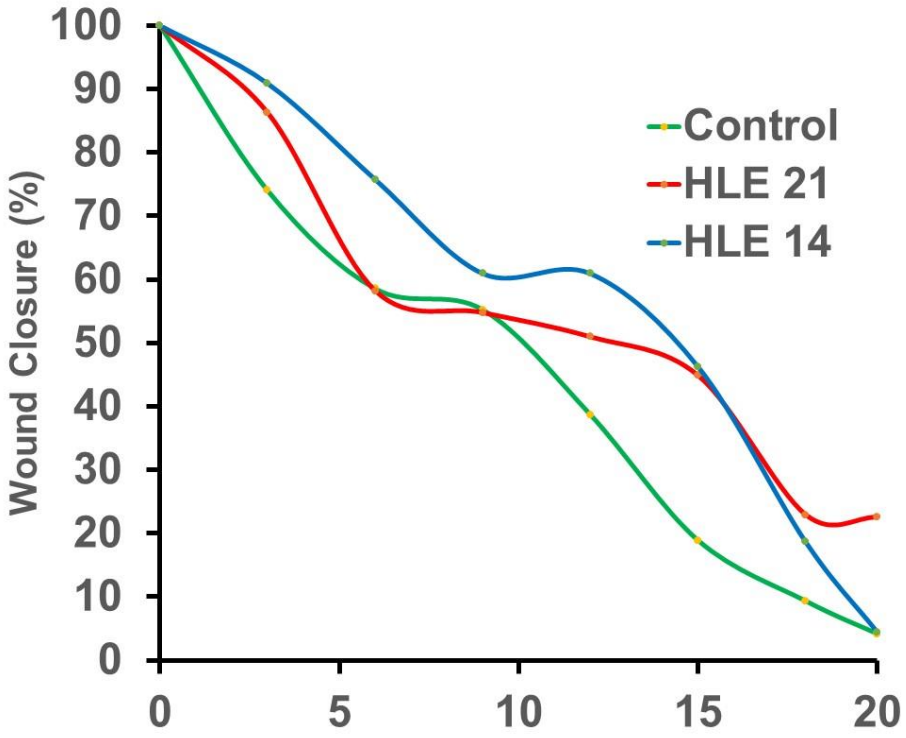

Figure 4.7(c) Quantification of wound closure. Pictures of wounds were taken every other day. Area of the wounds was analysed using imageJ.

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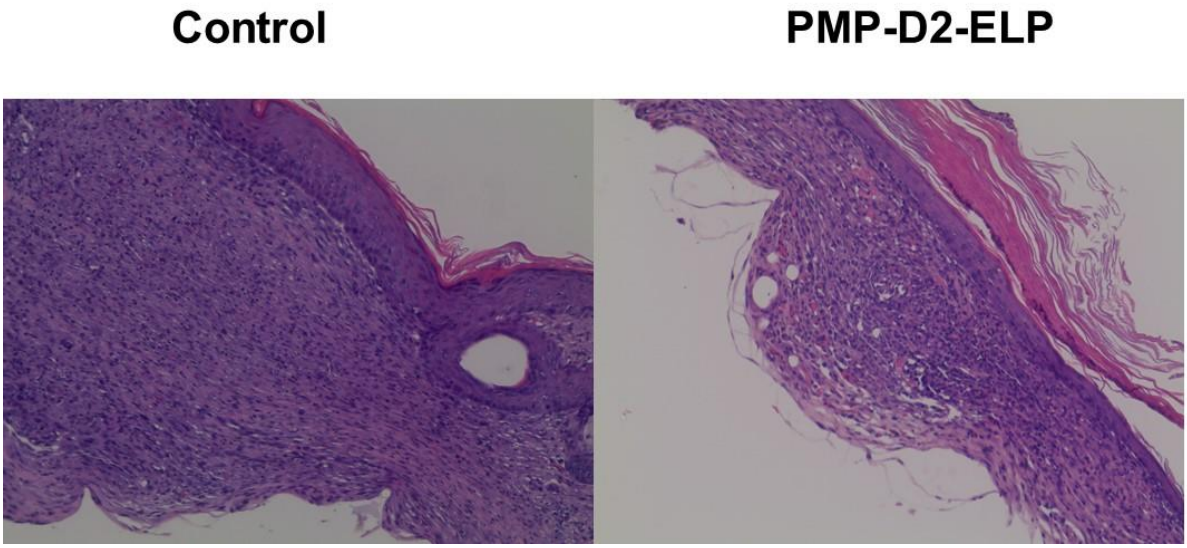

Figure 4.8 Histology of PMP-D2-ELP on diabetic mice wound showed that it didn't affect the healing process.

