THE UNIVERSITY OF CHICAGO

ENGINEERING FIBRIN BIOMATERIALS WITH A2-ANTIPLASMIN AND GROWTH FACTORS IN REGENERATIVE MEDICINE

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路漫漫其修远兮，吾将上下而求索

- 屈原

Long, long had been my road and far, far was the journey;

I would go up and down to seek my heart's desire

- Qu Yuan

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THESIS ABSTRACT

Fibrin is a natural biomaterial that plays an essential role in hemostasis upon injury, as being the main component of the blood clot. In the clinic, fibrin is widely used in regenerative medicine as a hemostatic agent to prevent blood leakage and as a sealant agent to glue tissues. In addition, fibrin-based biomaterials have been extensively explored as delivery carriers for therapeutics, including growth factors (GFs), to improve tissue regeneration. In these applications, premature degradation of fibrin, called fibrinolysis, leads to recurrent bleeding, tissue dehiscence and limited regenerative efficacy, which strongly hindered the potential of fibrin. Physiologically, fibrinolysis is slowed by α2-antiplasmin (α2PI), a protease inhibitor that crosslinks into fibrin during polymerization and prevent proteolytic degradation by plasmin.

In this thesis, we first engineered fibrin biomaterials with human α2PI to protect them from fibrinolysis. We highlighted the clinical translation potential of α2PI as a recombinant protein drug, as well as its superiority as compared to the current clinical drug aprotinin, a bovine-derived fibrinolysis inhibitor. We additionally showed that α2PI can protect fibrin materials made of low fibrinogen concentration, which could substantially reduce their cost and manufacturing challenges. Moreover, this could further support the development of patient-derived fibrin biomaterials using unpurified plasma, in which fibrinogen concentration is naturally low as compared to the clinical fibrinogen. Indeed, we then demonstrated that α2PI can be used to effectively prevent fibrinolysis of endogenous fibrin in healthy and diabetic conditions.

We particularly explored the use of α2PI-engineered fibrin as a carrier material for the delivery of growth factors in skin diabetic wound healing. We found that α2PI co-delivered with engineered variants of the vascular endothelial growth factor-A (VEGF-

A) and the platelet-derived growth factor-BB (PDGF-BB) in exogenous fibrin or directly from the endogenous fibrin clot can significantly improve the healing of diabetic wounds, despite their high proteolytic environment. In addition of these two GFs, we aimed to engineered a potent chemokine called the stromal-derived factor-1 (SDF-1), broadly involved in tissue regeneration and blood vessel formation, for increased affinity to fibrin and other extracellular matrix proteins. We found that fibrin-mediated delivery of SDF-1 increased angiogenesis in diabetic wounds, although the chemokine remained challenging to engineer and produce.

As another application of endogenous fibrin protection, we evaluated the hemostatic efficacy of α2PI to reduce bleeding during surgery. Indeed, the excessive activity of plasmin during coronary artery bypass graft surgery (CABG) is known to lead to excess of blood loss which increase the need of patient blood transfusion. We here proved that α2PI was able to reduce bleeding time and volume as effectively as the clinical aprotinin. Based on these findings, we believe that engineering of fibrin biomaterials and endogenous fibrin with α2PI and growth factors can have a strong impact in regenerative medicine, leading to improvements in the clinical use of fibrin.

Keywords:

Fibrin biomaterials, Protease inhibitors, α2-antiplasmin, Diabetic wound healing, Growth factors, Hemostasis.

I. INTRODUCTION

I. 1. Thesis Motivations

Regenerative medicine focuses on developing methods for re-growing, replacing and repairing tissues, cells or organs that are damaged or diseased [1]. Wound healing and hemostasis remains two important current challenges in regenerative medicine, particularly upon surgery and in patients with pre-existing conditions such as diabetes, which have poor healing capabilities. Indeed, chronic wounds, which are wounds that do not heal in less than 3 months, are one of the most serious complications of diabetes and impact about 25% of diabetic patients [2]. On the other hand, hemostatic dysregulation can lead to severe hemorrhages during surgery and increase the need of patient blood transfusion.

In these two challenges, fibrin has a key role to play. Fibrin is the main component of the blood clot, which stop bleeding and control the initial steps of tissue regeneration upon wounding[3]. Therefore, prematured degradation of fibrin impairs hemostasis and wound healing. Fibrin is physiologically degraded by plasmin, which level and activity have been shown to be increased during certain types of surgery, such as in coronary artery bypass graft (CABG) surgeries [4], as well as in diabetic wounds [5][6][7]. In addition, the highly proteolytic environment of diabetic wounds further degrades the extracellular matrix (ECM) and the growth factors (GFs), both important to orchestrate proper tissue repair [5][6][7].

Fibrin is also commonly as a biomaterial in regenerative medicine. Fibrin sealant, composed of fibrinogen and thrombin, works as an adhesive, sealant, and hemostat [8]. Approved by FDA in 1998, they are now available clinically both as patch and glue [9]. In these clinical products as well, premature degradation of fibrin reduces its efficacy and leads to tissue dehiscence, recurrent bleeding and poor regeneration.

I. 2. Thesis Aims and Structure

As many challenges of fibrin biomaterials in regenerative medicine are associated with the premature fibrinolysis and the subsequent lack of controlled release of growth factors, in this thesis, we aimed to engineer fibrin biomaterials with fibrinolysis inhibitor and growth factors. We particularly hypothesized that incorporating a fibrinolysis inhibitor into fibrin during polymerization would slow its degradation rate upon protease exposure, thus resulting in increased performance of fibrin biomaterials. In addition, we asked how to effectively supplementing fibrin with biomolecules that promote tissue regeneration to enhance its regenerative potential and its use as a carrier biomaterial for drug delivery. Therefore, this thesis aimed to improve the efficacy and regenerative properties of fibrin biomaterials for clinical applications in hemostasis and wound healing.

In **Chapter II**, we provided background information relevant to this thesis. We first introduced fibrin as a physiological biomaterial, covering its role in wound healing and hemostasis, and how it serves as a natural reservoir to control the delivery of growth factors. Next, we focused on the clinical use of fibrin biomaterials in regenerative medicine, including its applications as tissue sealant and as carrier vehicle for growth

factor delivery. Lastly, we present the current challenges and limitation of clinically used fibrin, and the techniques attempted to enhance fibrin accordingly.

In **Chapter III**, we extensively characterized the efficacy of α2PI in protecting fibrin from plasmin-mediated degradation. We found that α2PI was able to resist fibrinolysis effectively both *in vitro* and *in vivo* and outperformed the current clinical fibrinolysis inhibitor aprotinin. In addition, we highlighted that α2PI allowed protection of fibrin biomaterials made of low fibrinogen concentration. Lastly, we showed that α2PI was able to stabilize fibrin gels for growth factor delivery in diabetic wound healing, which improved tissue regeneration.

In **Chapter IV**, we reasoned that α2PI can be used to protect endogenous fibrin, and studied therapeutic use of α2PI for both diabetic wound healing and hemostasis. We proved that α2PI was able to protect endogenous fibrin effectively. We then developed appropriate models and showed that α2PI improved diabetic wound healing in a carrier-free growth factor delivery system and reduce bleeding during surgery.

In **Chapter V**, we focused on the engineering of the SDF-1, a chemokine that has been shown to enhance tissue regeneration by recruiting endothelial progenitor cells and mesenchymal stem cells, aiming at incorporating it into fibrin to enhance diabetic wound healing. We found that engineering SDF-1 was particularly challenging in terms of protein production and purification, yet that SDF-1 modulated angiogenesis in diabetic wounds upon delivery via fibrin biomaterials.

In **Chapter VI**, we summarize the main conclusions of the thesis and discuss its significance, limitations and future perspectives toward the clinical translation of fibrin biomaterials engineered with α2PI and growth factors.

I. 3. References

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II. GENERAL BACKGROUND

In this Chapter, we focus on fibrin biomaterials, first as a physiological material naturally produced by the body and its role in hemostasis and wound healing, and then as a clinical biomaterial, highlighting its applications and current limitations.

II. 1. Fibrin as a provisional matrix during wound healing

II.1.1. Fibrin as a physiological biomaterial

Fibrinogen is a fibrous soluble protein primarily synthesized from liver and secreted in the blood, with concentration of 2-4 g/L in human plasma [1]. Fibrinogen is the precursor of fibrin, an insoluble crosslinked matrix that is the main component of blood clots. Fibrin is involved in many physiological functions, such as hemostasis, wound healing, inflammation, and angiogenesis [2]. For example, upon blood vessel injury, fibrin helps aggregate platelets and forms clots to reduce bleeding. In addition, in wound healing, fibrin provides a provisional scaffold for cell migration and tissue growth. Lastly, fibrin can also prevent microbial invasion and proliferation by creating a mesh into the wound [3].

Upon injury or trauma, concentration of fibrinogen in plasma increases to as high as over 7 g/L as an acute-phase protein, and fibrinogen converts to fibrin clots via catalyzation of thrombin[4]. In this conversion, the peptides in fibrinogen are first cleaved by thrombin to generate fibrin monomers. The monomers then self-assemble and aggregate to form clots, and the clots are further stabilized via covalent crosslinking by FXIIIA (Figure 1).

Formed fibrin clots can be degraded by proteases in a process called fibrinolysis. The main enzyme responsible for fibrin degradation is plasmin, a serine protease activated in tissues from its precursor plasminogen, present in the blood, by the tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Figure 1).

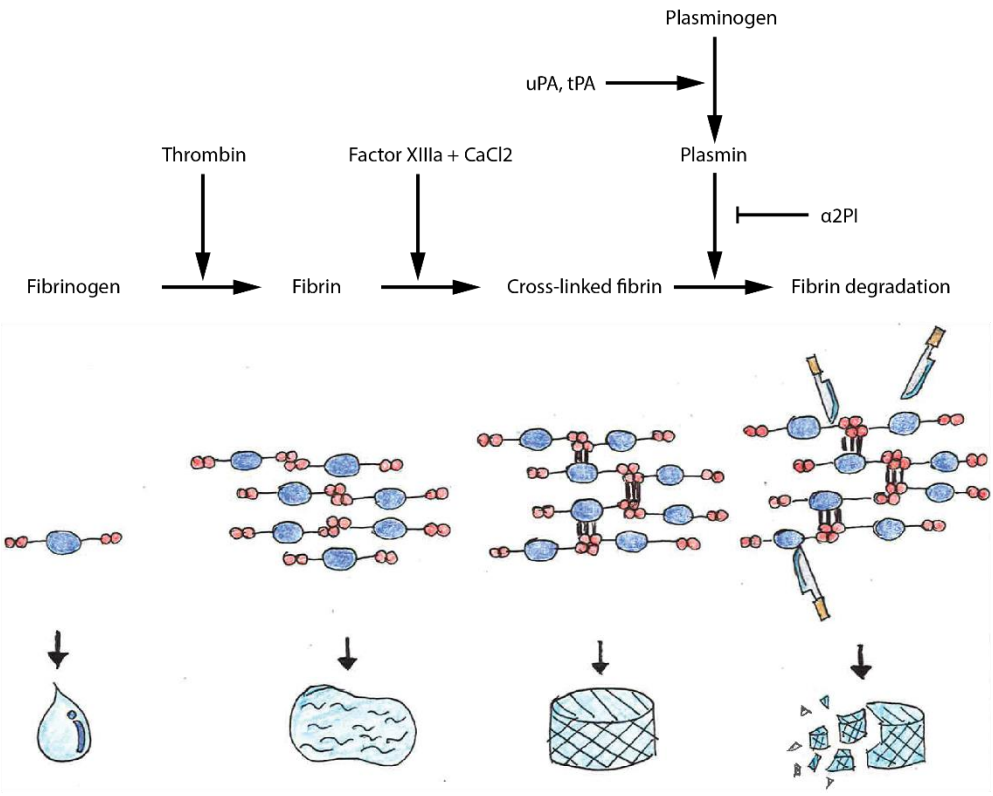

Fig 1. **Formation and degradation of fibrin.** Enzymatic activities both lead to fibrin formation and degradation. With addition of thrombin, fibrinogen assembles and forms oligomers of fibrin. Fibrin oligomers then crosslink under the activity of Factor XIIIa and $CaCl_2$ to form an insoluble fibrin matrix. Upon activation by tPA or uPA, plasminogen converts to plasmin, which degrades the fibrin. Plasmin can be inhibited by α2PI to regulate the degradation rate of fibrin.

The equilibrium of fibrin formation and fibrinolysis is crucial for maintaining normal physiological functions: excess fibrinolysis can cause bleeding, while reduced fibrinolysis lead to thrombosis, which is the formation of clots in blood vessels, either cases could be fatal [2]. Besides, the abnormal regulation of fibrinogen closely relates to various diseases. For example, patients with promyelocytic leukemia or liver diseases often have low fibrinogen level, leading to bleeding disorders [5][6]. In addition, some cancer cells produce fibrinogen, and the excess fibrinogen binds to growth factors, provides matrix for cancer cell adhesion and migration, and protect cancer cells from immune surveillance, leading to overall poor survival of patients [7][8]. Fibrinogen is also highly correlated to diabetes, in which correlations have been found between high fibrinogen level and low insulin level [9]. Fibrinogen from diabetes patients also forms denser clots more resistant to fibrinolysis [10]. On the other hand, diabetic patients have also been shown to have increased level of proteases, such as a higher plasmin activity that overall dysregulates this fibrinolysis balance[11][12].

Therefore, fibrin is a double-sided sword for health. The appropriate level of fibrinogen and balance between fibrin formation and fibrinolysis is the key for maintaining normal physiological activities.

II.1.2. Fibrin in hemostasis

Endogenous fibrin also has significant effects in hemostasis. The equilibrium between endogenous fibrin formation and fibrinolysis regulates the coagulation process [13]. Studies showed that fibrinogen knock-out mice developed over-bleeding event after birth, of which female mice all underwent fatal uterine bleeding during pregnancy, implying the importance of fibrinogen in hemostasis [14].

In the hemostasis process, fibrinogen can link platelets via binding to integrin αIIbβ3, a receptor on platelets, leading to aggregation of platelets at injury site to stop bleeding [2][15]. Fibrinogen in plasma also converts to fibrin clots, and the fibrin connects platelets, plasma proteins and red blood cells to form hemostatic plug and seal the injured blood vessel, which leads to coagulation of the blood [16][4]. Another role fibrin plays in hemostasis is blood clot retraction, through which blood clots shrink and draw injured tissue together for reparation of the damaged blood vessel. In the blood clot retraction process, fibrin interacts with myosin cytoskeleton of platelets, and the platelets transduce contractile force on fibrin, leading to the retraction [17]. Both the polymerized fibrin clots and the fibrinogen are critical to hemostasis. Previous research showed that pathological bleeding events occur in transgenic mice whose fibrinogen was not able to be cleave by thrombin, indicating that soluble fibrinogen only is not enough for the hemostasis process [18].

Many quantitative and qualitative changes in fibrinogen or relative enzymes can lead to bleeding disorders. Decrease or absence of plasma fibrinogen causes afibrinogenemia or hypofibrinogenemia; abnormality of fibrinogen leads to defective fibrin formation and causes dysfibrinogenemica; impaired thrombin gene causes Hemophilia A and B [3][19]. These bleeding symptoms for these disorders can be mild or severe, but all lead to abnormal bleeding in various physiological activities. Factor XIII deficiency is another bleeding disorder, rare to occur but usually causes serious bleeding [19]. The fibrin formation related bleeding disorders can be inherited or acquired: for example, mutations in genes can cause these disorders, as can do liver disease, leukemia, or medications impairing liver proteins [3].

Overall, fibrinogen and fibrin formation are essential for hemostasis. Pathological change impairing fibrin formation can leads to abnormal bleeding. Thus, enabling appropriate fibrin formation would be one direction of hemostatic drug.

II.1.3. Fibrin matrix during wound healing

Fibrin plays an important role in wound healing. Literature shows that in fibrinogen deficient wounds, granulation tissue formation is not adequate for closing wound gap, and cells are not able to organize and migrate properly in the wounds [20].

In the wound healing process, fibrin provides the initial matrix to stabilize wound fields, and is able to protect the tissue from external infections and support proliferation and migration of cells to regenerate the tissue [20]. Particularly, fibrin is able to promote deposition and migration of monocytes, macrophages, endothelial cells and fibroblasts around wound site through integrin receptors of the cells [3][21]. These cells clean the wound site, decrease inflammation, generate extracellular matrix and collagen networks, promote vascularization, and help the wound contract and remodel.

Besides promoting cell migration, fibrin is also able to bind growth factors and release them in a spatio-temporal controlled way to promote tissue healing [22]. Many growth factors have been shown to be involved for angiogenesis, such as the vascular endothelial growth factor-A (VEGF-A), the fibroblast growth factors (FGFs) or the platelet-derived growth factor-BB (PDGF-BB). Angiogenesis in wounds is essential for expanding blood supply in the wounded tissue to bring oxygen and nutrients, to increase clearance of the damaged tissue metabolic waste and to promote cell recruitment and circulation, all of these mechanisms favoring a good healing process. Fibrin is also able to activate neutrophil in wound healing process. The fibrinopeptides cleaved from fibrinogen by

thrombin and the degradation product of fibrin by plasmin can work as chemoattractants to activate neutrophils, which decontaminate the wound to prevent infection [23][24].

The clearance of fibrin clot from the wound is also crucial for wound healing. In the wound, the fibrin clots are degraded by plasmin, activated from plasminogen. The appropriate fibrinolysis can facilitate further remodeling of the wounds and the migration of keratinocytes, which are in charge of epithelization of the wounds [25]. Previous research showed that plasminogen deficient mice showed delayed wound healing and prolong inflammation stage after wounding, implying the importance of appropriate fibrinolysis in the wound healing process [25].

Therefore, endogenous fibrin is essential for wound healing. Proper equilibrium of fibrin formation and fibrin degradation is crucial for the wound healing process. Imbalance in either direction could lead to delayed wound healing.

II.1.4. Fibrin as a reservoir of growth factors

Growth factors are proteins with effects on cell proliferation, migration, differentiation, adhesion or gene expression, thus playing very important roles during wound healing. Some examples of growth factors commonly involved in wound healing include VEGF-A, PDGF-BB, FGFs. These growth factors have different functions in the wound healing process. VEGF-A is able to induce vasodilation and vascular permeability, degradation of basement membrane, and endothelial cell migration and proliferation, and thus promotes angiogenesis and favors epithelization of wounds [26]. PDGF is able to improve angiogenesis by attracting mesenchymal cells and regulating their cell growth and division [27]. FGFs stimulate the migration of proliferation of many cell types in the wound healing process, including endothelial cells, fibroblasts, keratinocytes and epithelial cells [28]. FGFs are also able to promote collagen synthesis, epithelization and

fibronectin synthesis [28]. Besides, stromal derived factor-1 (SDF-1), a chemokine in charge of recruiting cells and enhancing tissue growth is also involved in the wound healing process. SDF-1 has effects in recruiting endothelial progenitor cells (EPCs), epithelial cells, mesenchymal stem cells (MSCs), and is able to promote re-epithelialization and revascularization of the wounds[29][30]

Fibrin also serves as reservoir for growth factors, especially some angiogenic growth factors, such as FGF-2, PDGF and VEGF [31][32][33][22]. Fibrin binds to these growth factors via its heparin binding domain[34]. Through this binding, growth factors are able to retain in fibrin and gradually release into the extracellular matrix during wound healing, with a proper biochemical gradient supporting the angiogenesis process [34]. Growth factors are secreted in the wound by infiltrated cells or released upon platelets degranulation, after what they bind to the fibrin clot. Upon binding, the growth factors are sequentially released depending on their specific affinity for fibrin to the recipient cells in a sustainable way, to efficiently promote tissue healing. The growth factors retained in fibrin clots bind to ECM proteins during the regeneration process, who further bridges cells to fibrin, supporting healing of the wounds [35].

Therefore, fibrin has excellent capability in binding with many growth factors and serves as reservoir for them, controlling their release kinetics to regulate their effects on cells. This function is not only important for wound healing, but also highlights the potential of fibrin as a biomaterial delivering growth factors.

II. 2. Fibrin biomaterials in regenerative medicine

II.2.1. Fibrin as a tissue sealant agent

Mimicking the physiological role of fibrin clots, fibrin sealant have been developed as a two-component material made by fibrinogen mixed with thrombin, and works as a hemostat, sealant, and tissue adhesive [36]. Factor XIII is also added to fibrin sealant sometimes for improving cross-linking and clot-strength[37][38]. Fibrin sealant can both be in liquid formulation as glue and in patch form [39]. It creates a clot to seal tissues and degrades naturally over a few days [40].

Fibrin sealant has application in many different areas. First, fibrin can be used as a hemostat in surgeries to stop bleeding, such as in cardiovascular surgeries, in thoracic surgeries, in neurosurgeries, and in plastic and reconstructive surgeries [41][37]. Secondly, fibrin can be used as adhesive to reattach tissue, such as cartilage, nerve, mesh, dermis; it can be applied in skin graft and facial flap attachment [42]. Lastly, fibrin can be used as a sealant to create a barrier for leakage prevention, for example, colon sealing [42]. It is reported that clinicians are more satisfied with fibrin sealants than the standard of care in ease to use, product properties and efficiency [36].

Liquid formulation of fibrin sealant was initially approved by the U.S. Food and Drug Administration (FDA) in 1998, and fibrin sealant as patch was approved in 2010 [39]. Currently, there are 7 commercially available fibrin sealants approved by the FDA: 5 of them are liquid form for topical use (Artiss, Evicel, Fibrin Sealant (Human), Raplixa, Tisseel), while 2 of the approved fibrin sealant products are absorbable patch (Evarrest (Fibrin Sealant Patch), TachoSil). Most of these products are approved for usage as hemostat, with a few others approved for adhesive or sealant.

The risks associated with fibrin sealants are mainly caused by the origin of fibrinogen combined from multiple plasma donors, which potentially cause transmission of blood borne viral diseases and prion diseases [39]. Other risks could be associates with the usage of bovine thrombin in some fibrin sealant products, which could lead to immunogenic response. Moreover, fibrin sealants need to be handled with caution to avoid them from entering cell saver and cardiopulmonary bypass circuit due to the presence of thrombin, which can causes fatal thrombosis [43].

Besides its utilization in fibrin sealants, another application of fibrin in hemostasis is fibrinogen infusion. This method is usually applied in coronary artery bypass graft (CABG) surgery for patients with low plasma fibrinogen level (<3.8 g/L) [44].

Overall fibrin is widely used clinically as hemostat, adhesive and sealant. To further improve the safety and manufacturing of fibrin-based products, utilization of low dosage of fibrinogen or recombinantly expressed fibrinogen and thrombin could be considered.

II.2.2. Fibrin as a carrier vehicle for growth factor delivery

Besides clinical usage, fibrin sealants are also used in some pre-clinical research studies for tissue engineering and drug delivery purpose [42]. Particularly, fibrin biomaterials have been widely explored for GF delivery. They are appealing biomaterials due to their biocompatibility, controllable degradability, and ability to deliver cells and biomolecules via the presence of multiple binding sites. Moreover, as the fibrinogen and thrombin are both naturally secreted in human blood, fibrin also shows potential in being an autologous scaffold derived from the patients [45].

Fibrin biomaterials properties can be tuned to tailor growth factor loading and release profile [41]. Commonly, GFs can be passively released by diffusion from the biomaterial or actively released based on specific interactions with fibrin and upon material

degradation. There are multiple strategies that have been developed to immobilize GFs into fibrin biomaterials. Particularly, GF retention can be achieved via physical-chemical interactions with the material, by physical encapsulation, adsorption or electrostatic interactions [46]. For example, GFs delivery via fibrin biomaterials has been achieved by the fabrication of GFs loaded gelatin microspheres entrapped in fibrin [45][47]. In addition, GFs can be incorporated into fibrin via covalent conjugation using chemical or enzymatic-based crosslinking, or via non-covalent interactions when the GF has a natural or engineered specific affinity for the biomaterial. Previously, our laboratory have developed many technologies to improve the delivery of GFs from fibrin, resulting in enhancement of the GFs efficacy on tissue healing. For example, we found that fibrin is able to bind to multiple growth factors via its heparin-binding domain, particularly to PlGF-2, PDGF-BB, FGF-2 and BMP-2 [34]. Besides utilizing the native growth factor binding capabilities of fibrin, we engineered GFs for covalent incorporation into fibrin by fusing them to a transglutaminase (TG) substrate domain derived from the human α2-antiplasmin ($\alpha 2PI_{1\text{-}8}$), which crosslink into fibrin upon factor XIIIa activity. This strategy has been shown successful for the engineering of VEGF-A, PDGF-BB and IGF-1 for example[48].

Similarly, fibrin can be engineered to incorporate additional GF-binding sites to further enhance interactions with the GFs [41]. In this case, strategies can be inspired from interactions that naturally occurs between the ECM and GFs [46]. For example, our group has developed controllable GF delivery system by covalently immobilized heparin or heparin-binding peptides into fibrin [41][49][50]. By conjugating heparin to fibrin, BMP-2, a heparin-binding growth factor could also be immobilized electrostatically [45]. In addition, other growth factor binding domains that have strong and promiscuous affinity for GFs could be crosslinked into fibrin, as for instance, the 12^{th}-14^{th} type III repeats of

fibronectin (FNIII12-14). Engineering FNIII12-14 with the TG domain allowed its efficient incorporation into fibrin matrix to form a growth factor delivery system [51]. Following this approach, many other researchers tend to engineer fibrin by crosslinking GFs binding-peptides into fibrin to construct growth factor delivery systems [52].

More recently, our laboratory has engineered growth factors with super-affinity for ECM proteins, including to fibrin to improve their delivery from fibrin biomaterials as well as from the native ECM of the tissues to regenerate. Indeed, we discovered that the domain aa123-aa144 from the placental growth factor-2 (PlGF-2), here named PlGF-2_{ECM}, possess a very high and promiscous affinity for multiple ECM proteins. By fusing PlGF-2_{ECM} to VEGF-A, PDGF-BB, or BMP-2, growth factor variants with super-affinity to the ECM can be generated. These engineered growth factors displays strong retention into exogenous fibrin and into the endogenous ECM upon delivery, which controls their pharmacokinetics release in the wounds and improve their therapeutic effects. Indeed, delivery of VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM} in diabetic wounds accelerated healing as compared to treatment with wild-type growth factors [53], in a diabetic wound healing model in the type 2 diabetic db/db mouse.

Overall, utilizing these strategies, fibrin biomaterials can be successfully engineered for the effective delivery of GFs to improve tissue regeneration.

II. 3. Challenges in clinical use of fibrin

II.3.1. Source and dosing of fibrin(ogen)

Currently, the fibrin utilized clinically are purified from whole human plasma with cryoprecipitation or from Cohn fraction I [54]. Then the plasma undergoes a series of steps for further purification, concentration and sterilization. There are two preparation methods for fibrin products: human pooled plasma, and individually obtained human plasma [39]. In human pooled plasma fibrinogen products, fibrinogen is highly concentrated from plasma donated by multiple donors, which creates a potential risk for pathogen transmission, such as viral diseases transmitted by blood. While individually obtained human plasma has much lower risk related to disease transmission, it requires more intensive labor for the centrifuge and process of plasma after each unit of plasma donation, and thus is more expensive [39]. Besides obtaining fibrinogen from donors, autologous fibrinogen is also a source for fibrin in treatment usage. Usage of autologous fibrinogen can lower the infectious risks and risks of antigencity caused by foreign proteins [55].

Fibrinogen can also be expressed recombinantly using CHO cells. Due to the complex structure of fibrinogen, the expression level of fibrinogen had been a challenge for many years. After optimizing the expression system and culture conditions, the recombinant expression yield of human fibrinogen in CHO DG44 could be improved to as high as 1.3 g/L [56]. Importantly, the recombinant fibrinogen has similar functions in the polymerization process as the plasma extracted fibrinogen, and works effectively in hemostasis and adhesion [57][56]. These results raise the possibility of the use of recombinant fibrinogen in larger scale for commercial usage.

For the fibrin products, the maximum fibrinogen dosage allowed in fibrin sealant patch is 2.3 mg to 3.5 mg and per person [58][59]. For the fibrinogen dosage for liquid form fibrin sealant, as the dosage is related to the application area, most of the products do not specify the dose, with only one product limits the dose used at 237 mg fibrinogen per surgery [60]. Concentration of fibrinogen in liquid formulated in general remains high in the range of 40 and 100 mg/mL, as compared to the physiological fibrinogen concentration which is 2-4 mg/mL. Interestingly, there are studies that showed that profound hypertension could occur in patients delivered with a large dose of fibrin on parenchymal tissue [61]. Thus, dose control is still important to reduce adverse effects caused by fibrin. Besides, dose reduction would also results in significant cost reduction of fibrin-based products.

II.3.2. Fibrinolysis and inhibitors of fibrinolysis

Protease inhibitors are used clinically as a supplement into fibrin to stabilize fibrin biomaterials from proteolytic degradation. One of the most commonly used clinical protease inhibitor is aprotinin, a bovine-origin broad spectrum serine protease inhibitor[62]. Aprotinin has been used in cardiac surgeries in Europe for about 50 years. In 1993, it was approved for intravenous use in coronary artery bypass graft (CABG) surgeries in the United States [63]. However, aprotinin application is associated with risks of severe anaphylactic reactions due to its bovine-origin; it can cause skin symptoms, fever, cardiovascular reactions, gastrointestinal disturbance, respiratory disturbance, shock, or even cardiac/respiratory arrest [63][64][65]. The risk of anaphylaxis is as high as 2.8% for patients re-expose to aprotinin within 21 months[63]. As an alternative, small molecules inhibitors have been used as antifibrinolytic agents in the clinic, for example,

tranexamic acid and aminocaproic acid. However, these inhibitors are less effective in resisting fibrinolysis as compared to aprotinin [66].

Previously, our group has engineered the Kunitz-type protease inhibitor (KPI) derived from the amyloid-β A4 precursor protein, a human-derived protease inhibitor candidate that could be a substitute for aprotinin. To improve the local delivery of KPI in fibrin, our group fused a TG domain ($\alpha2PI_{1-8}$) at the C-terminus of KPI. Although KPI has a lower inhibitory power than aprotinin, the engineered KPI was able to extend longevity of fibrin more effectively than aprotinin [67]. Due to the commonly use of fibrin biomaterials in tissue engineering recent years, the development of safe antifibrinolytic agents with high efficacy are worth investigation [68].

Physiologically, the main protease inhibitor for plasmin is α2-antiplasmin (α2PI), which naturally contains the transglutaminase (TG) substrate domain and crosslinked into fibrin clots during polymerization [69]. α2PI is a single chain glycoprotein synthesized by the liver, majorly exists in plasma with 0.7 – 1.0 μM physiological [70][71].

α2PI is the main inhibitor of plasmin. α2PI is able to form a plasmin-α2PI complex with C-terminus binding to the lysine-binding site of plasmin, and crosslink to fibrin via factor XIIIa (FXIIIa) on its N-terminus [71]. Thus, α2PI inhibits plasmin competitive binding to fibrin. α2PI can also inhibit activators of plasmin: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)[71]. Thus, α2PI can be a competitive candidate for an anti-fibrinolytic agent, as it is both of human-origin and is able to bind to and protect fibrin. In this thesis, we will particularly use α2PI as an anti-fibrinolytic and hemostatic agent for the engineering of fibrin biomaterials and endogenous fibrin.

II.3.3. Fibrin-mediated growth factor delivery in diabetic wounds

Diabetes is a disease widely influencing the world. It is estimated that the global diabetes prevalence is 463 million people, about 9.5% of the total population, and the prevalence is projected to increase to 10.2% in 2030[72]. Specifically, United States is seriously influenced by diabetes. As of 2015, 9.4% of US population (30.3 million people) had diabetes[73]. In both types, diabetes results in uncontrolled hyperglycemia, leading to major complications such as cardiovascular diseases, neuropathy, kidney failure, blindness and chronic wounds[74][75]. Chronic wounds are amongst the more serious complications of diabetes; it affects about 4-6% of diabetic patients each year, and it has been estimated that 25% of diabetic patients will develop chronic wounds in their lifetime[76]. Chronic wounds are defined as wounds that are unable to heal for more than 3 months, which include those that never heal spontaneously. Therefore, they are painful, represent a major risk of infections and require excessive medical care. Moreover, chronic wounds often occur in the foot of diabetic patients, so called diabetic foot ulcers, which further reduces their mobility and social activities. For 20% of these patients, non-healing ulcers will ultimately lead lower-limb amputation[76]. For all these reasons, chronic wounds strongly impair life quality of the patients. Lastly, from an economical point of view, the costs of chronic wounds in the United States has been estimated at $9-$13 billion dollars per year, which represents a burden for our society[77][78].

Diabetic wounds are characterized by a high proteolytic environment as compared to healthy wounds. The increased presence of inflammatory cells such as neutrophils and macrophages leads to overexpression of many proteases in the wound bed, including matrix metalloproteinases (MMP) -1, 2, 8, and 9, as well as elastase and plasmin [11][12]. In addition, the level of urokinase plasminogen activator (uPA) is also elevated in diabetic wounds, which leads to an excessive activity of plasmin [79]. In parallel, levels of several

protease inhibitors are reduced, particularly, the levels of the tissue inhibitor of metalloproteinase (TIMP) -1 and -2, and the α1-protease inhibitor [11][79]. Overall, the imbalance between proteases and proteases inhibitors results in excessive proteolytic activities in the wound microenvironment, causing cleavage and destruction of the ECM and GF in diabetic wounds, which strongly impair wounds healing (Figure 2) [80][81]. Furthermore, the levels of many growth factors are reduced in diabetic chronic wounds. The contents of insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF) are all diminished [80][81]. Reduced amounts of growth factors impairs wound angiogenesis and tissue formation, resulting in poor tissue regeneration.

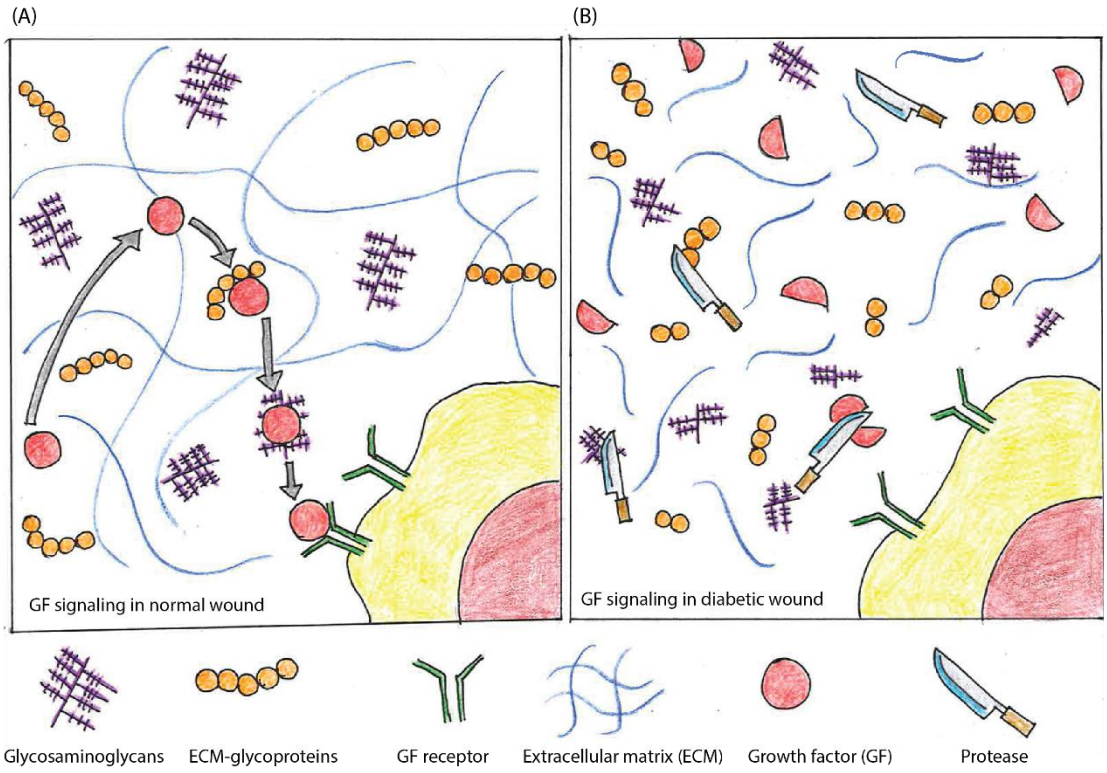

Fig 2. **Impaired growth factor signaling in diabetic wounds.** (A) Growth factor signaling in normal wound. In healthy wound environment, growth factors interact with multiple molecules of the ECM, before reaching their cognate receptors at the cell surface. (B) Impaired growth factor signaling in diabetic wounds. In diabetic wounds, increased proteases degrades the ECM and associated molecules, as well as the growth factors. Thus, the growth factor signaling is substantially reduced, leading to delayed wound healing.

Due to their strong ability to control tissue morphogenesis processes, such as cell proliferation, migration, differentiation, adhesion or gene expression, exogenous delivery of GFs have been widely explored as therapeutic drugs to promote tissue regeneration in various clinical applications [82]. In context of chronic wounds, some growth factors are currently undergoing clinical trials or have already been clinically approved for chronic wound treatment. Indeed, Regranex® (Smith & Nephew) is a drug containing the recombinant human PDGF-BB (rh-PDGF-BB) that was approved by FDA in 1997 for the treatment of diabetic foot ulcers [83]. Regranex® is able to increase diabetic foot ulcer healing by as much as 39% comparing to the placebo group [27]. There are also trials conducted for application of Regranex® on pressure ulcers [83]. As to other GFs, the delivery of plasmid encoding for the human VEGF-A165 gene was tested in clinical trials in patients with diabetic and ischemic wounds [27]. Besides, topical application of rh-VEGF, under the name of Telbermin®, is currently in phase II of the clinical trial to test its efficacy and safety in diabetic foot ulcers [83]. In addition, the use of FGF-2 (also known as bFGF) has been approved in the clinic, in a commercially available product called Fiblast® Spray for application in skin ulcers. Delivery of FGF-2 has been also approved in diabetic foot ulcers in Japan [84]. Another related protein, FGF-1 (also known as aFGF) is currently in the process of clinical trials for chronic skin wound healing [27]. Lastly, there are some commercially available products containing recombinant human EGF (rh-EGF) for diabetic foot ulcer treatment, in the products called Heberprot-P®, Regen-D™ 150, and Easyef® [84], which could be applied intralesionally or topically to the ulcer site [27].

Nevertheless, some important concerns have raised regarding the safety of growth factors in the clinic. Importantly, PDGF-BB in the product Regranex® received a safety warning from the FDA as it increases tumor formation and the risk of death from malignant tumors

[83]. As another example, delivery of VEGF-A led to blood vessel hyperpermeability issues that prevented its approval in the clinics [85]. Similarly, other growth factors used in other applications than diabetic ulcers have been correlated with serious side-effects, such as BMP-2 in the product InFuse® Bone Graft [86]. Such safety concerns are related to the uncontrolled delivery of supra-physiological doses of the GFs, often used at milligrams doses (whereas growth factors are naturally secreted at nanograms doses). Indeed, upon bulk delivery, high doses of GFs are fast released from the wound site into the surrounding tissues and the systemic circulation, which lower the treatment efficacy while increasing the side-effects [87]. In addition, the high doses required for growth factors in the clinic substantially raises the costs of these therapies.

Therefore, spatio-temporal controlled growth factor delivery system is necessary to make growth factors therapies cost-effective and safe in the clinic. Furthermore, as the efficacy of wild-type growth factors is often limited by their natural properties such as stability, localization, and cellular internalization, engineering of growth factor delivery systems can improve the benefits of growth factor therapies [87]. As we discussed above, fibrin has been extensively explored as a carrier material for GFs delivery and engineering of fibrin biomaterials and GFs can efficiently improve tissue regeneration in diabetic wound healing[53][34][48]. To date, however, fibrin-mediated GFs delivery remained in development in pre-clinical stages. Therefore, optimization of fibrin-based GF delivery systems in view of their clinical translation remains highly relevant.

II. 4. References

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III. α2-ANTIPLASMIN EXTENDS LONGEVITY OF FIBRIN BIOMATERIALS AND ENHANCES GROWTH FACTOR DELIVERY IN DIABETIC WOUND HEALING

III. 1. Abstract

Fibrin is widely used as biomaterial in clinic, as a tissue sealant to stop bleeding and stick tissues together during surgery. In preclinical research, fibrin is also extensively studied as a carrier material for the delivery of growth factors to promote tissue regeneration. Prematured degradation of fibrin leads to important complications, such as recurring bleeding, tissue dehiscence or poor regeneration. Consequently, anti-fibrinolytic agents have been used to stabilize fibrin, one of them being aprotinin, a bovine-origin broad spectrum protease inhibitor. However, due to its xenogenic origin, aprotinin is immunogenic and can cause severe anaphylactic reactions. In this project, we propose to use the human protease inhibitor α2-antiplasmin (α2PI) to engineer fibrin biomaterials and prevents fibrinolysis. We expressed recombinant α2PI in mammalian cells and demonstrated its high anti-fibrinolytic efficacy as compared to aprotinin, even in protecting fibrin biomaterials made of low fibrinogen dose. Lastly, we used α2PI to stabilize fibrin gels for the delivery of fibrin-binding growth factors (GFs) in diabetic wounds healing, which resulted in enhanced tissue regeneration. Therefore, α2PI is a competitive substitute to aprotinin for clinical use and in regenerative medicine.