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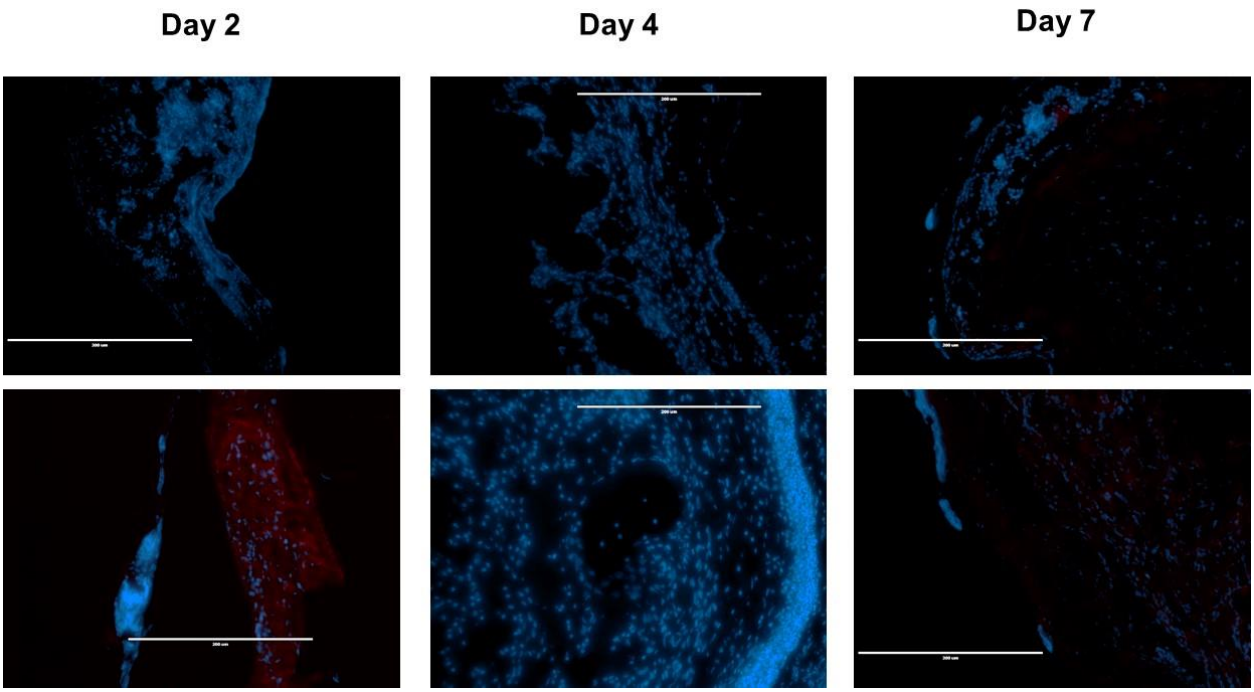

Figure 4.9 ELP stays in diabetic mice wounds for 2 days. Full thickness wounds were treated biotin labeled ELP. Mice were euthenized at day 2, 4, and 7 for histology study. Backgroud cells were stained with hoechst dye.

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CHAPTER 5: SUMMARY AND CONCLUSIONS