Keywords: *Fibrin, protease inhibitor, plasmin, growth factors, diabetic wound healing.*

III. 2. Introduction

Fibrin biomaterials are widely used in many applications in tissue engineering and regenerative medicine, as a tissue sealant and as biodegradable carrier materials[1]. Particularly in the clinic, fibrin is used to stop bleeding and reattach tissues during cardiovascular surgeries, thoracic surgeries, neurosurgeries, and plastic and reconstructive surgeries[2][3]. In addition, fibrin has be used as scaffolding material to deliver cells or biomolecules in tissue engineering, for example in adipose, cardiovascular, ocular, muscle, liver, skin, cartilage, and bone engineering[4][5][6]. In these applications, biologically active molecules, such as GFs, are commonly be incorporated in fibrin to promote growth of tissues and structures, for example angiogenesis or neurite extension[6].

Currently, the fibrinogen used for the preparation of clinical-grade fibrin is purified from human blood, which creates some important limitations. Particularly, the source of fibrinogen is limited because derived from human, the cost is high, and there are batch-to-batch variability and risk of pathogen transmission. In addition, clinical fibrin uses high concentration of fibrinogen in the range of 50-100 mg/mL in order to prevent rapid degradation upon application. Indeed, improper degradation of fibrin caused by various proteases, for example, plasmin and matrix metalloproteases (MMPs) can lead to recurrent bleeding, loss of tissue adhesion and impede tissue regeneration progress[2]. In addition, in some pathologies characterized by chronic inflammation, such as diabetic wounds, protease levels are highly elevated and have been shown to accelerate degradation of endogenous ECM, including the fibrin clot, as well as of GFs, thus limiting their effects on tissue healing[7]. In such cases, the delivery of exogenous fibrin as a carrier material for wound healing is similarly limited by fast proteolytic degradation.

To stabilize fibrin biomaterials from prematured proteolytic degradation, protease inhibitors are used as a supplement into fibrin. For example, aprotinin, a small bovine-origin broad spectrum serine protease inhibitor peptide is clinically used to effectively extend the longevity of fibrin[8]. However, due to its bovine-origin, aprotinin causes immune responses, in some cases leading to severe anaphylactic reactions, including skin rashes, fever, cardiovascular reactions, gastrointestinal disturbance, respiratory distress and shock, or even cardiac/respiratory arrest[9][10][11]. The risk of severe anaphylaxis upon re-exposure caused by aprotinin is about 2.8%[9]. As an alternative, small molecules inhibitors, such as tranexamic acid and aminocaproic acid, have been used as antifibrinolytic agents in the clinic but are less effective in resisting fibrinolysis as compared to aprotinin[12].

Previously, our group have developed a strategy to highly increase the efficacy of aprotinin by fusing it to the transglutaminase substrate sequence (TG) to cross-link aprotinin into fibrin, thus preventing its diffusion out of fibrin and increasing its local protective effects. While such strategy can allow strong reduction of dosing, and reduce immunogenicity issue, it still relies on the use of the bovine aprotinin. Our group has then identified a human-derived protease inhibitor using Kunitz-type serine protease inhibitor (KPI) derived from the amyloid-β A4 precursor protein (APP) that has a very homologous structure to aprotinin[13]. However, the intrinsic inhibitory effects of KPI were lower than the aprotinin ones, requiring further engineering with the TG sequence (TG-KPI) and uses of high doses to be a competitive alternative to aprotinin.

In this project, we then aimed to search for an effective and human origin antifibrinolytic protein as a substitute to aprotinin. Interestingly, the TG sequence used by our group is naturally derived from a human protease inhibitor, namely α2PI. α2PI has a key role in the fibrinolytic system and is the most important physiologic inhibitor of plasmin, which

is the principal protease of the fibrinolytic pathway[14]. α2PI is a single chain glycoprotein synthesized in the liver and secreted in the blood, where it is found at a physiological concentration of 0.7-1.0 μM[15][16].

As a main inhibitor of plasmin, α2PI regulates fibrinolysis by binding to the lysine-binding site of plasmin using its C-terminus to form the plasmin-α2PI complex. At its N-terminus, α2PI is enzymatically crosslinked into fibrin via its TG sequence by factor XIIIa (FXIIIa)[16]. In this way, α2PI is able to inhibit binding of plasmin to fibrin competitively. α2PI can also inhibit activators of plasmin, including tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)[16]. The human-origin of α2PI and its ability to bind to and protect fibrin makes it a potential powerful candidate for use as an antifibrinolytic agent.

Since α2PI is the principal inhibitor of plasmin, and naturally contains a TG substrate enabling its binding with fibrin, we have laid down the hypothesis that α2PI is able to extend longevity of fibrin gel effectively as compared to the clinically used protease inhibitor aprotinin, and the engineered inhibitor TG-KPI[14]. Here, we studied the efficacy of α2PI in protecting fibrin in subcutaneous implants, modeling an application in fibrin sealants, as well as for the delivery GFs in diabetic wound healing. We overall demonstrated a very strong efficacy of α2PI in both applications, and further showed that α2PI can protect fibrin gels made of low fibrinogen concentration, potentially improving the cost-effectiveness of fibrin-based material. Finally, we have also shown that α2PI can be produced recombinantly in mammalian cell, setting a promising basis of supply for clinical applications. These together indicate that α2PI could be effectively used as an antifibrinolytic substitute to aprotinin in the clinic.

III. 3. Materials and Methods

Cloning, expression, and purification of α2PI

Human α2PI DNA sequence was purchased from GenScript and cloned into pXLG expression vector under a CMV promoter and using an IgGκ signal peptide. α2PI was his-tagged at the N-terminus. Suspension-adapted Human Embryonic Kidney (HEK) 293-F cells were transfected with the α2PI plasmid DNA using PEI. Cell culture supernatant was collected 7 days after the transfection and was purified by His-tag affinity-mediated purification using

HisTrap HP his tag purification column (GE Healthcare). The column was pre-equilibrated with buffer A (20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4). The cell culture supernatant was then loaded through column. The column was washed with buffer A after the sample loading was completed. After washing, the protein binding to the column was eluted in 3 steps: 98% buffer A with 2% buffer B (20 mM NaH_2PO_4, 0.5 M NaCl, 1M imidazole), 50% buffer A and 50% buffer B, and 100% buffer B. Fractions of the elution were collected, dialyzed against Tris-Buffered Saline (TBS; 150 mM NaCl, 20 mM Tris, pH 7.4), sterile-filtered at 0.22 μm and stored at -80°C until use.

Fluorescent fibrinogen preparation

Lyophilized human fibrinogen (FIB3, pg, vWF & Fn depleted, Enzyme Research) was dissolved at 37 °C and dialyzed against HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6). The fibrinogen after dialysis was concentrated using Amicon Ultra Centrifugal Filters (Millipore) and stored at -80°C. Fluorescent fibrinogen was prepared by mixing 0.1 mg Alexa Fluor 680 NHS ester (Invitrogen) with 100 mg of fibrinogen in 0.1 M sodium bicarbonate buffer. The reaction was incubated for 2 h at room temperature under

continuous shaking. The fluorescent fibrinogen was purified using Zeba Spin Desalting Columns (Thermo Scientific) to remove unconjugated free dyes. The fluorescent fibrinogen was quantified by nanodrop and stored at -80°C.

Proteolytic inhibition of fibrinolysis *in vitro*

Fibrin gels (70 µL) were made of 10 mg/mL fibrinogen of 25% w/w fluorescent fibrinogen, 2 U/mL thrombin, 4 U/mL Factor XIIIa, and 5 mM $CaCl_2$ in HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6), in which 1 µM inhibitors α2PI (Sigma-Aldrich), aprotinin (Roche), and TG-KPI (in-house production)[13] were added. After these materials were mixed and polymerized on paraffin films, the gels were incubated for 1 h at 37°C with 5% CO_2 to ensure complete polymerization. The fibrin gels were then transferred into a 24-well cell culture plates and incubated in 1 ml release buffer (Tris 20 mM, NaCl 150 mM, 0.1% BSA, Pen/Strep, pH 7.4) containing 2.5 nM plasmin (Roche). The plate was kept at 37 °C with 5% CO2 until the gels were fully degraded. The plasmin-containing buffer was daily refreshed. The gel volume were quantified over time by IVIS Spectrum system via fluorescence measurements. Total radiant efficiencies of the gels were measured using the recommended software (PerkinElmer, Caliper). The percentages of gel remaining as then calculated by normalizing the total radiant efficiency of a gel to the one measured at day 0.

The same methods were used for *in vitro* fibrinolysis assessment of different fibrin gel volumes (70 µL, 100 µL), different inhibitor concentrations (0.1 µM, 0.32 µM, 1 µM, 3.2 µM, 10 µM, and 15 µM), different fibrinogen concentrations (4 mg/mL, 6 mg/mL, 8 mg/mL, 10 mg/mL, and 12 mg/mL), and different plasmin concentrations (2.5 nM, 25 nM).

Characterization and activity assays of in-house produced α2PI

Purified α2PI was analyzed using SDS-PAGE and western blot under reducing conditions and compared with commercial α2PI (Sigma-Aldrich). Western blot was performed using anti-his-tag antibody and α2PI antibody (Abcam). *In vitro* fibrinolysis test (100 µL fibrin gel with 25% w/w fluorescent fibrinogen, 1 µM inhibitors, 2.5 nM plasmin) was performed as described previously for in-house produced and commercial plasma-purified α2PI to compare their proteolytic inhibition activity.

Proteolytic inhibition of fibrinolysis *in vivo* in a subcutaneous implantation model

Fibrin gels (100 µL) were prepared as detailed above using 15 µM of the inhibitors α2PI, aprotinin or TG-KPI. All *in vivo* experimentation was approved by the IACUC of University of Chicago. Ten female BALB/c mice at 8-10 weeks age were used. Isoflurane was applied to the mice by inhalation as anesthetization, with 4% at beginning, and 2% for maintenance. The mice were placed on a heating pad. Buprenorphine was injected intradermal at dosage of 0.1 mg/kg as analgesia, and artificial tears ointment was applied to the eyes of mice. The back of the mice was shaved, and disinfected with betadine wipes, followed by 70% ethanol wipes. Two incisions of about 8 mm were created on the skin, one on each side of the spine, and subcutaneous pockets were created using sterile scissors and forceps. The fibrin gels were implanted subcutaneously in the back of the mice, for a total of 2 gels per mouse. Prolene 4-0 sutures (Ethicon) were used to close the incisions.

The degradation of the fibrin gels were quantified by IVIS Spectrum system via fluorescence detection. Total radiant efficiencies of the gels were measured using the recommended software (PerkinElmer, Caliper). Percentages of gels remaining were

quantified by normalization of the total radiant efficiency to the one measured at day 0 (after implantation of the gel).

Proteolytic inhibition of fibrinolysis *in vivo* on wound

9 male db/db mice were included in this experiment, approved by IACUC of University of Chicago. The wounding procedure was the same than described above. After the wounds were created, fibrin gels (70 µL) were made by mixing 10 mg/mL or 4 mg/mL fibrinogen of 25% w/w fluorescent fibrinogen, 2 U/mL thrombin, 4 U/mL Factor XIIIa, 5 mM $CaCl_2$ in HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6) and α2PI or aprotinin inhibitors (15 µM or 3 µM), and directly polymerized on wounds. The degradation of the fibrin gels on wounds were quantified by IVIS Spectrum system via fluorescence detection as described previously.

Histological analysis of wounds

The wounding surgical procedures were the same as in the proteolytic inhibition of fibrinolysis *in vivo*, except that Adaptive non-adhering dressing (Systagenix) was applied on top of the wounds before covering wounds with the hydrofilm (Hartmann).

The mice were euthanized on day 7 after the surgery, with wounds extracted and fixed using 4% PFA in PBS. The fixed wounds were embedded in paraffin and cut into 5 µm thick sections to show cross-sections of the wounds. The slides stained using H&E staining. Images were taken using microscope (Leica) to analyze wound closure and granulation tissue formation using Fiji software (ImageJ).

Skin wound healing of db/db mice using fibrin gel

Db/db male mice 10-12 weeks old were anesthetized by isoflurane and placed on heating pad. Artificial tear ointment was used to protect eyes of the animals. Animal were injected with buprenorphine at dosage of 0.1 mg/kg subcutaneously as analgesia. The back of mice was shaved and disinfected with betadine wipes followed by 70% isopropyl alcohol wipes. After disinfection, 4 wounds were created on the back of mice using 6-mm biopsy punch with two wounds on each side of the spine. After wounding, fibrin gels (50 µL) were polymerized directly on the wounds by mixing 4 mg/mL fibrinogen, 2 U/ml thrombin, 4 U/mL Factor XIIIA, 5 mM $CaCl_2$ in HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6) with α2PI (1 µM or 3 µM), VEGF-A-PlGF-2_{ECM} (200 ng/wound), and PDGF-BB-PlGF-2_{ECM} (200 ng/wound). After polymerization of fibrin gel, splints (6 mm or 8 mm internal diameter) were sticked to the surrounding of the wound using 1 x Histoacryl BLUE glue (B. Braun Surgical). The top of the splints was covered by hydrofilms (Hartmann), with a few holes created by needle enabling ventilation.

Preparation of fibrin gel containing plasmin

The fibrin gel preparation procedure was the similar as described in the previous section. 50 nM of plasmin (Roche) was added together with 4 mg/mL fibrinogen, 2 U/ml thrombin, 4 U/mL Factor XIIIA, 5 mM $CaCl_2$ in HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6) with α2PI (3 µM or 15 µM), and then mixed with VEGF-A-PlGF-2_{ECM} (200 ng/wound), and PDGF-BB-PlGF-2_{ECM} (200 ng/wound) on wound site for polymerization to generate fibrin gel containing plasmin.

Histology analysis for wound closure and granulation

The mice were euthanized 10 days after the surgery, with the wounded tissues dissected and fixed using 4% PFA in PBS overnight at 4°C. The fixed wounds were embedded into paraffin and sectioned at 5 μm to obtained cross-sections at the center of the wounds. The slides were stained using hematoxylin and eosin (H&E) staining. Images were taken using microscope (Leica DMi8) and wound closure and granulation tissue formation in wounds were analyzed using Fiji (ImageJ Open Source Software). The distance between the wound edges and between the regenerated epithelium edges were measured to calculate the percentage of wound closure. The area of granulation tissue was measured and normalized using the distance between wound edges to assess the amount of granulation tissue in the wound.

Immunohistochemistry staining on wounds and quantification

Paraffin sections of the wounds were dewaxed and rehydrated using serial washes in xylene, ethanol (100%, 96% and 70%) and water. The antigens of the tissues were than retrieved in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in a steam bath. After antigen retrieval, the slides were washed using TBS (150 mM NaCl, 20 mM Tris, pH 7.4). The tissues on the slides were blocked with 5% casein in PBS (Blocking buffer) for 2 hours at room temperature, and then stained with anti-CD31 (Abcam) and anti-smooth muscle actin (SMA; Sigma-Aldrich) in blocking buffer overnight at 4°C. After washes in TBS, secondary antibodies and DAPI were incubated for 2 h at room temperature. Finally, the slides were washed again and mounted after staining for fluorescent microscopy imaging.

III. 4. Results

α2PI can be expressed as a recombinant protein in HEK293-F mammalian cells

Human α2PI can be isolated at reasonable quantities from human plasma, and commercially purchased. Nevertheless, human-derived products have multiple limitations, such as sourcing, risks of blood borne infections and quality issues affected by the processing of starting materials after collection[17][18]. As we would like to explore application of α2PI clinically, it is meaningful to assess whether α2PI can be produced as a recombinant protein.

Here, we tested expression of human α2PI in HEK293-F mammalian cells. We transfected the cells with a plasmid encoding α2PI containing an N-terminus his-tag and expressed it for 7 days. SDS-PAGE and western blot analysis using anti-his-tag antibody have shown the presence of recombinant α2PI in cell culture supernatant (Fig. 3A). We then purified α2PI by his affinity-mediated purification column and were able to obtained highly pure α2PI, visualized at the same molecular size as the commercially purchased plasma-derived one from Sigma-Aldrich (St. Louis, MO USA), as assessed by SDS-PAGE. The identity of the recombinant α2PI was further confirmed by a western blot probed with anti-α2PI antibody (Fig. 3B). As to the yield of expression, about 10 mg of α2PI was obtained per liter of transfection.

We then confirmed the bioactivity of the recombinantly produced α2PI in a fibrinolysis assay *in vitro*. To do so, physiological concentration of both plasmin (2.5 nM) and α2PI (1 μM) was used. Fluorescently labeled fibrin gels containing recombinant or plasma-derived α2PI were incubated in buffer containing plasmin, and the gel degradation was evaluated from fluorescence decay via IVIS spectrum fluorescent imaging every 2-3 days. The activity test showed that recombinant α2PI have similar efficacy in protecting

fibrin gels as the commercially purchased α2PI purified from human plasma. Both of them were able to extend longevity of fibrin gel to around 20 days, which is 8 days longer than the gel longevity without inhibitors (Fig. 3C).

These results indicate that bioactive human α2PI was able to be produced and purified recombinantly with high yield, which provides an alternative to the use of human plasma-derived source, which represents a strong advantage for potential clinical applications.

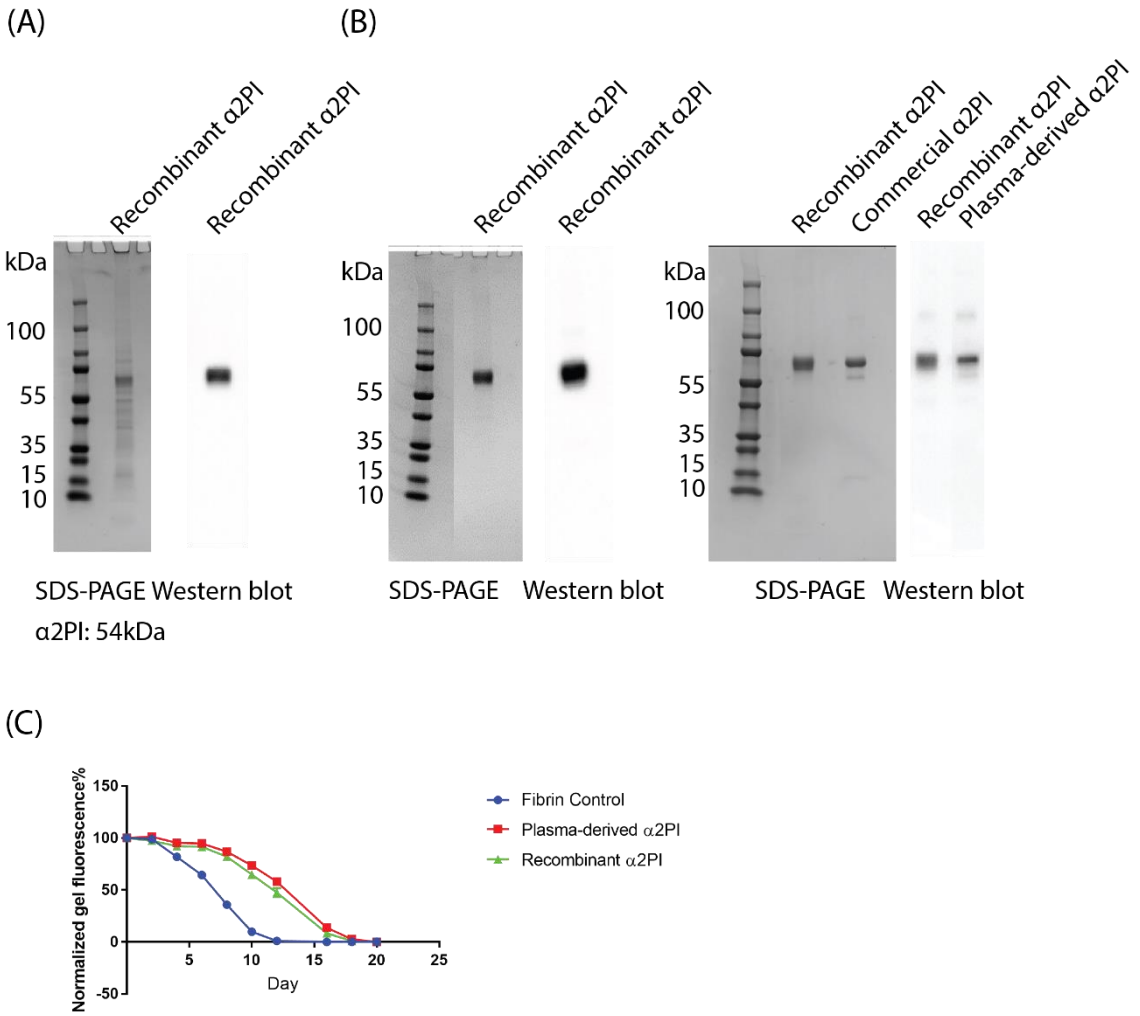

Fig 3. **Expression of α2PI in HEK293-F mammalian cells. (A)** SDS-PAGE and anti-his-tag western blot of the recombinant α2PI expressed in HEK293F cells before purification. A band at expected size (54 kDa) could be observed on both the SDS-PAGE and the western blot. **(B)** SDS-PAGE and anti-α2PI western blot of recombinant α2PI after his-affinity mediated purification and comparison to the commercially available plasma-derived α2PI. **(C)** Comparison of the activity of 1 μM of the recombinant and the plasma-derived α2PI in inhibiting fibrin gel degradation *in vitro* in presence of 2.5 nM of plasmin. Recombinant α2PI shows similar efficacy as the plasma-derived α2PI purified from human blood.

α2PI extended the duration of fibrin biomaterials more effectively than other inhibitors *in vitro*

We then examined how effective α2PI is in extending longevity of fibrin gels *in vitro* as compared to the clinical aprotinin, or to another engineered protease inhibitor previously developed by our group[13], made by the fusion of the transglutaminase substrate domain TG and a human Kunitz-type protease inhibitor domain (KPI), named TG-KPI. To do so, we first evaluated the efficacy of α2PI and the other inhibitors when incorporated into fluorescent fibrin gels at 1 μM, which is close to the physiological concentration of α2PI[15][19]. In addition, we studied fibrinolysis of these gels in presence of physiological concentration of plasmin (2.5 nM), by measuring gel degradation via fluorescence decay using IVIS spectrum fluorescent imaging. We observed that the fibrin gels supplemented with aprotinin or TG-KPI did not stay longer than the fibrin only control group at a 1 μM dose, and lasted for about 8 days, whereas fibrin gels with α2PI have a significantly longer longevity of 19 days (Fig. 4A).

We then performed a dose study *in vitro* to evaluate the efficacy of α2PI in protecting fibrin gels, prior to *in vivo* experimentations. We kept a physiological concentration of plasmin and tested 5 different concentrations of α2PI, being 10 μM, 3.2 μM, 1 μM, 0.32 μM and 0.1 μM. As expected, we observed that the higher the α2PI concentration, the slower the fibrin gel degradation. At the highest concentration of α2PI tested (10 μM), the longevity of the fibrin gel was extended to about 35 days (Fig. 4B). Moreover, the efficacy of α2PI did not yet reach a plateau at the 10 μM dose, suggesting that further increase in dose could prolong fibrin duration even more.

Considering the overexpression of plasmin in some applications of regenerative medicine, particularly in diabetic wounds, we then repeated the fibrin gel degradation assay using a 10-fold increased concentration of plasmin (25 nM). Here, we compared

the degradation of fluorescent fibrin gels that contain 15 μM of α2PI, aprotinin or no inhibitor. In high plasmin concentration environment, the fibrin gels containing aprotinin degraded in 3 days, with a longevity increase of only 1 day as compared to fibrin only. In contrast, α2PI still showed good efficacy in resisting fibrinolysis and lasted for about 16 days (Fig. 4C). Therefore, even at high plasmin concentration, α2PI was vastly superior to the other inhibitors in protecting fibrin. Based on this result, we proceeded to *in vivo* experiments using a high concentration of 15 μM inhibitor.

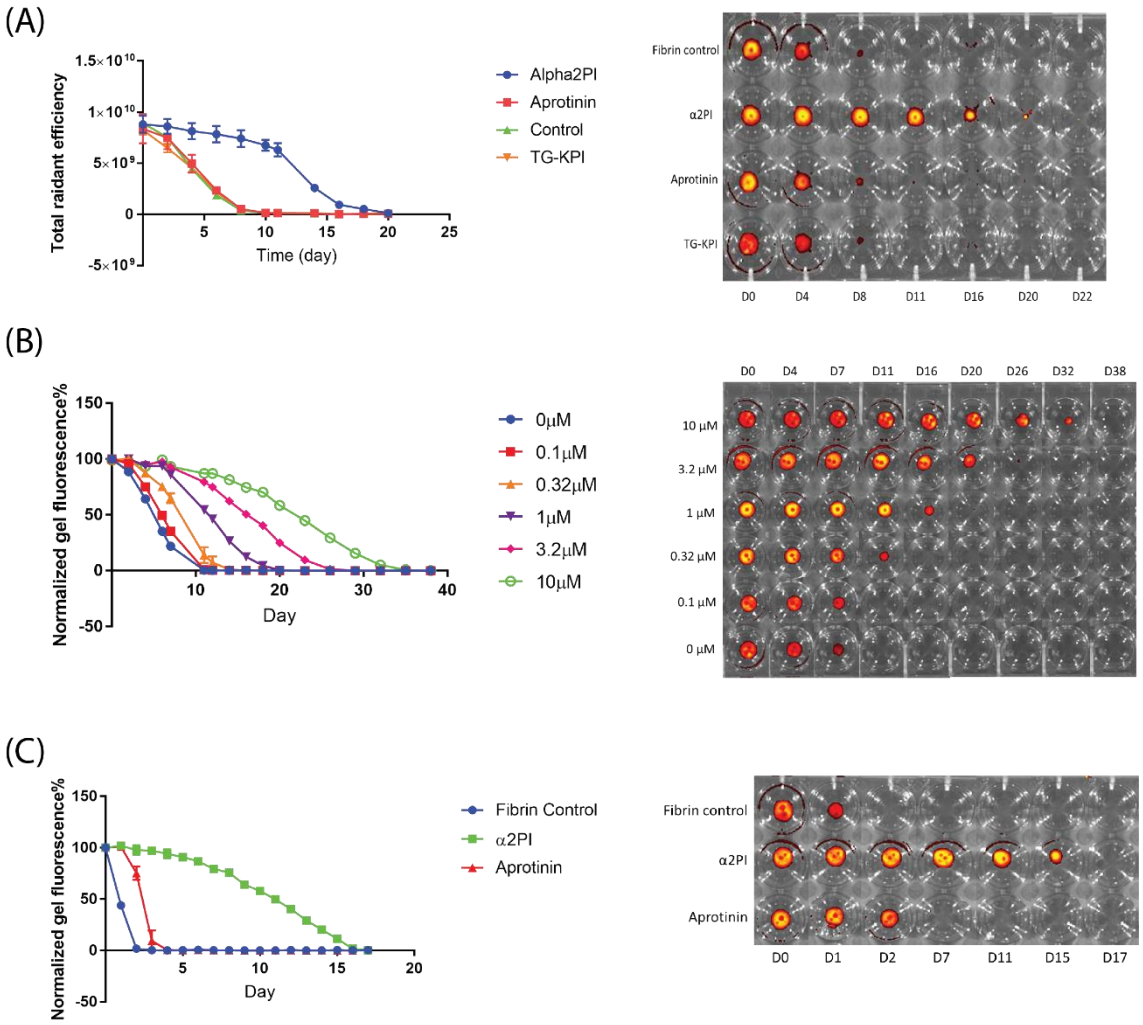

***Fig 4.* α2PI protects fibrin biomaterials *in vitro*. (A)** Comparison of α2PI, aprotinin or TG-KPI (1 μM) efficacy to protect fibrin gels from degradation in presence of 2.5 nM of plasmin. Fibrin gel containing α2PI had longer longevity than all the other groups. **(B)** Dose study of α2PI was performed by incubating fluorescent fibrin gels containing doses of α2PI in 2.5 nM plasmin. The longevity of fibrin gel extended with the increase of α2PI dose. **(C)** Efficacy of 15 μM of α2PI, aprotinin or TG-KPI in protecting fibrin gels in presence of high plasmin concentration (25 nM). High dose of α2PI protected fibrin gels very effectively as compared to the other inhibitors.

α2PI increased longevity of fibrin biomaterials *in vivo* in models of subcutaneous implants and diabetic wounds

After having demonstrated the efficacy of α2PI in protecting fibrin gels *in vitro*, we then evaluated the longevity of α2PI-containing fibrin gels upon subcutaneous implantations in the back of mice. Fluorescently labelled fibrin gel with 15 μM α2PI or other inhibitors were implanted, and fibrin gel degradation was evaluated via IVIS spectrum fluorescence imaging up to day 56. The fibrin gels containing aprotinin or TG-KPI mostly degraded around day 18, as did the fibrin only control group. α2PI, however, was able to stay for about 40 days, and one of the gels in the α2PI group still had 5% remaining on day 56 (Fig. 5A). Therefore, these results indicated that α2PI was able to extend the longevity of fibrin gels of at least twice as compared to all other groups, in this model of subcutaneous implantation.

As we aimed to explore application of α2PI in diabetic wound healing, we also performed *in vivo* fibrinolysis in diabetic wounds in the type 2 diabetic db/db mice. Fluorescently labelled fibrin gels containing 15 μM α2PI, aprotinin or no inhibitor were polymerized on the top of the wounds, and covered with a transparent non-fluorescent hydrofilms to protect the wounds and the fibrin gels. Fibrin gel degradation was measured via IVIS spectrum fluorescence imaging until the wounds closed and the gel degradation stopped, which occurs after 15-18 days. After 18 days, some of the hydrofilms covering the wounds detached and some of the gels got removed from the wounds, which prevent further quantification of these gels. Once again, we observed that the fibrin gels containing α2PI had the longest longevity. Indeed, the gels with α2PI degraded about 20% slower than the fibrin gels with aprotinin, and was able to have more than 50% remaining at day 18 (Fig. 5B). Surprisingly, the aprotinin containing fibrin gels degraded around 10% faster than the fibrin only control.

Therefore, we concluded that α2PI was able to protect fibrin gels better than other inhibitors, including the clinical aprotinin, when used in fibrin subcutaneous implants or as a topical material for diabetic wound healing.

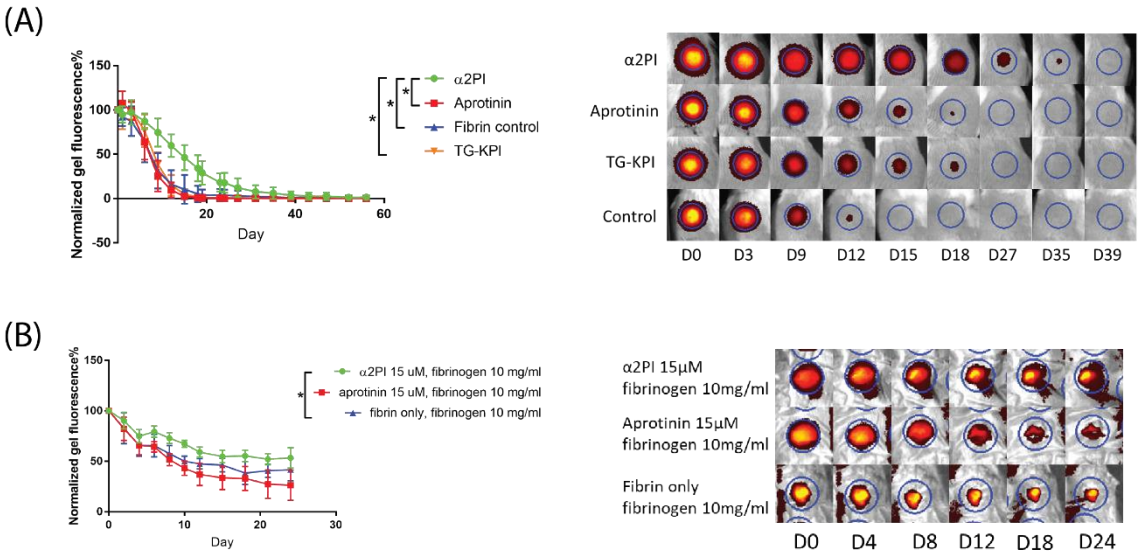

***Fig 5.* α2PI protects fibrin materials *in vivo* as compared to other inhibitors. (A)** *In vivo* degradation of fibrin subcutaneous implants (10 mg/mL fibrinogen) supplemented with 15 μM of α2PI, aprotinin or TG-KPI in Balb/C mice (N=5, *p < 0.05, Friedman with Dunns post-hoc test) α2PI was able to prolong gel longevity more effectively than the other groups. **(B)** *In vivo* degradation of fibrin gels, supplemented with 15 μM of α2PI or aprotinin, applied topically on diabetic wounds in db/db mice (N=6, *p < 0.05, Friedman with Dunns post-hoc test). α2PI was able to extend gel longevity more effectively than aprotinin or fibrin gel without inhibitor.

α2PI extends longevity of fibrin biomaterials made of low concentration of fibrinogen *in vitro* and *in vivo*

In the clinic, fibrinogen is purified from human blood and, as such, has limited sourcing and relatively high cost. Therefore, we asked whether the high efficacy of α2PI in protecting fibrin biomaterials can permits reduction of fibrinogen concentration.

To examine efficacy of α2PI in protecting fibrin gels with different fibrinogen concentrations, we first performed *in vitro* fibrinolysis test. Fluorescently labelled fibrin gels with 5 different doses of fibrinogen ranging from 4 mg/ml to 12 mg/ml were compared. The fibrin gels were supplemented with a physiological concentration α2PI (1 μM) and were incubated in physiological concentration plasmin (2.5 nM).

The longevity of the fibrin gels in absence of inhibitors varies from 6 to 12 days for gels made of 4 mg/mL to 12 mg/mL fibrinogen; as expected, the gel longevity extended with increased concentrations of fibrinogen. Impressively, the fibrin gels containing α2PI had an extended longevity between 22 and 24 days (Fig. 6A, B), with a minimal influence of the fibrinogen concentration. Indeed, α2PI protected fibrin gels made with low concentration of fibrinogen (4 mg/mL) very efficiently, to similar extent that gels made with 12 mg/mL fibrinogen (Fig. 6A, B). Therefore, α2PI was able to protect fibrin gel even at low fibrinogen concentration *in vitro*, allowing for an important reduction of fibrinogen concentration in fibrin biomaterials.

Based on this results, we assessed the potential of α2PI to protect low-dose fibrin biomaterials *in vivo*, in the subcutaneous implantation model. Fluorescently labelled fibrin gel with 4 mg/ml or 10 mg/ml fibrinogen containing 15 μM of α2PI were implanted under mice back skin, and compared to fibrin gels without inhibitors. IVIS spectrum fluorescence imaging was used every 3 to 5 days to monitor the degradation of the fibrin gels implanted until the gels were fully degraded. In absence of inhibitor, the 4 mg/mL

fibrin gels degraded in about 8-10 days, which is about 50% faster than the 10 mg/ml fibrin gels, which lasted about 15-20 days (Fig. 6C). In contrast, both the 4 and 10 mg/ml fibrin gels containing α2PI degraded at similar speed and lasted around 35 days for both α2PI containing groups (Fig. 6C).

Finally, we performed a similar experiment in the diabetic wound model. Although in this model the differences were more difficult to visualize, we showed that low-concentration fibrin gels without inhibitor degraded in about 12 days, whereas 60% of the gels were still present when α2PI was added (Fig. 6D). In addition, the degradation rates of the 4 mg/mL and 10 mg/mL fibrin gels supplemented with α2PI were the same. With α2PI, more than 50% of the fibrin gels remained in the wounds at the end of the experiments when fibrin degradation stopped (due to wound closure), around day 18 (Fig. 6D).

Overall, these *in vitro* and *in vivo* results demonstrated the very high potency of α2PI to protect fibrin gel even at low fibrinogen concentration.

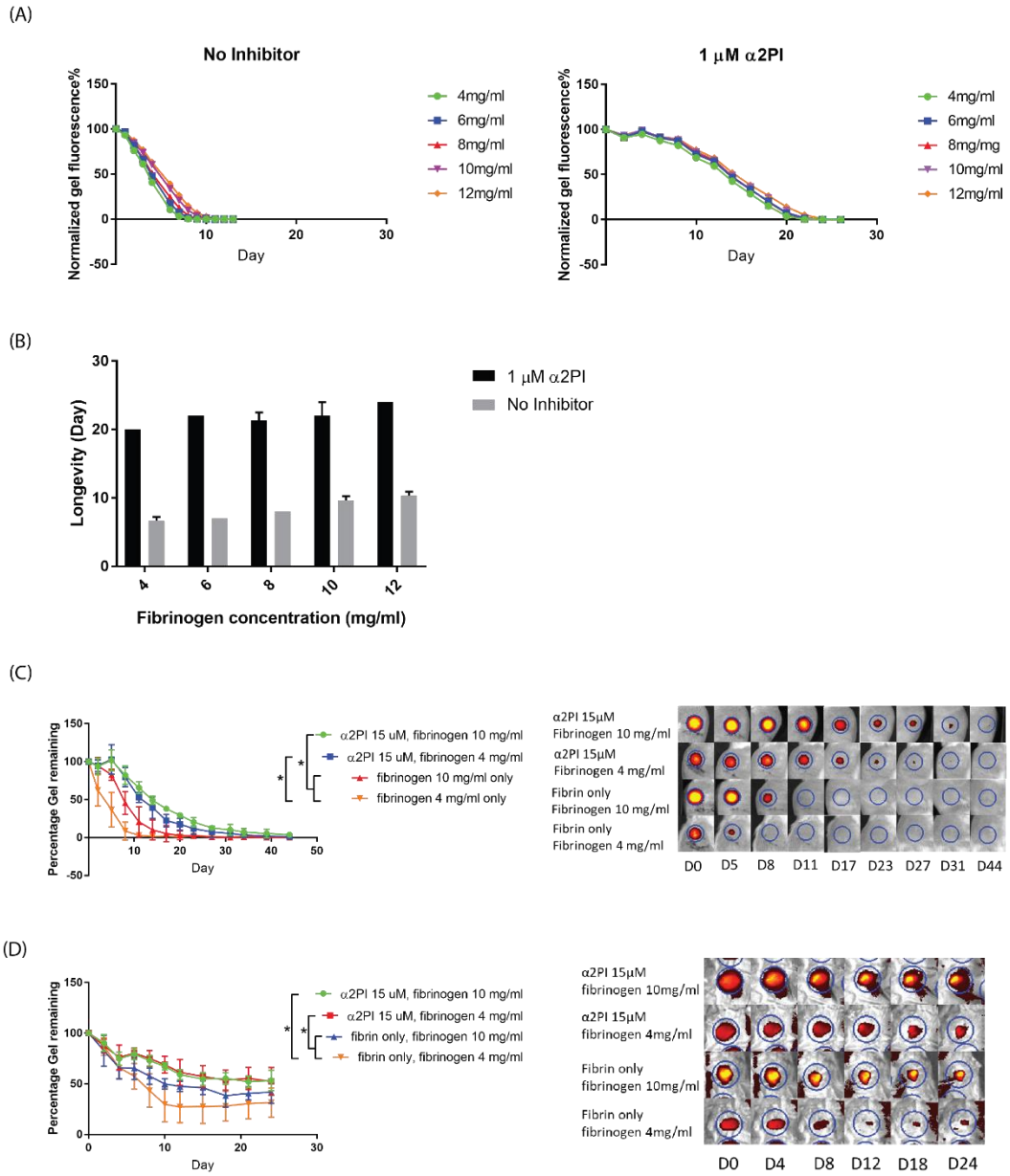

***Fig 6.* α2PI effectively protects fibrin biomaterials at low fibrinogen concentration.** **(A)** Efficacy of α2PI (1 μM) in protecting fibrin gels made of 4-12 mg/mL fibrinogen concentrations in presence of 2.5 nM plasmin *in vitro*. Comparing with fibrin gel without inhibitor, α2PI was able to extend longevity of all groups to similar extent. **(B)** *In vitro* longevities of fluorescent fibrin gels with or without α2PI, determined from the experiment presented in (A). **(C)** *In vivo* degradation of fibrin subcutaneous implants made of 4 or 10 mg/mL fibrinogen and containing 15 μM of α2PI, in Balb/c mice (N=5, *$p < 0.05$, Friedman with Dunns post-hoc test). α2PI was able to effectively prolong longevity of 4 mg/ml gels to the similar extent as the 10 mg/ml gels. **(D)** *In vivo* degradation of fibrin gels made of 4 mg/ml or 10 mg/ml fibrinogen and containing 15 μM α2PI upon topical delivery onto wounds of db/db diabetic mice (N=6, *$p <$ 0.05, Friedman with Dunns post-hoc test). α2PI efficiently extended longevity of 4 mg/ml fibrin gels to the same extent as the 10 mg/ml fibrin gels.