5.1 Summary and Conclusions

In this dissertation, I have shown that the regulation of the proliferative activity of ELP on fibroblasts through the manipulation of phase transition and its application in chronic wound healing. An interesting property of ELPs is their ability to undergo phase transition at transition temperatures. When the temperature is below their inverse transition temperature, also known as the lower critical solution temperature (LCST), they assume a random coil structure and are soluble in aqueous solution. However, at temperatures higher than the LCST, ELPs undergo an entropy driven self-assembly rendering them insoluble. This physical property of ELP are dependent on the chain length and the chosen amino acid at the guest residue position. In our study, we investigated the potential bioactivity of ELP that could be beneficial for tissue regeneration. Indeed, we found out that ELP induces fibroblasts proliferation through interaction with HSPG, which is a complete new mechanism comparing to Elastin receptor for EDP-cell interaction. We further found out that the ELP fibroblast interaction is driven by the secondary structure change which is caused by phase transition. Consequently, we are able to control this proliferative activity the same way as we regulating ELP physical property, through the chain length and guest residues. Previous studies have shown that growth factor ELP fusion proteins not only could retain their phase transition property but also they are able to preserve the biological activity of the growth factors. However in chronic wound treatment, a delivery method is needed for growth factors to stay active in the presence of excessive protease. Here, we have created PMP-D2-ELP fusion protein which

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can form heterogeneous multifunctional nanoparticles when mixed with other growth factor ELP fusion proteins. The inhibition activity of PMP-D2 allow growth factors retain their activity in the presence of elastase. Also we have developed a modified animal chronic wound model represents a quick and easy approach to re-create the HNE levels similar to human chronic wounds and block healing and can be used as an initial screening tool to test the efficacy of the proposed multifunctional NPs.

In chapter 2, we have demonstrated ELP induces the proliferation and mmp-1 expression of fibroblasts. Further we showed that these two functions of ELP were achieved through different interaction mechanism. ELP induced MMP-1 expression is through binding with elastin receptor complex similar to EDP-cell interaction; while ELP induced proliferation is close related to its phase transition property and is through HSPG. Crosslinking reversed the induced proliferation suggesting that the structure change associated with phase transition is crucial to this proliferative effect.

In chapter 3, our work demonstrates that the biological activity of ELPs can be modulated by simply tweaking their physical properties. Specifically, we show for the first time that ELPs induce fibroblast proliferation which is dependent on cell surface HSPGs. We further show that this interaction can be modulated by designing ELP sequences having different phase transition behavior or charge; thereby controlling the mitogenic activity of ELPs. Fibroblast proliferation and function is essential for granulation tissue formation in not only chronic wound healing but in healing of injuries of other tissues such as bone or neural tissue. Thus, ELP based materials with differential proliferative activity on fibroblasts can be designed and will have broad application in the development of therapeutics for tissue regeneration.

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In chapter 4, we developed a multifunctional nanoparticle that comprised of PMP-D2-ELP fusion protein and different functional peptide ELP fusion proteins to preserve the bioactivity of the functional group with the existence of elastase. These heterogeneous particles will be beneficial for the delivery of combination therapy to solve multiple problems that often existed in chronic wound healing or other tissue regeneration process. We also tested a new animal chronic wound model by increasing the local elastase concentration to mimic human chronic wound. The healing process has been delayed significantly. The development of this chronic wound model may help the translation of therapeutics that tested in animal research to clinical approved drug.

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APPENDICES

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Appendix A: List of Abbreviations

7-AAD	7-Aminoactinomycin D
AGRP	Agouti related peptide
AKT	Protein kinase B
ANOVA	Analysis of variance
BMP-2	Bone morphogenetic protein 2
Brdu	Bromodeoxyuridine
cDNA	Complimentary Deoxyribonucleic acid
DI Water	Deionized water
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EBP	Elastin binding protein
ECM	Extracellular matrix
EDP	Elastin derived peptides
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
ELP	Elastin like peptides
ERC	Elastin receptor complex
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor

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FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly	Glycine
H_2O_2	Hydrogen peroxide
HLE	Human leukocyte elastase
HSPG	Heparan sulfate proteoglycan
IGF	Insulin-like growth factor
ITC	Inverse temperature cycling
KCPS	Kilo count per second
KDa	Kilo Dalton
KGF	Keratinocyte growth factor
LCST	Lower critical solution temperature
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
mNP	Multifunctional nanoparticle
NOD	Non-obese Diabetes
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Poly(ethylene glycol)
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C

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Pro	Proline
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real time-Polymerase chain reaction
SEM	Standard error of the mean
SDC	Syndecan
SDS	Sodium dodecyl sulfate
TIMP-1	Tissue inhibitor of metalloproteinase
Tt	Transition temperature
Val	Valine
VEGF	Vascular endothelial growth factor

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