α2PI increased the efficacy of fibrin-delivered growth factors in diabetic wound healing

In addition to its use as a tissue sealant, fibrin has been extensively explored as a carrier materials to deliver growth factors in regenerative medicine. Based on our findings that α2PI can extend longevity of fibrin in wounds, we here explored the use of α2PI for fibrin-mediated growth factors (GFs) delivery in diabetic wounds. Indeed, it has been shown that a slow release kinetics of GFs in wounds strongly enhance their efficacy[20]. Here, we reasoned that the release of GFs from fibrin in wounds is modulated by the rate of plasmin-mediated fibrin degradation, which can be modulated by α2PI.

Therefore, we assessed the efficacy of fibrin-delivered growth factors, with or without α2PI, in the diabetic wound healing model in db/db mice. We treated the wounds with 4 mg/mL fibrin gels containing 200 ng of the vascular endothelial growth factor A (VEGF-A) and 200 ng of the platelet derived growth factors BB (PDGF-BB), both being engineered for super-affinity to ECM proteins, including to fibrin[21]. In addition, the gels were supplemented with 0, 1 or 3 μM of α2PI. At the time of surgery, the fibrin gels were directly polymerized on the wounds, and tissue regeneration was evaluated 10 days later by histomorphometric analysis. The results showed that both 1 μM and 3 μM of α2PI enhanced the effect of growth factors and improved diabetic wound healing. Gels containing 3 μM of α2PI with the growth factors significantly improved wound closure, while the 1 μM α2PI dose led to a trend towards improved wound closure (Fig. 7A). In addition, both these groups significantly improve granulation tissue formation (Fig. 7B, C). Importantly, α2PI in fibrin did not improve wound healing in absence of the growth factors, highlighting that the improved healing was not due to a direct therapeutic effect of α2PI (Fig. 7A-C). Surprisingly, both the wound closure and granulation tissue formation for the α2PI in fibrin treatment group was even less than the fibrin only control,

this could be caused by excess fibrin presence in the wound that cannot be properly degrade during the healing process, or this could due to a lower randomization of the conditions on mice for this group. Thus this result would have to be further repeated to clarify the causes.

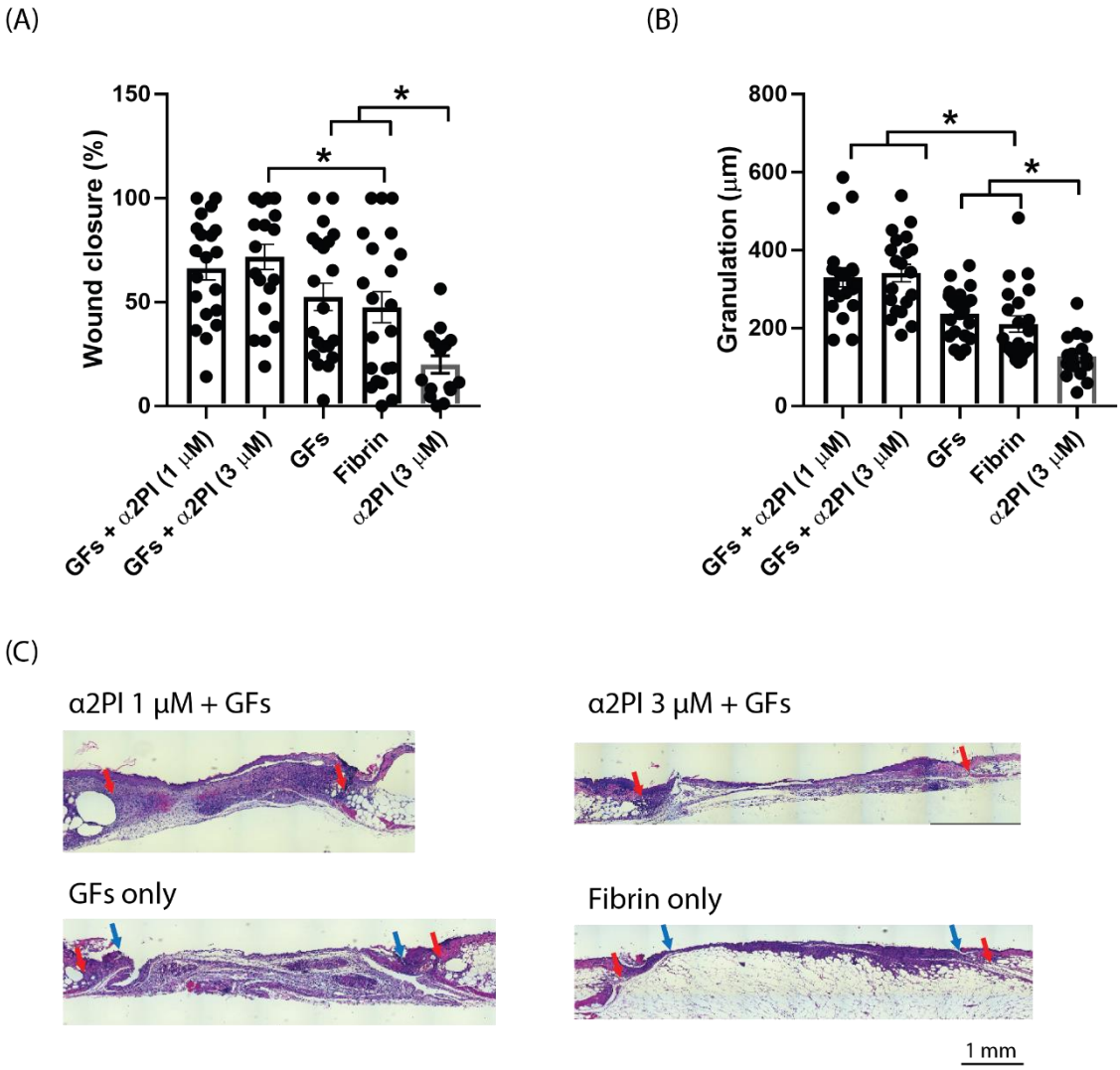

Fig 7. **Effects of α2PI co-delivered with growth factors in fibrin gel in diabetic wounds in db/db mice.** Two different doses (1 and 3 μM) of α2PI was co-delivered with VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM} (here labelled GFs) in 6 mm diameter skin wounds in db/db mice. **(A)** Wound closure and **(B)** granulation tissue formation at 10 days after wounding was quantified with histomorphometric analysis (N=15-20 for each group, mean±SD, *$p<0.05$, one-way ANOVA test). **(C)** Representative histology of the wounds treated with or without α2PI and GFs at day 10 (red arrow: wound edge; blue arrow: epithelium tongue).

α2PI and growth factors co-delivery improves angiogenesis in diabetic wounds

After confirming effects of α2PI co-delivered with growth factors in accelerating diabetic wound healing and improving granulation tissue formation, we further analyzed effects of α2PI and growth factor co-delivery on diabetic wound angiogenesis. We stained the wounds for $CD31^+$ ECs and SMA^+ SMCs. We observed that more neovascularization (stained by CD31 in red) and relatively mature blood vessels (co-stained by SMA in green) were generated for wounds treated by α2PI and growth factor co-delivery at both α2PI concentrations (1 μM and 3 μM) (Fig. 8A and B). Of these two α2PI doses, 3 μM α2PI showed slightly better effects in blood vessel formation than 1 μM α2PI. Specifically, α2PI and growth factor co-delivery improves angiogenesis in the whole wound area, including wound edges and middle of the wounds, while the other groups generally have less blood vessels in middle of the wounds. Since angiogenesis improvement was not observed in the wounds treated with α2PI only, we further confirmed that the angiogenesis effects were due to the delivery of GFs (Fig. 8C). Compared with the group treated with fibrin only, the GFs group also showed improved angiogenesis (Fig. 8D and E). However, efficacy of GFs only in angiogenesis was lower than the α2PI and growth factor co-delivery.

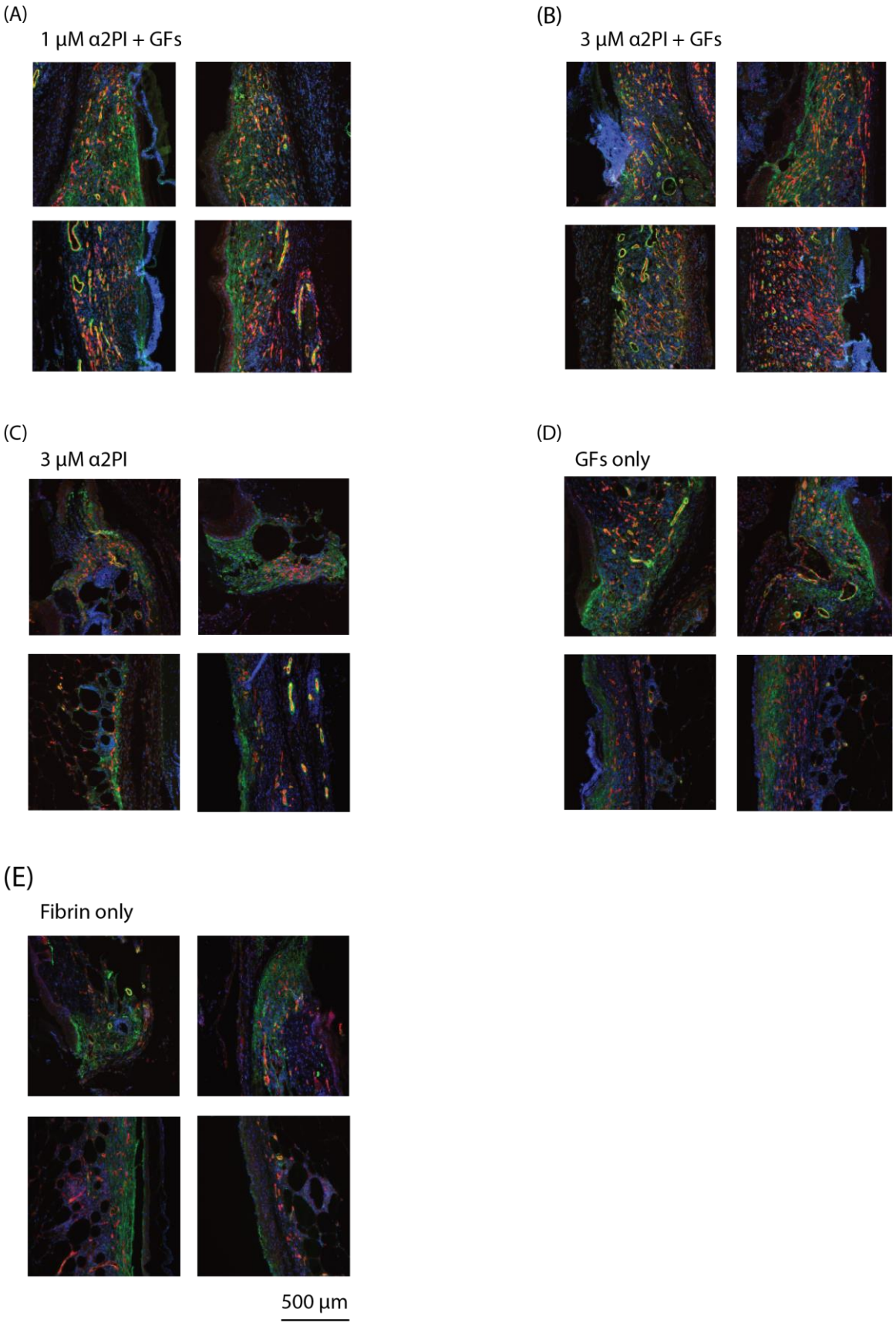

Fig. 8. **Diabetic wound angiogenesis upon co-delivery of α2PI and growth factors.** Back skin wounds in db/db diabetic mice were treated with two different doses of α2PI (1 µM and 3

Figure 8, continued. μM) and VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-$2_{ECM\backslash\backslash\backslash}$ (labeled as GFs). Angiogenesis were assessed via immunostaining of ECs ($CD31^+$), and SMCs (α-SMA^+). Representative images of angiogenesis and blood vessels in immunohistochemical stained wounds of db/db mice are shown for **(A)** 1 μM α2PI co-delivered with GFs; **(B)** 3 μM α2PI co-delivered with GFs; **(C)** 3 μM α2PI only; **(D)** GFs only; **(E)** fibrin only (red: CD31+ ECs, green: α-SMA^+ SMCs, blue: DAPI).

α2PI protected growth factors efficacy upon delivery in fibrin in presence of high plasmin concentration in diabetic wounds

We next explored performance of α2PI in fibrin gel in excessive presence of plasmin, considering that plasmin levels are elevated in diabetic wound, and that in human patients, there could be more wound fluid than in mice wound[22]. We added 50 nM plasmin (Roche) in the 4 mg/mL fibrin gel during the gel preparation, and tested co-delivery of 3 μM and 15 μM α2PI with 200 ng of VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM}. We showed that α2PI co-delivered with growth factors in fibrin gels still showed some positive effects in accelerating diabetic wound healing even at high plasmin concentration. Particularly, 3 μM of α2PI delivered with growth factors showed trends toward improvement of wound closure (Fig. 9A). In addition, the amount of granulation tissue formed in the wounds was significantly higher in the groups containing 3 and 15 μM of α2PI (Figure 9B, C).

In conclusion, we proved that α2PI was able to protect fibrin biomaterials from plasmin-mediated degradation very effectively, and enhanced diabetic wound healing when added to the fibrin carrier used to deliver growth factors in wounds, even in the presence of high concentration of plasmin.

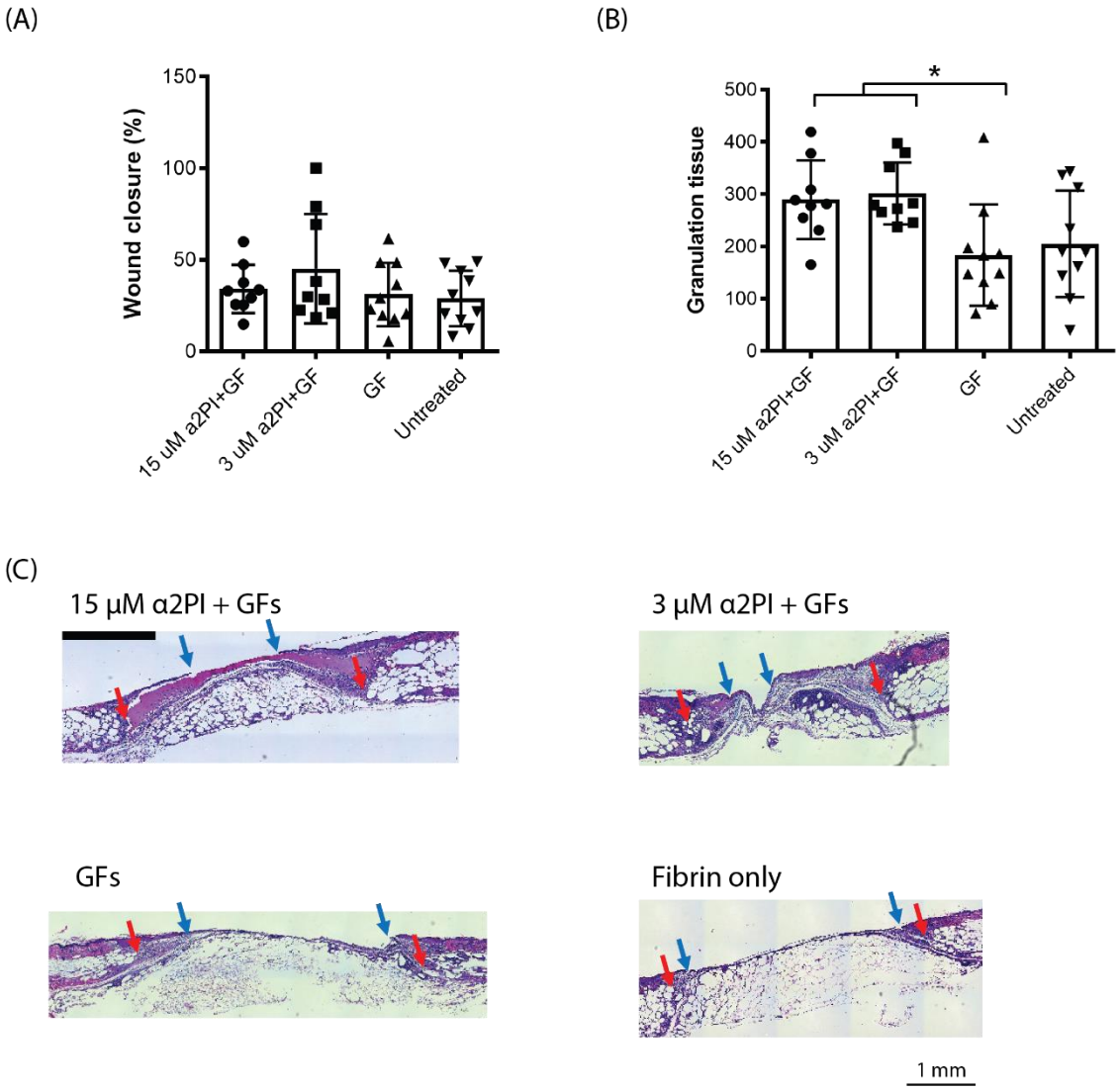

Fig 9. **Efficacy of α2PI and growth factor co-delivered in fibrin gel in diabetic wound healing in presence of high plasmin in the db/db mice.** Two doses (15 and 3 µM) of α2PI were co-delivered with VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM} (here labelled GFs) in fibrin gels supplemented with 50 nM of plasmin, onto 6 mm diameter diabetic wounds in db/db mice. **(A)** Wound closure and **(B)** granulation tissue formation were quantified by histomorphometric analysis at day 10 after wounding (n=10 for each group, mean±SD, *$p<0.05$, one-way ANOVA test). **(C)** Representative histological images of wounds at day 10 after the surgery (red arrow: wound edge; blue arrow: epithelium tongue).

III. 5. Discussion

In this study, we aimed to develop a protease inhibitor that could effectively slow down fibrin matrix degradation. Specifically, we searched for a protease inhibitor that was at least as good as the current clinical protease inhibitor aprotinin and of human origin to solve immunogenicity issue related to the use of aprotinin[9].

Interestingly, the TG sequence extensively used by our group for the cross-linking of biomolecules into fibrin[13] was derived from the human α2-antiplasmin, which is main inhibitor of plasmin in human. Therefore, we reasoned that α2PI was evolutionary developed to be cross-linked into fibrin clot and regulates its degradation, making it an interesting candidate to evaluate as an alternative to aprotinin. Moreover, α2PI has some inhibitory effects on trypsin and chemotrypsin, in addition to plasmin, giving it a relatively broad inhibitory spectrum.

To increase clinically relevance and translatability, it would be valuable if α2PI could be expressed and produced as a recombinant protein at reasonably good yields, rather than being purified from human plasma. Indeed, human-derived products have limited sourcing and an unneglectable risk of pathogen transmission. Therefore, we first focus on recombinant α2PI production and showed successful expression at relatively high yield of 10 mg pure protein per liter of culture in HEK293-F mammalian cells (Fig. 3A, B). Importantly, we demonstrated that our recombinant α2PI has similar bioactivity in inhibiting plasmin as the commercially purchased plasma-derived one (Fig. 3C).

We then compared *in vitro* the ability of α2PI to extend longevity of fibrin gels to the other protease inhibitors, namely aprotinin and TG-KPI. We mimicked the physiological conditions of both the plasmin content and the protease inhibitors concentration to evaluate the efficacy of α2PI. We found that at a concentration of 1 µM, which is a low dosage as compared to the concentrations of TG-KPI and aprotinin previously used in

other literature[13], α2PI was able to extend longevity of fibrin gels of at least twice as compared to the other inhibitors (Fig. 4A).

We then proceed to test the effect of α2PI in resisting fibrinolysis *in vivo*, in a model of subcutaneous implants mimicking potential use in fibrin sealants. Here we confirmed the superiority of α2PI as compared to aprotinin, of about 2-fold extension in fibrin longevity. Interestingly, α2PI implants stays visible for about 35-40 days, which is higher or comparable to the longevity of fibrin stabilized with TG-aprotinin[8] (Fig. 5A). In addition, we studied *in vivo* degradation in diabetic wounds, envisioning an application of fibrin as a carrier material for the delivery of growth factors. In this model, the gels stopped degrading around day 15-18, at which we anticipated the wound to be closed. Despite of that, α2PI slowed degradation of fibrin more efficiently than aprotinin (Fig. 5B).

After confirming the efficacy of α2PI in preventing fibrinolysis both *in vitro* and *in vivo*, we explored the potential of α2PI in protecting low fibrinogen concentration gels. Fibrinogen as a biomaterial is expensive and could generate large clinical costs, and is isolated from human which limits its availability and leads to batch-to-batch variations. Therefore, lowering the concentration of fibrinogen in fibrin gel is important to increase cost-effectiveness. We tested both *in vitro* and *in vivo* on the effects of α2PI in extending longevity of low concentration fibrin gels and we found α2PI protected efficiently gels made of 4 mg/mL fibrinogen against fibrinolysis for about 35 days, to similar extent as gel of 10 mg/mL (Fig. 6C). In the clinic, current fibrin sealant products use a fibrinogen concentration of 50-100 mg/mL, for example in Tisseel (Baxter) and Evicel (Johnson & Johnson), and reported full resorption of the fibrin sealing clot in about 6 days in absence of aprotinin (Evicel) and 14 days when supplemented with aprotinin (Baxter). While it is likely that the use of α2PI could be competitive to these performances and would permit

a reduction of fibrinogen dose, a side-by-side comparison with the clinical products would be valuable. Indeed, degradation in human might occurs at faster speed than in the mice models, in which our 10 mg/mL fibrin gels, rather than 50-100 mg/mL clinical ones, already lasted for 10 days in absence of aprotinin (Fig. 6C).

Finally, we assessed use of α2PI to protect fibrin for delivery of growth factors in diabetic wound healing. We co-delivered α2PI with VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM}, two GFs that our group previously engineered for increased retention in fibrin, in low-dose fibrin gels on diabetic wounds in the db/db mouse, and found that the presence of α2PI improved tissue regeneration, both in terms of wound closure and granulation tissue formation in the wound bed (Fig. 7). The α2PI and growth factor co-delivery also improved angiogenesis as compared to GFs only and to α2PI only (Fig. 8). The lack of effects in absence of the GFs suggests that α2PI did not have a therapeutic effect on wound healing per se but rather indirectly enhanced the efficacy of the GFs by controlling their sustainable release in the wound, via slowing fibrinolysis. In addition, we found that α2PI effectively protected fibrin and the delivered GFs in an environment with high plasmin concentration (Fig. 9), which could better mimics human diabetic wounds.

Overall, we concluded that α2PI was able to be used in fibrin biomaterials to efficiently protect them from plasmin-mediated degradation *in vitro* and fibrinolysis *in vivo*, and could enable lowering the concentration of fibrinogen in fibrin gel while maintaining an extended longevity of fibrin gel. Based on these efficacy data, α2PI could be a competitive drug to aprotinin for use in fibrin sealants, in addition to be more safe because less immunogenic due to its human origin. In addition, α2PI could be used to control the sustained delivery drugs via fibrin to improve their efficacy, as we here exemplified with the delivery of GFs in diabetic wound healing.

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IV. α2-ANTIPLASMIN PROTECTS ENDOGENOUS FIBRIN AND ENHANCES DIABETIC WOUND HEALING AND HOMEOSTASIS DURING SURGERY

IV. 1. Abstract

Physiologically, fibrin plays important role in blood coagulation and wound healing. In presence of elevated levels of plasmin, fibrin degradation occurs prematurely which can lead to hemorrhages and healing complications. While we previously demonstrated that the use of α2PI, a potent inhibitor of plasmin, can effectively protect exogenous fibrin in tissue sealants or in carrier biomaterials, we here asked whether α2PI can be used for endogenous fibrin protection. Particularly, we explored α2PI uses in diabetic wound healing and in hemostasis during surgery. We first showed that α2PI bound to and effectively protected endogenous fibrin clots, both in healthy and diabetic conditions. In diabetic wounds, we demonstrated that the co-delivery of α2PI with GFs improved granulation tissue formation, thus enhancing the regenerative effects of the GFs. Finally, we highlighted a promising efficacy of α2PI as a hemostatic agent to reduce bleeding loss during surgery, thus addressing one of the major cause of blood transfusion in coronary artery bypass graft surgery (CABG). Overall, we believed that α2PI could become an effective clinical drug in multiple applications of regenerative medicine, in which protection of endogenous fibrin positively affects the outcomes.

Keywords: *Endogenous fibrin, α2-antiplasmin, hemostasis, diabetic wound healing*

IV. 2. Introduction

Fibrin plays an important role in both wound healing and hemostasis. In the wound healing process, fibrin provides the initial matrix to support proliferation and migration of cells[1]. Fibrin also serves as reservoir for growth factors and cytokines, especially some GFs involved in angiogenesis, such as FGF-2, VEGF and PDGF-BB[2][3][4], which improve vascularization and healing. Literature shows that in fibrinogen deficient wounds, granulation tissue formation is not adequate for closing wound gap, and cells are not able to organize and migrate properly in the wounds[1]. Therefore, endogenous fibrin is key for wound healing, especially for the formation of granulation tissue.

Diabetic wounds are highly proteolytic comparing to normal wounds. Specifically, the level of plasmin, which closely related to fibrin degradation, are elevated. Plasmin is overexpressed in diabetic wounds due to increase neutrophils and macrophages[5]. Levels of activators of plasminogen are also enhanced in diabetic wounds, for example, the urokinase plasminogen activator (uPA)[6]. Besides, the decrease of plasmin inhibitors α1-protease inhibitor also leads to the proteolytic environment in diabetic wounds[6]. With the elevation of plasmin level in diabetic wounds, endogenous fibrin, as well as other endogenous matrix proteins and growth factors are fast degraded, which cause delayed wound healing.

In addition to its role in wound healing, endogenous fibrin also has significant effects in hemostasis. The rate of endogenous fibrin degradation regulates to thrombosis and coagulation processes[7]. In the coagulation process, fibrinogen in plasma converts to fibrin upon enzymatic processing by thrombin and factor XIIIa, to form the fibrin clot and induce platelet aggregation, which leads to coagulation of the blood[8].

In some circumstances, due to pathologies or medical procedures, the rate of fibrinolysis is unbalanced which leads to hemorrhages or thrombosis. For example, an increase in plasmin activity is a major reason that causes bleeding in cardiac surgeries. In CABG surgery, the tissue plasminogen activator (tPA) level is elevated, leading to 11-fold increase of plasmin generation[9]. The increased plasmin activity caused degradation of fibrin that prevents coagulation during the surgery. Besides, surgical blood loss further causes depletion of fibrinogen in blood[10]. Overall, the abnormal endogenous fibrinolysis by plasmin causes excessive blood loss during CABG that increases the need of patient blood transfusion[11].

Currently, aprotinin, a broad spectrum serine protease inhibitor of bovine origin is used clinically as a hemostatic agent. Trasylol (aprotinin injection) was approved by FDA in 1998 as treatment for blood loss during CABG surgery. However, as aprotinin is not of human origin, it has the risk of causing side effects related to its immunogenicity. For patients re-exposed to aprotinin within 6 month of the first administration, incidence of anaphylactic reaction is 5%, and this incidence is still as high as 2.8% for re-exposure in 12 months[12][13]. The anaphylactic reaction includes skin eruptions, itching, dyspnea, nausea and tachycardia, and can be as serious as fatal anaphylactic shock with circulatory failure[14]. Due to these side effects, supplies of Trasylol was removed from hospitals and pharmacies in 2008, and Trasylol is now only available for use as investigational drug under special treatment protocol.

In previous studies, we already found that α2PI is able to effectively protect fibrin biomaterials both *in vitro* and *in vivo*. Thus, we here hypothesized that α2PI can also protect endogenous fibrin, and we explored its use in applications where fast endogenous fibrin degradation impair the outcomes, particularly in diabetic wound healing and for bleeding reduction during surgery. In this study, we found that α2PI can protect

endogenous fibrin in healthy and diabetic plasma, and showed that α2PI co-delivery with GFs to the diabetic wound site improves granulation tissue formation in absence of exogenous carrier material. In addition, we demonstrated that intravenously injected α2PI was able to reduce both bleeding time and bleeding volume in a bleeding model in mouse. The hemostasis effects of α2PI was comparable to the clinical hemostasis drug aprotinin. These together indicates that α2PI could be used in multiple applications of regenerative medicine in which endogenous fibrin protection is needed, particularly in diabetic chronic wound healing and bleeding reduction during surgery.

IV. 3. Materials and Methods

Fluorescent conjugation of α2PI and albumin

Fluorescent α2PI and albumin were prepared by mixing Alexa Fluor 647 NHS ester (Invitrogen) with α2PI or albumin at 10:1 molar ratio in 0.1 M sodium bicarbonate buffer. The mixing continued at room temperature for 2 h with continuous shaking. The fluorescent labeled protein was purified using Zeba Spin Desalting Columns (Thermo Scientific) to eliminate unconjugated free dyes, and then stored at -80°C.

Binding of α2PI to fibrin on mice ear wounds

All the animals used in the experiments were approved by University of Chicago IACUC. NOD mice were used in this experiment. Isoflurane was applied to the mice by inhalation as anesthetization, with 4% at beginning, and 2% for maintenance. 150 µL of 10 mg/mL 488 labelled fibrinogen was intravenously injected to each mice prior to the surgery. The surgery started 10 minutes after the injection of fluorescent fibrinogen. During the surgery, the top skin and cartilage of the mice ears were removed with blade. 20 µL of the fluor AF647 labeled α2PI or albumin at a concentration of 15 µM was pipetted on the ear and incubated for 15 minutes under coverslips to prevent drying. After the 15 minutes, the mice were euthanized and the ears were dissected and washed with 50 mL TBS buffer for 2 h at 4 °C. The wash process was repeated twice to remove unbound fluorescent proteins. After the washes, the mice ears were fixed overnight using a zinc fixative buffer. The fixed ears were washed with TBS, cleared and mounted with BABB (Benzyl Alcohol/Benzyl Benzoate) for imaging.

Binding of α2PI to endogenous fibrin on mice back skin wounds

Wounding was performed on C57BL6 mice in this experiment. Isoflurane was applied to the mice by inhalation as anesthetization. Mice were injected with 0.1 mg/kg of buprenorphine as analgesia. Two wounds were created on the back of each mouse using 6 mm punch biopsy, with one wound on each side of the spine. After the wounds were created, 40 µL of AF647 labeled α2PI or albumin (control) at concentration of 15 µM were pipetted to the wounds, and stayed on the wound for 5 minutes. The mice were then euthanized, and the wounds were washed with 50 mL of PBS on ice for 1 h. The wash process was repeated for 3 times. After the wash process, the wounds were fixed with 4% PFA in PBS and then washed again with PBS. The fixed wounds were mounted for fluorescent imaging.

Plasma extraction from mice

To obtain plasma, blood was withdrawn from heart of the mice with syringe right after the mice were euthanized. The whole blood of mice was mixed with sodium citrate (100 mM, pH 7.4-7.8) at 9:1 ratio to prevent coagulation. After the mixing, the blood was centrifuged at 1500 xg for 10 min. The plasma supernatant was collected and frozen at -80 °C for long-term preservation.

Proteolytic fibrinolysis of plasma gel *in vitro*

Plasma gels (100 µL) were made of mice plasma (from C57BL6 mice or db/db mice) added with 0.18 mg/mL fluorescent fibrinogen to enable visualization in fluorescent imaging. To induce clotting, 25 mM $CaCl_2$ in HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6) was mixed with the fluorescent plasma mix, containing the plasma, flourescent fibrinogen and in 5 µM of α2PI (Sigma-Aldrich). In some conditions, 4 U/mL

thrombin, 8 U/mL Factor XIIIa were also added to potentially increase clotting. All the materials above were mixed and polymerized on paraffin films, and incubated for 1 h at 37°C with 5% CO_2 to ensure complete polymerization. The plasma gels were then transferred into a 24-well cell culture plate, 1 gel per well, and incubated in 1 ml release buffer (Tris 20 mM, NaCl 150 mM, 0.1% BSA, Pen/Strep, pH 7.4) containing 25 nM plasmin (Roche). The plate was kept at 37 °C with 5% CO2 until all the gels were fully degraded. The plasmin-containing buffer was daily refreshed. The quantification of the plasma gel volume was the same as described in Chapter 1 (proteolytic fibrinolysis of fibrin gel *in vitro*).

Skin wound healing of db/db mice without fibrin gel

The surgical process and creation of the wounds were the same as described in Chapter 1. After the wounds were created, 30 μL of HEPES buffer containing VEGF-A-PlGF-2_{ECM} (500 ng/wound), PDGF-BB-PlGF-2_{ECM} (500 ng/wound), and α2PI (15 μM) was pipetted onto the wound sites. Five minutes after application, the top of the wounds was covered by hydrofilm (Hartmann), with edge of the hydrofilm sealed by 1 x Histoacryl BLUE glue (B. Braun Surgical).

Tail bleeding model in mice for *in vivo* coagulation test

C57BL/6 mice were used in this model. Isoflurane was applied to animal by inhalation as anesthetization, with 3.5% concentration. The animal was placed on a 32 °C heating plate during the surgery to maintain body temperature. At the beginning of the surgery, 100 μL of protease inhibitor (178 μM) or HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6) was given to the mice by retro-orbital intravenous injection. 30 s after the injection, the mice tails were fully transected at 1 mm from tip of the tail. The tail was

then submerged in room temperature PBS. Two minutes after transection of the tail, another 100 µL of protease inhibitor (178 µM) or the HEPES buffer dose was injected to the other eye of the mice via retro-orbital injections. Bleeding of the tail was observed up to 20 min after the transection. A bleeding time of 20 min was assigned to the mice that were not able to stop bleeding after 20 min.

Blood volume quantification

During the *in vivo* coagulation experiment, the blood loss upon transection of the tail was collected into PBS. The total number of red blood cells (RBCs) was counted using a hemocytometer. The total number of RBCs was used as the indication of blood volume loss.

Diabetic wound healing model in db/db mice using plasma gel

Plasma gel was prepared 1 to 2 h before the surgery, as described previously. The plasma gels were kept in humid chamber until use. The details of the surgery process and animal care are the same than described in Chapter 1 on db/db mice. After the wounds were created, the plasma gel was transferred onto the wounds to completely cover the wound. 6 mm splints were then glued to the surrounding of the wounds using 1 x Histoacryl BLUE glue (B. Braun Surgical) with hydrofilm (Hartmann) covering the top of the splints. Some holes were created on the hydrofilm to enable ventilation.

IV. 4. Results

α2PI bound to endogenous fibrin *in vivo*

We first verified if α2PI could cross-link into endogenous fibrin *in vivo* upon topical application on wounds. In these studies, we used albumin, a protein that cannot crosslink to fibrin as a control for unspecific binding. To visualize co-localization of α2PI with fibrin, we injected non-obese diabetic (NOD) mice intravenously with fluorescently labeled fibrinogen prior to wounding. After injecting fluorescent fibrinogen, fluorescent AF647-labelled α2PI or albumin was applied to the ear wounds and stayed on the wounds for 15 min. The binding was then assessed via fluorescent microscopy imaging after the ear wounds were washed and fixed. We found that α2PI showed strong binding to fibrin, while the binding was not observed in the albumin control group (Fig. 10A). Importantly, we aimed to confirm binding to diabetic mice endogenous plasma envisioning future application in diabetic wound healing.

As a next step, we investigated if α2PI was able to bind to and protect endogenous fibrin in the model of back skin wounds using wild-type C57BL6 mice. In this study, fluorescent AF647 labeled α2PI, albumin or PBS control was applied to the wounds immediately after the surgery, and stayed on wounds for 5 minutes. After fixing and extensive washing to remove unbound proteins, we performed fluorescent microscopy imaging to assess the presence of α2PI and albumin. Similarly to in diabetic mice, we found that α2PI is able to bind to the endogenous fibrin and stay at the wound site, while the albumin treatment group does not show any difference from the PBS control (Fig. 10B). Together, these results indicated that α2PI was able to retain into fibrinogen upon topical application *in vivo* in healthy and diabetic mice, setting the rationale for its use to protect endogenous fibrin from proteolysis.

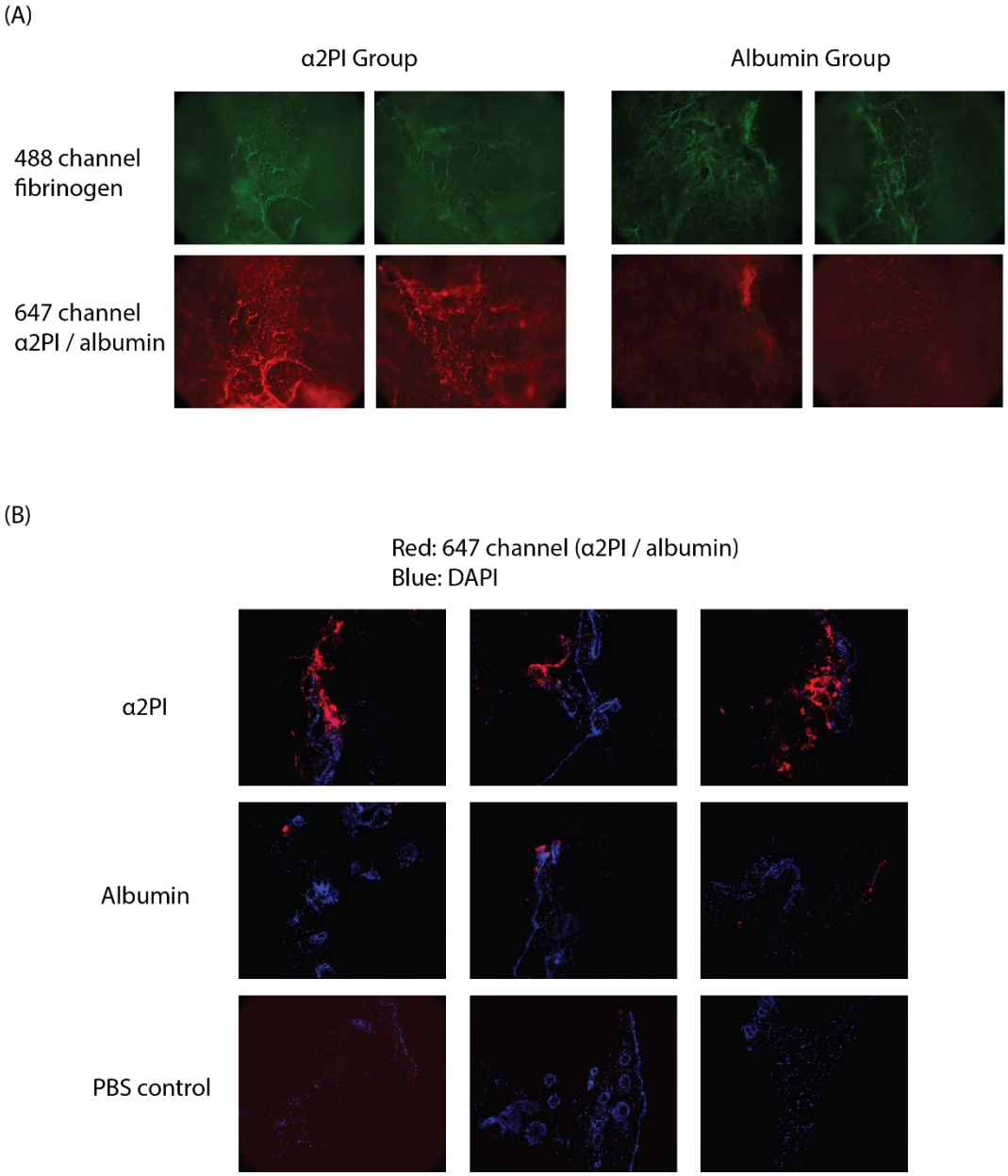

Fig 10. **Binding of α2PI to endogenous fibrin *in vivo* in mice wounds.** Fluorescent labeled α2PI was applied to NOD and C57BL6 mice wounds topically immediately after wounding to assess its binding to endogenous fibrin *in vivo* (N=3-5 wounds per experiment). **(A)** Immunohistochemistry image showing the binding of α2PI and albumin control to fibrinogen on NOD mice ear wound. Fibrinogen was conjugated with 488 fluorescence and shown in green; α2PI and albumin were conjugated with 647 fluorescence and were shown in red. α2PI bound better than albumin (here used as a control for unspecific binding). **(B)** Immunohistochemistry image showing the binding of α2PI and albumin control to endogenous fibrin in back skin wound in C57BL6 mice. DAPI staining was shown in blue; α2PI and albumin were conjugated with 647 fluorescence and were shown in red. α2PI retained in the fibrin clot in wounds in contrast to albumin.

α2PI is able to protect endogenous fibrin from degradation

After confirming the binding of α2PI to endogenous fibrin *in vivo*, we investigated effects of α2PI in protecting endogenous fibrin from plasmin-mediated fibrinolysis *in vitro*. In this study, we made plasma gels directly polymerized from the mice plasma, and assess efficacy of α2PI to prevent their degradation via fibrinolysis assays.

We first evaluated the efficacy of α2PI in plasma gels with or without addition of the enzymes (Thrombin, Factor XIIIA), considering some endogenous enzymes are already present in the plasma, and compared the effects of α2PI on fibrin gels made of purified fibrin. We observed that even without addition of protease inhibitor, plasma gels has a longer longevity than the fibrin gels of comparable fibrinogen concentration (2-3 mg/mL). Besides, though with enzyme added, longevity of plasma clot is 3 days longer than the plasma clot made without addition of enzyme, after the addition of α2PI, the longevity of both types of plasma gel were extended to similar extent (Fig. 11A).

We then assessed and compared performance of α2PI on C57BL6 mice plasma and diabetic db/db mice plasma to see if the pathology impacts endogenous fibrin stability. In the study, we found that db/db mice plasma gels had a 2 days shorter longevity than the C57BL6 mice ones, which could be caused by an excess of plasminogen (the precursor of plasmin) content in db/db mice plasma (Fig. 11B). With the addition of α2PI, the longevity of the plasma gels were both extended. The plasma gels made of C57BL6 mice plasma stayed for 9 to 11 days with α2PI, and the db/db plasma gels with α2PI stayed for 8 to 9 days (Fig. 11B). Thus, α2PI showed its efficacy in protecting endogenous fibrin clots from both healthy and diabetic mice.

Next, we performed a dose study to explore impact of α2PI dosage on its efficacy to protect endogenous fibrin. We compared longevity of plasma gels with 5 μM α2PI and 9 μM α2PI *in vitro* using the same fibrinolysis assay. We found that 9 μM α2PI can extend

plasma clot longevity to longer than 5 μM α2PI, but that this difference was relatively small, of about 2 days (Fig. 11C). Besides, the remaining of the plasma clot through the degradation process was also similar for the 5 μM α2PI group and the 9 μM α2PI group. These results showed that relatively small dosage (5 μM) α2PI could be adequate for protection of endogenous fibrin.

Overall, based on the strong protective effects of α2PI on endogenous fibrin, we further aim to explore applications for α2PI as diabetic wounds healing as well as in hemostasis upon wounding.

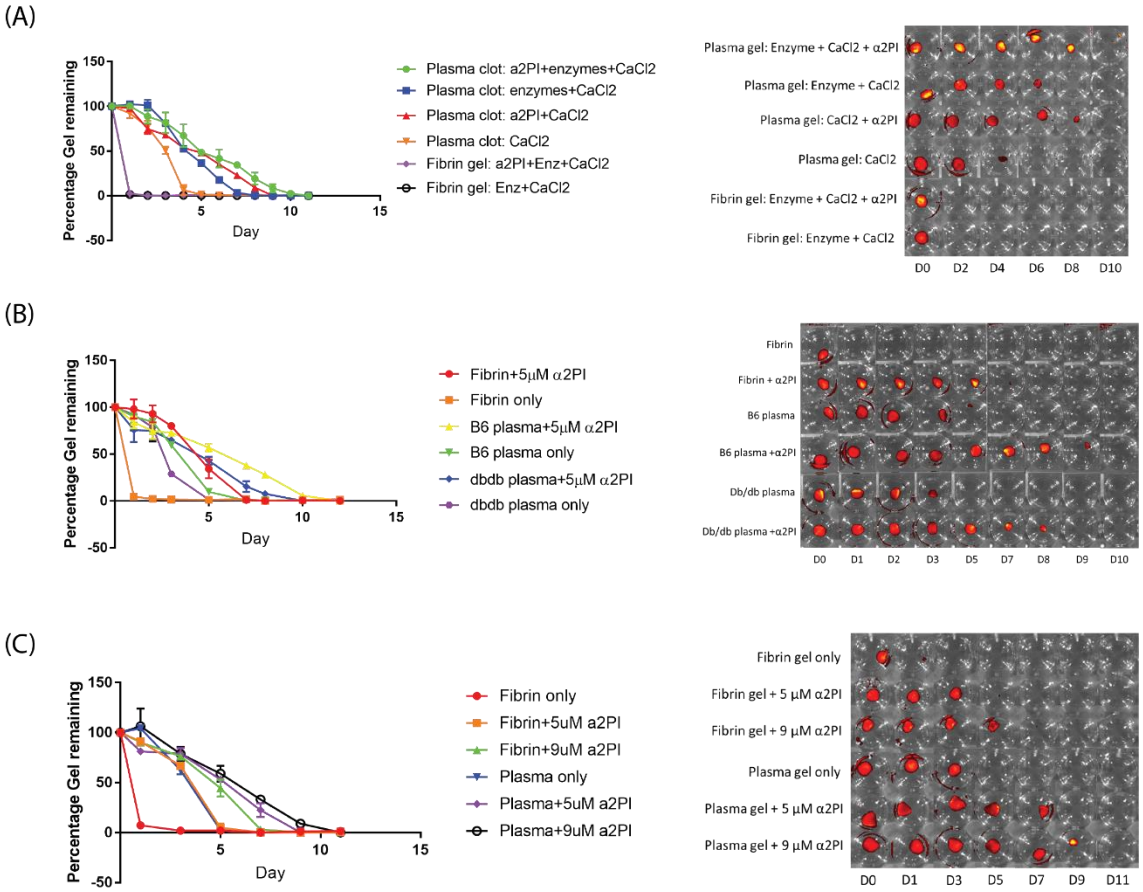

***Fig 11.* α2PI protects endogenous fibrin from plasmin-mediated fibrinolysis *in vitro*.** **(A)** Fluorescent C57BL6 mice plasma gels and fluorescent fibrin gels containing 5 μM α2PI were incubated in 25 nM plasmin, in which plasma gels were made with addition of thrombin, Factor XIIIA and $CaCl_2$, or with addition of $CaCl_2$ only. Gel degradation was quantified via fluorescence decay measurements. Independently of the addition of the enzymes, the plasma gels had longer longevity than fibrin gels, further improved by the presence of α2PI. **(B)** Fibrinolysis of plasma gels made by C57BL6 mice plasma and db/db mice plasma were compared. Both types of plasma gels were incubated in 25 nM plasmin with or without addition of 5 μM α2PI. The longevity of db/db mice plasma gels was shorter than the C57BL6 mice ones, and α2PI was able to effectively extend longevity of both plasma gels types. **(C)** Dose study of α2PI was performed on the C57BL6 mice plasma gels containing 5 μM or 9 μM of α2PI. The longevity of fibrin gel extended with the increase of α2PI dose, but the difference between the two doses remained limited.

α2PI enhanced the delivery of GFs from endogenous fibrin and supported granulation tissue formation in diabetic wounds

In diabetic chronic wounds, both the plasmin content and the plasminogen activator levels are elevated, which leads to excessive plasmin activity[15][5]. The plasmin activity could cause degradation of endogenous fibrin in wounds, impairing the wound healing process. In the previous section, we have also shown that endogenous fibrin in diabetic plasma degraded faster. Therefore, we would like to examine if α2PI co-delivered with growth factors could improve diabetic wound healing.

In this experiment, we delivered VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM} with α2PI in HEPES buffer onto db/db mice back wounds immediately after wounding. The wounds were dissected and analyzed via histology on day 7 after the surgery. We found that α2PI co-delivered with the growth factors did not improve wound closure (Fig. 12A), however, increase of granulation tissue formation can be observed for the α2PI and growth factors co-delivery group (Fig. 12B). The increased granulation tissue can be visualized from the representative images as shown by the purple layer between the wound edges: the thickness of the granulation tissue layer is larger for the α2PI and growth factor co-delivery group than for the other two groups (Fig. 12C).

These results showed that α2PI supported the delivery of the GFs in the wounds and enhanced their effects in increasing granulation tissue, which is a proper sign of healing. Nevertheless, further optimization in dosing and frequency of treatments would be needed to evaluate whether the co-delivery of α2PI and GFs in absence of additional carrier material could ameliorate wound closure.

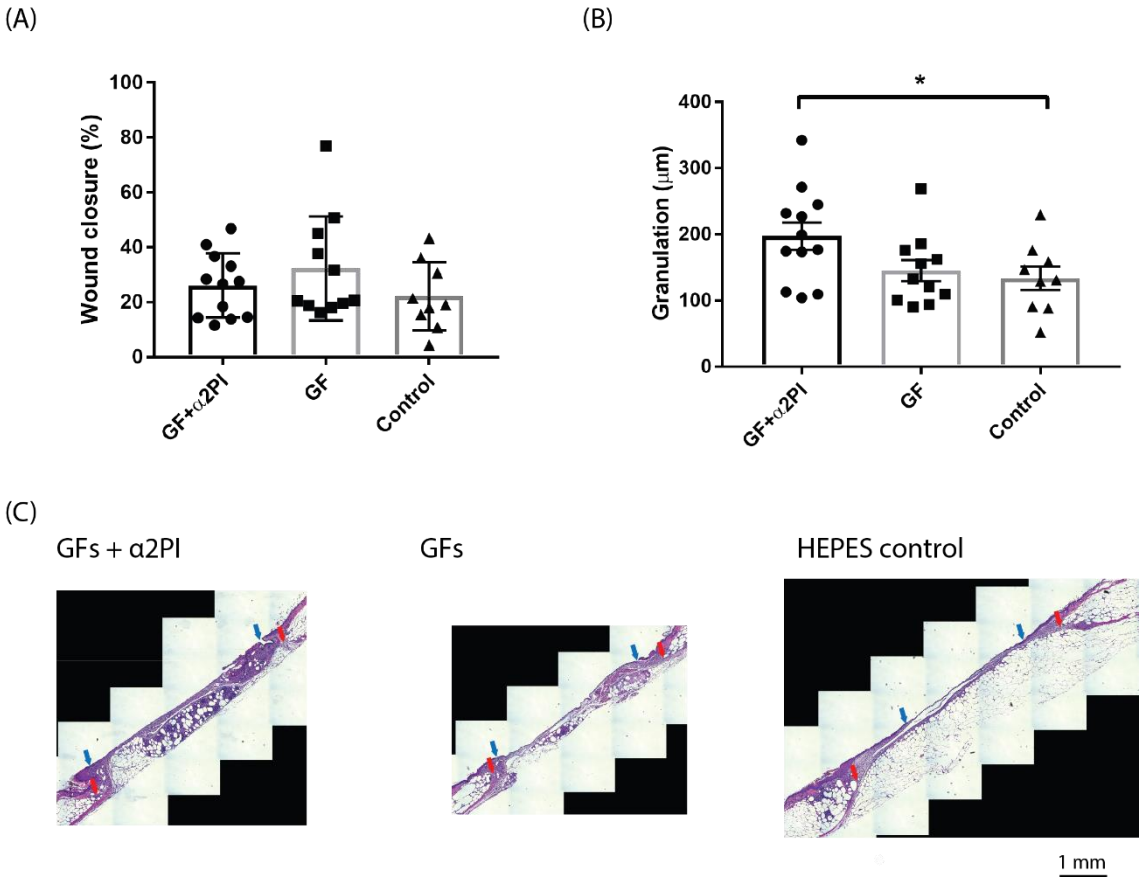

Fig 12. **Efficacy of α2PI in delivering growth factor from endogenous fibrin in diabetic wound healing in db/db mice.** α2PI was co-delivered with VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM} (= GFs) to 6 mm diameter skin wounds in db/db mice. **(A)** Wound closure and **(B)** granulation tissue formation 7 days after the surgery was quantified by histomorphometric analysis (n=8-12 for each group, mean±SD, *p<0.05, one-way ANOVA test, Control = HEPES-treated wounds). **(C)** Representative histology for the db/db mice wounds at day 7 after surgery (red arrow: wound edge; blue arrow: epithelium tongue)

α2PI reduced bleeding when used as a hemostatic agent during surgery

Another application of α2PI we explored was its effects in hemostasis. Indeed, while we previously found that α2PI was a competitive drug to aprotinin for application in fibrin sealants, one of the main application of the clinical aprotinin is as a hemostatic agent for intravenous delivery during CABG. In fact, the elevated plasmin activity during CABG causes blood loss and increase the need for blood transfusion to the patients[11]. Thus, we would like to investigate if α2PI could be used to protect endogenous fibrin clotting and reduce bleeding in such context.

To do so, we established a tail bleeding model using C57BL6 mice to measure coagulation upon vein transection. In this model, we delivered α2PI, the clinically used aprotinin, or HEPES buffer as a control via retro-orbital intravenous injections. The bleeding time and bleeding volume were assessed. We observed that α2PI was able to significantly reduce bleeding time, a little more effectively than the clinical hemostatic aprotinin drug (Fig. 13A). We also quantified blood loss volume via the total red blood cell count in the lost blood, and found that α2PI significantly reduced blood loss volume to similar extent than aprotinin (Fig. 13B). The effects of α2PI in reducing blood loss can also be visualized via the intensity of red coloring of the collected blood at the same dilution volume (Fig. 13C).

In these bleeding tests, we decided to set the experimental endpoint at 20 min after the tail was cut for practical reasons, although at this time many of the HEPES control group mice were still bleeding strongly. By doing so, we tended to underrepresent the efficacy of the drug, and thus wanted to assess the actual bleeding time for mice in the control group in absence of hemostatic agents, in a small number of animals. Therefore, in the next sets of bleeding tests, we allowed 5 mice in the HEPES control group to bleed until the bleeding stop. We found that without hemostasis agent, all mice bled for much longer

than 20 min; the average bleeding time was actually more than 90 min, and 3 out of the 5 mice tested kept bleeding until death (Fig. 13D). We also measured the corresponding bleeding volume. The bleeding volume has high variance among the groups, but the median bleeding volume is still as high as 716 million cells, around 5 times of the average bleeding amount of the α2PI group (Fig.13E).

In conclusion, we here demonstrated in mice that α2PI could be as effective as the clinical aprotinin in inducing hemostasis by acting directly on endogenous fibrin upon intravenous injection. We believe that this finding is particularly important considering the human origin of α2PI, which could be then developed as a safe substitute to the bovine aprotinin to prevent the associated adverse immunological reactions.

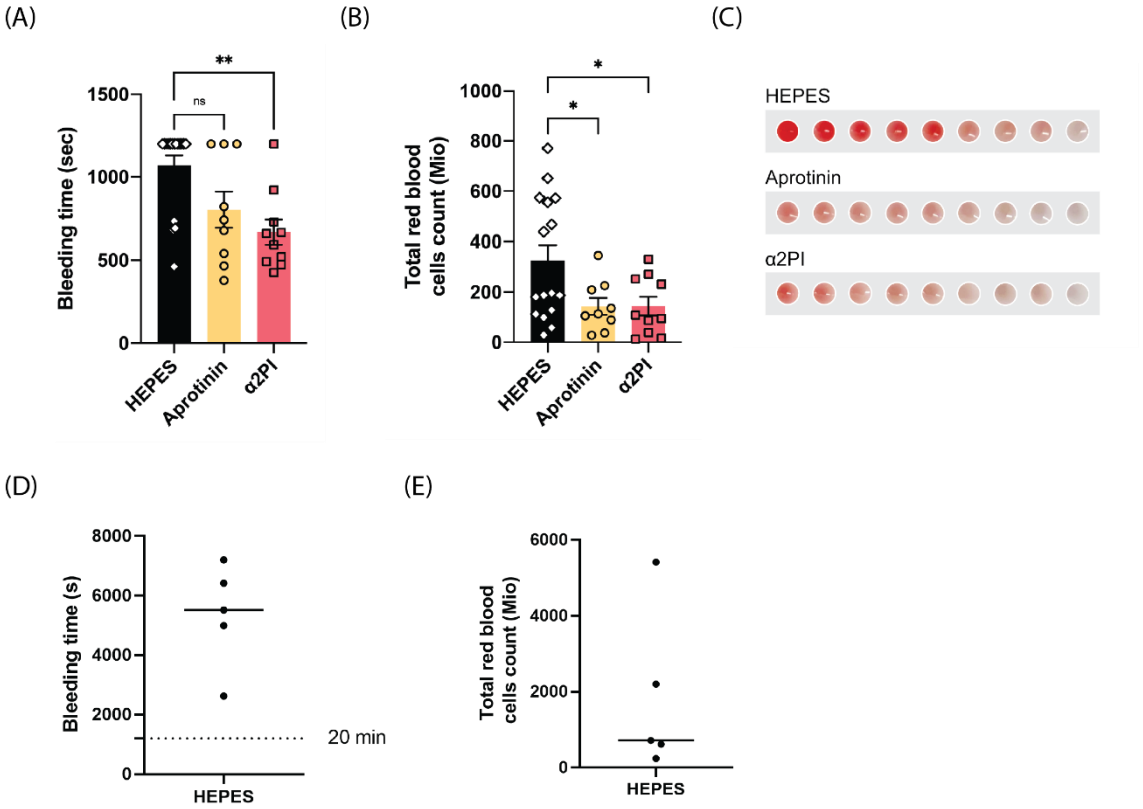

***Fig. 13.* Effects of α2PI in reducing bleeding in a tail bleeding model in C57BL6 mice.** α2PI or aprotinin were intravenously injected retro-orbitally in C57BL6 mice, whose tails were cut at 1 mm from the tip and the bleeding time and loss volume were measured. **(A)** Bleeding time was measured from the time of the tail cut until the bleeding stopped or up to 20 min (N=9-10, *p < 0.05, Kruskal-Wallis with Dunns post-hoc test). α2PI was able to effectively reduce the bleeding time. **(B)** Bleeding volume was quantified by the total red blood cell numbers counted the lost blood of the mice. The experiment stopped 20 min after the tail was cut even if the bleeding did not stop. Both α2PI and aprotinin were able to reduce bleeding volume. **(C)** Representative image of the collected blood in PBS. Each well represent the collected blood from a mouse belonging to the corresponding group. **(D)** Bleeding time was measured for 5 mice in the HEPES control, without a stopping timepoint at 20 min. The bleeding generally continued for over 1.5 h, with the longest bleeding time at over 2 h. **(E)** Bleeding volume was quantified by total red blood cells count for the 5 mice in HEPES groups presented in (D). There was a large variation for the bleeding volume, with the maximum bleeding as high as over 5 000 million RBCs.

IV. 5. Discussion

In this study, our goal was to investigate effects of α2PI on endogenous fibrin protection, and then explore potential applications of α2PI based on these effects. As in Chapter 1, we have shown that α2PI was able to protect fibrin biomaterials from degradation, we reasoned here that α2PI should also be able to similarly protect endogenous fibrin and that we could use α2PI in the applications where the endogenous fibrin needs to be protected.

To prove binding of α2PI to endogenous fibrin *in vivo*, we applied fluorescence conjugated α2PI both on mice ear wounds and on back skin wounds and found that after staying at the wound site for 5 to 15 min, α2PI was able to show binding to both the intravenously injected fibrinogen and the endogenous fibrin *in vivo* (Fig. 10A, B). Although these results were expected considering that α2PI physiological function is protection of fibrin clots, confirming them upon delivery of recombinant α2PI was meaningful to prove its potential to be used as a drug when delivered exogenously.

We then proceeded to test efficacy of α2PI in protecting endogenous fibrin via *in vitro* fibrinolysis assays on gels made from unpurified mouse plasma, showing protection of endogenous fibrin in plasma by α2PI. Interestingly, we found that α2PI is able to protect endogenous fibrin in plasma without the addition of exogenous crosslinking enzymes (thrombin and factor XIIIa), suggesting that their natural physiological concentrations of were sufficient to induce clotting *ex vivo* too. In addition, we found that plasma gels degraded slower than gels made of purified fibrinogen of similar concentration, which could be explained by the presence of endogenous α2PI naturally contained in the plasma at a concentration of about 1 μM, and which is likely removed during the fibrinogen purification process. Performing a dose study, we showed that efficacy of α2PI increased

with the increased dose, yet that a relatively small dose of 5 μM could already show strong effects in resisting plasma gels degradation (Fig. 11C). Finally, we compared sustainability of gels made of plasma derived from diabetic and non-diabetic healthy mice, and observed that plasma gels from diabetic mice degraded faster upon plasmin exposure than ones of the non-diabetic mice. This observation can be explained by the dysregulation of the proteolytic environment in diabetic wounds, as described in literature[15]. Nevertheless, addition of α2PI in the diabetic plasma gels still showed efficacy in extending their longevity (Fig. 11B).

While doing these experiments, we envisioned a scenario in which patient-derived plasma gels could be used as a biomaterial for clinical application. In contrast to exogenous clinical fibrin which is purified from human, expensive and associated with risk of pathogens transmission, plasma clots derived from the patient might provide some advantages, such as the presence of endogenous clotting enzymes and α2PI, the easiness of the blood sampling procedure to collect the plasma, the availability of the material and the absence of risk of disease transmission. Nevertheless, such patient-based materials might have some high variability in clotting properties depending on the patient conditions, e.g. age, pre-existing pathologies. In a future goal, we aim to use the plasma gels as a scaffold for the delivery of growth factors in diabetic wound healing specifically, and so we here performed some preliminary evaluation on the comparison of healthy vs. diabetic mouse-derived plasma and to prove that addition of α2PI can protect diabetic plasma gels. In addition, we demonstrated that α2PI can crosslink in fibrin which questioned if the co-delivery of growth factors with α2PI on the wound without exogenous plasma or fibrin could improve their release from the endogenous plasma clot. Therefore, we tested co-delivery of α2PI and growth factors (VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM}) in type 2 diabetes db/db mice back skin wounds. Different from

the last chapter, we directly delivered α2PI and growth factors in HEPES buffer to the wound site rather than using fibrin gel as the carrier system. We found that co-delivery of α2PI and growth factors was able to improve wound healing via protection of endogenous fibrin in diabetic wounds, especially in granulation tissue formation (Fig. 12B). This finding indicates interesting effects of α2PI in diabetic wounds other than protecting only exogenous fibrin biomaterials. More characterizations on the difference in release kinetics of the GFs in the wounds would be important to further optimize such carrier-free delivery approach.

Another application of α2PI on endogenous fibrin protection is bleeding reduction. In CABG surgeries, the major reason that causes bleeding is the increase of plasmin activity[11]. Thus, we wanted to assess the effects of α2PI in reducing bleeding, and compare its effects with aprotinin, the clinically used hemostatic drug. To do so, we established a tail bleeding model in mouse in which α2PI or aprotinin were applied to mice twice via intravenous injection, and the tip of the mice tail was cut to evaluate the bleeding. We found that α2PI was able to reduce both bleeding time and bleeding volume as effective as aprotinin (Fig. 13A, B, and C). After application of α2PI, the bleeding time and bleeding volume were reduced tremendously comparing to the group without protease inhibitor treatment (Fig. 13D, E). These results indicates that α2PI could be a good candidate as a hemostastic drug in CABG surgeries as compared to aprotinin. In addition, the human origin of α2PI would not create the side effects of aprotinin caused by immunogenic responses. In this experiment, we used 178 μM α2PI, over 150 times of the physiological concentration. With the relatively high dose of α2PI, there are some risks we need to consider: high blood level of α2PI is associated with risks of ischemic stroke[16]. α2PI also enables deep vein thrombosis, which is related to cancer risks[17].

Overall, we concluded that α2PI is able to bind to and protect endogenous fibrin *in vivo*. Through this protection, α2PI could be utilized in diabetic wound healing with growth factor co-delivery, and in bleeding reduction for CABG surgeries as a substitute of aprotinin.

IV. 6. References

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V. ENGINEERING THE DELIVERY OF STROMAL-DERIVED FACTOR-1α FOR DIABETIC WOUND HEALING

V. 1. Abstract

Stromal-derived factor (SDF)-1α is a chemokine able to recruit a variety of cells types, including mesenchymal stem cells and endothelial progenitor cells. Therefore, SDF-1α has been explored for therapeutic use to promote tissue regeneration, and has been shown to accelerate the healing of acute surgical wounds and chronic diabetic wounds, although its efficacy remains limited. Here, we aim to increase the therapeutic efficacy of SDF-1α by engineering a variant with super-affinity to the extracellular matrix to increase its retention at the wound site, based on an approach already validated by our group. We found that SDF-1 variants were produced via bacterial expression at higher yields than via mammalian expression. However, purification of bacteria-produced SDF-1α variants remained challenging despite the use of common histidine or gluthatione-S-transferase affinity-tags. As an alternative, we compared the efficacy of SDF-1γ, a natural matrix-binding isoform of SDF-1 that is commercially available, to SDF-1α for diabetic wound healing. We found that neither SDF-1γ nor SDF-1α accelerated wound closure or granulation tissue formation when delivered via fibrin biomaterial. However, though not directly improving wound healing, SDF-1 variants increased the presence of endothelial cells in wounds, thus modulating angiogenesis. Finally, we observed that SDF-1 was fast degraded by plasmin, a proteolytic enzyme overexpressed in diabetic wounds, which might abrogate its efficacy. Based on that, combined delivery of SDF-1 with protease

inhibitors or other engineering approaches would be worth to evaluate to improve the therapeutic potential of SDF-1 in diabetic wound healing.

Keywords: stromal-derived factor (SDF-1), extracellular matrix, fibrin, plasmin, angiogenesis, diabetic wound healing

V. 2. Introduction

SDF-1, also called C-X-C motif chemokine 12 (CXCL12), was first discovered as a soluble ligand secreted by bone marrow stromal cells, which can promote B-cell progenitor growth[1]. Later characterizations have then revealed that many cell types can express the SDF-1 receptor C-X-C chemokine receptor 4 (CXCR4), and can therefore respond to SDF-1 gradients, notably mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), muscle-, neural- and liver-tissue-committed stem/progenitor cells[2]. Upon SDF-1 binding, CXCR4 activation regulates $CXCR4^{+}$ stem cell migration and recruitment into damaged tissues[3][4]. Considering the central role of stem cells during tissue repair, the role and therapeutic use of SDF-1 during wound healing has been explored in various tissues. Among the 6 natural isoforms of SDF-1, SDF-1α has been the most studied one[5][6]. Previous studies have shown that SDF-1α has positive healing effects in bone, muscle and nerve regeneration, as well as in diabetic wound [2][7][8].

In diabetic wounds, the level of SDF-1α is reduced as compared to non-diabetic wounds, which impairs the recruitment of EPCs during the healing process[9]. Therefore, it has been shown that the delivery of SDF-1α encoding-plasmids in diabetic wounds can increase the level of SDF-1α and restore the speed of wound closure to normal rate[10]. Similarly, direct injection of SDF-1α protein or lentiviral construct containing SDF-1α into the wound bed was able to reduce wound size on diabetic mice[11][12]. However, therapeutic benefits of SDF-1α in many studies on diabetic wound healing were not significant or required repeated high dose applications, likely due to the sort life of SDF-1α and its protease sensitivity.

Consequently, effective delivery systems for SDF-1α are still needed to improve its therapeutic efficacy. Over the past years, many different materials have been used to

deliver SDF-1α. For example, self-assembled nanoparticles have been used to shield SDF-1α, which resulted in a sustained controlled release and effective protection of SDF-1α against proteolysis[13]. Similarly, delivery of SDF-1 encapsulated in liposomes has been assessed in diabetic wounds[14]. As another example, alginate hydrogel patches have been developed to deliver SDF-1α locally at the wound site, which accelerated healing and reduced scarring in acute surgical wounds[15].

Despite the multiple delivery systems that have been evaluated for SDF-1α, none has yet stand out for clinical translation in wound healing. Therefore, we questioned whether engineering SDF-1α for super-affinity to the extracellular matrix (ECM) can provide an effective and translatable solution for its clinical use. Indeed, our laboratory has previously demonstrated that engineering other growth factors, particularly VEGF-A and PDGF-BB for super-affinity to the ECM increased their local retention in diabetic wounds and enhanced their therapeutic efficacy as compared to the wild-type counterparts[16]. Similarly, we will here fuse SDF-1α to the heparin-binding domain of PlGF-2, here called PlGF-2_{ECM}, which displays super-affinity to the ECM. We hypothesized that increased affinity of SDF-1α to the ECM would extend its retention at the wound site, allowing for better local efficacy and lower side effects, such as excessive angiogenesis [17][16]. In addition, sequestering SDF-1α in the ECM might additionally reduce its degradation upon delivery in wounds. In this study, we aim to compare the efficacy of the SDF-1α-PlGF-2_{ECM} to the wild-type SDF-1α, as well as to SDF-1γ, a natural heparin-binding isoform that has been shown to bind to extracellular matrix molecules such as laminin and heparan sulfates[18]. Indeed, SDF-1γ contains an R/K-rich heparin-binding domain at its C-terminus which is very similar to the PlGF-2_{ECM} domain and that modulates its interactions with the ECM. The therapeutic efficacy of SDF-1 variants will be evaluated

in both the non-obese type 1 diabetes (NOD) mouse and the obese type 2 diabetes mouse models of wound healing.

V. 3. Materials and Methods

Bacterial expression of SDF-1γ and SDF-1α -PlGF-2_{ECM}

Human SDF-1γ sequence were purchased from GenScript and subcloned in pGEX-6P-1 vector with a GST-tag at N-terminus. Similarly, the human SDF-1α sequence was purchased from GenScript fused to the PlGF-2_{ECM} (PlGF-$2_{123-144}$) and inserted into the pRSET-A vector. SDF-1γ and SDF-1α-PlGF-2_{ECM} were than transformed into BL21DE3 competent cells. Single colonies of these two variants were picked and cultured in 5 mL of LB medium for 10-14 hours at 37 °C under 220 rpm agitation, and then transferred to 1 L of LB culture under same conditions. IPTG (1 mM) was added when the OD600 of the culture was between 0.4 and 0.8. The culture was then incubated at 25 °C under 220 rpm agitation overnight. The cell pellets were collected by centrifugation at 5000 xg for 20 min and resuspended in lysis buffer (50 mg lysozymes, 1 protease inhibitor tablet, 50 mL ice-cold PBS buffer). The lysates were sonicated for 30 s for 3 times on ice. After sonication, 1% triton X-100, benzonase (2.5 KU), and 20 mM $MgCl_2$ were added to the lysates. The lysates were then gently shaken for 30 minutes at 4 °C and stored at -80 °C for further purification.

GST-mediated affinity purification of SDF-1γ

The frozen lysate from bacterial production was thawed and centrifuged at 15'000 xg for 30 min at 4°C. The supernatant was purified via GST HiTrap column (GE Healthcare). The column was pre-equilibrated with binding buffer (PBS, pH 7.4). The lysate supernatant was then loaded through the column. After loading the lysate supernatant, the column was washed by binding buffer containing 0.1% Triton X-114, and then washed again with binding buffer. After washes, the proteins bound to the column were eluted

using elution buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM GSH, pH 8.0) via a 100% step elution, and the eluted fractions were collected for analysis. The eluted protein fractions were concentrated using Amicon tubes.

Heparin column purification for SDF1- γ and SDF-1α-PlGF-2$_{ECM}$

The frozen lysate from bacterial production was thawed and centrifuged at 15'000 xg for 30 min at 4°C. The supernatant was purified via HiTrap Heparin HP affinity column. The column was pre-equilibrated with binding buffer (10 mM sodium phosphate, pH 7.0). The lysate supernatant was then loaded through the column. After loading the lysate supernatant, the column was washed by binding buffer containing 0.1% Triton X-114, and then washed again with binding buffer. After washes, gradient elution was performed to elute protein bound to the column using the following elution buffer (10 mM sodium phosphate, 2 M NaCl, pH 7.0). The fractions of the elution were collected for further analysis using SDS-PAGE and western blot.

Size-exclusion purification for SDF-1γ and SDF-1α-PlGF-2$_{ECM}$

Following heparin purification, size exclusion was performed on the SDF1 variants, using a HiLoad 16/60 Superdex 75 pg column (GE Healthcare). After equilibration of the column using PBS, SDF-1 variants were loaded on the column and the eluted proteins were collected into fractions. The collected fractions were analyzed using SDS-PAGE and western blot.

Histidine-tag mediated purification for SDF-1α-PlGF-2$_{ECM}$

The procedures in this section was the same as described in Chapter 1.

Cloning and mammalian cell expression of SDF-1γ

Human SDF-1γ sequence was purchased from ViGene, and cloned into pSecTagA vector with histidine tag at C-terminus between NdeI and NotI restriction sites via Quick Change Polymerase Chain Reaction (PCR) under two different signal peptides: IgGκ signal peptide or the native signal peptide of SDF-1. Human Embryonic Kidney (HEK) 293-F cells were transfected with SDF-1γ DNA plasmids using PEI in OptiPro SFM medium, and cultured in FreeStyle293 medium for 7 days. Cell cultures were then collected, and centrifuged at 300 xg for 5 min to separate cells and supernatant. The cell pellets were lysed with RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl). Western blot with anti-his antibody was performed for both the cell culture supernatant and the cell lysate to detect SDF-1γ protein.

***In vivo* wound healing on db/db mice**

All animal experimentation was approved by the University of Chicago IACUC. Wounding experiments were performed on male db/db mice of 10-12 weeks old. Isoflurane was applied to the mice by inhalation as anesthetization, with 4% for induction and 2% for maintenance. The mice were placed on a heating pad for the duration of the surgery and artificial tears ointment was applied to the eyes of mice. Buprenorphine was injected subcutaneously at dosage of 0.1 mg/kg as analgesia. The back of the mice was shaved, and disinfected with betadine and 70% ethanol wipes. Four wounds were created using 6 mm-diameter biospy punch on the back of each mouse, with two wounds on each side of the spine.

After wounding, fibrin gels (70 μL) were made by mixing 4 mg/mL fibrinogen, 2 U/mL thrombin, 4 U/mL Factor XIIIa, 5 mM $CaCl_2$ in HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.6) and the SDF1 variants (from 200 ng to 5 μg). After the fibrin gel

polymerization, the back of the mice were covered with a hydrofilm (Hartmann), and the edges of the hydrofilms were sealed using Histoacryl BLUE glue (B. Braun Surgical). The mice were monitored until fully awaken, and analgesia was repeated every 12 h until no sign of pain was observed.

In vivo wound healing in NOD mice

Blood glucose was monitored for female NOD mice between 8 and 16 weeks old using a glucometer, which requires 1 μL drop of blood taken from tail vein. Mice with 2 consecutive blood glucose measurement higher than 250 mg/dL were considered diabetic. Diabetic NOD mice were treated with insulin by subcutaneous implantation of controlled-release insulin implants (LinBit, from LinShin Canada). The implantation process was performed under 1-3% isoflurane anesthesia using a 12G needle as a trocar. Two implants were administrated for the first 20 g body weight, and 1 additional implant for every additional 5 g of body weight to control the blood glucose level of mice. Mice glycemia was stabilized for 2 weeks around 250-300 mg/dL.

The surgery procedure for NOD mice was mostly same as for db/db mice, except that two wounds were created using 6 mm-diameter biopsy punch on the back of each mouse, with one wounds on each side of the spine. In addition, after the gel polymerization, donut-shape silicone splints were glued around the wounds using a non-toxic surgical glue, and the position of the splints were further stabilized by sutures (Nylon monofilament 5-0). The wounds were then covered by hydrofilm (Hartmann), and with position of the hydrofilm fixed by bandage.

Histological analysis for wound closure and granulation tissue formation

The procedures in this section was the same as described in Chapter 1.

Immunohistochemistry staining on wounds and quantification

The immunohistochemistry staining procedures in this section was the same as described in Chapter 1.

All the microscopy images were processed using the image processing software Fiji. Area of fluorescence was measured after background subtraction (based on the secondary only control slides) and normalized to the total area of tissue in each image. Quantification of the size of blood vessels was done by measuring the lumen area of the 10 largest blood vessels in the wound. The average areas of the largest 10 vessels were calculated for each image to assess the blood vessel sizes.

Flow cytometry analysis of skin wounds

The mice were euthanized on 10 days after surgery, and the wounds were harvested using an 8 mm-diameter biopsy punch to include the wound margin. The extracted wounds were cut into small pieces and incubated in 1 mg/ml collagenase with shaking at 37 °C for 1 h. The cells were then filtered through a 70 μm strainer and collected. The cells were stained for viability, blocked using CD16/32 Fc antibody and surface stained with anti-CD31, anti-CD34, anti-CXCR4 in 2% FBS in PBS (FACS buffer). The cells were then fixed and permeabilized with 2% PFA, 0.2% tween, and 0.5% saponin overnight. The cells were finally stained for intracellular markers using anti-SMA in FACS buffer and washed again. Cells were analyzed by flow cytometry on a BD LSRFortessa and analyzed using FlowJo software (FlowJo, LLC).

Plasmin digestion of SDF-1 variants

SDF-1γ (4 μg) were incubated with 0.5 mU of plasmin for 1 h at 37°C. Same amount of each protein untreated with plasmin, and plasmin only group were used as control. After

protein digestion, a SDS-PAGE gel was run to compared the digested proteins with control groups.

V. 4. Results

Expression of SDF-1γ and SDF-1α-PlGF-2_{ECM} in bacterial cells

We first designed SDF-1γ and SDF-1α-PlGF-2_{ECM} for expression in bacteria cells. For SDF-1γ, we used its native sequence of the isoform γ which is naturally composed of the SDF-1α added with a heparin-binding domain at its C-terminus (Fig. 14A). Using a similar design, we fused the PlGF-2_{ECM} domain at the C-terminus of SDF-1α to obtain the SDF-1α-PlGF-2_{ECM}. Since both proteins possess a heparin-binding domain that can be used for heparin-affinity mediated purification, we chose to not add any additional tags on the proteins. We subcloned the DNA sequences of the SDF-1 variants into pRSET-A and expressed them in BL21DE3 E.coli cells. Expression of the SDF-1 variants was analyzed via western blot in the supernatant of the bacterial expression lysate using an anti-SDF-1 antibody. While a band around 13 kDa was observed on the western blot for both variants, consistent with the theoretical size of the SDF-1 monomers (~13kDa), multiple bands of high molecular weights indicated multimerization, aggregation and unstability of the SDF-1 variants (Fig. 14B). We then used heparin-affinity mediated purification to purify expressed SDF-1 variants from contaminants, and found that both variants efficiently bound to heparin. Nevertheless, heparin-based purification did not enrich for SDF-1 monomers (Fig. 14C). However, an intense band at correct size of SDF-1 variants monomers for both SDF-1γ and SDF-1α-PlGF-2_{ECM} was still seen in the SDS-PAGE and western blot (Fig. 14C).

We further purified SDF1-γ and SDF1-α-PlGF-2_{ECM} by size exclusion to remove the multimers observed after heparin-based purification. Surprisingly, SDS-PAGE and western blot revealed that the SDF-1 variants co-eluted with the aggregates, thus not being efficiently separated as a monomer fraction (Fig. 14D). In addition, the highly

produced 13 kDa protein observed previously and eluted as a relatively pure protein after the size exclusion was not detected by western blot analysis (Fig. 14D). Overall, these results indicated that SDF-1 variants were expressed but not efficiently purified using these protein designs.

Therefore, we added tags to SDF-1 variants to improve the purification method. We first designed a SDF-1γ to be fused with a GST-tag at its N-terminus (Fig. 14E) and expressed it similarly as before. Western blot using an anti-GST antibody was performed on the expression lysate and showed that most of the GST-tags were already cleaved during expression, as indicated by a band around the size of the GST-tag without SDF-1 fusion (~26 kDa) (Fig. 14F). The prematured cleavage of GST-tag made this SDF1-γ fusion protein design unsuitable for GST-based.

Lastly, we tested a design using a histidine-tag added at the C-terminus of SDF1-α-PlGF-2_{ECM} so we could purify it using immobilized metal ion affinity chromatography (Fig. 14G). After expression in BL21DE3 E.coli, we analyzed the expression lysate supernatant and the cell pellet by western blot using an anti-his-tag antibody. In both cases, only very faint bands were detected, suggesting a low expression yield (Fig. 14H). Further purification by histidine-tag was not able to recover satisfying yield of pure proteins.

In conclusion, SDF-1 variants were able to be expressed in E.coli bacteria, but the yields were too low to further study the SDF-1γ and SDF-1α-PlGF-2_{ECM} proteins.

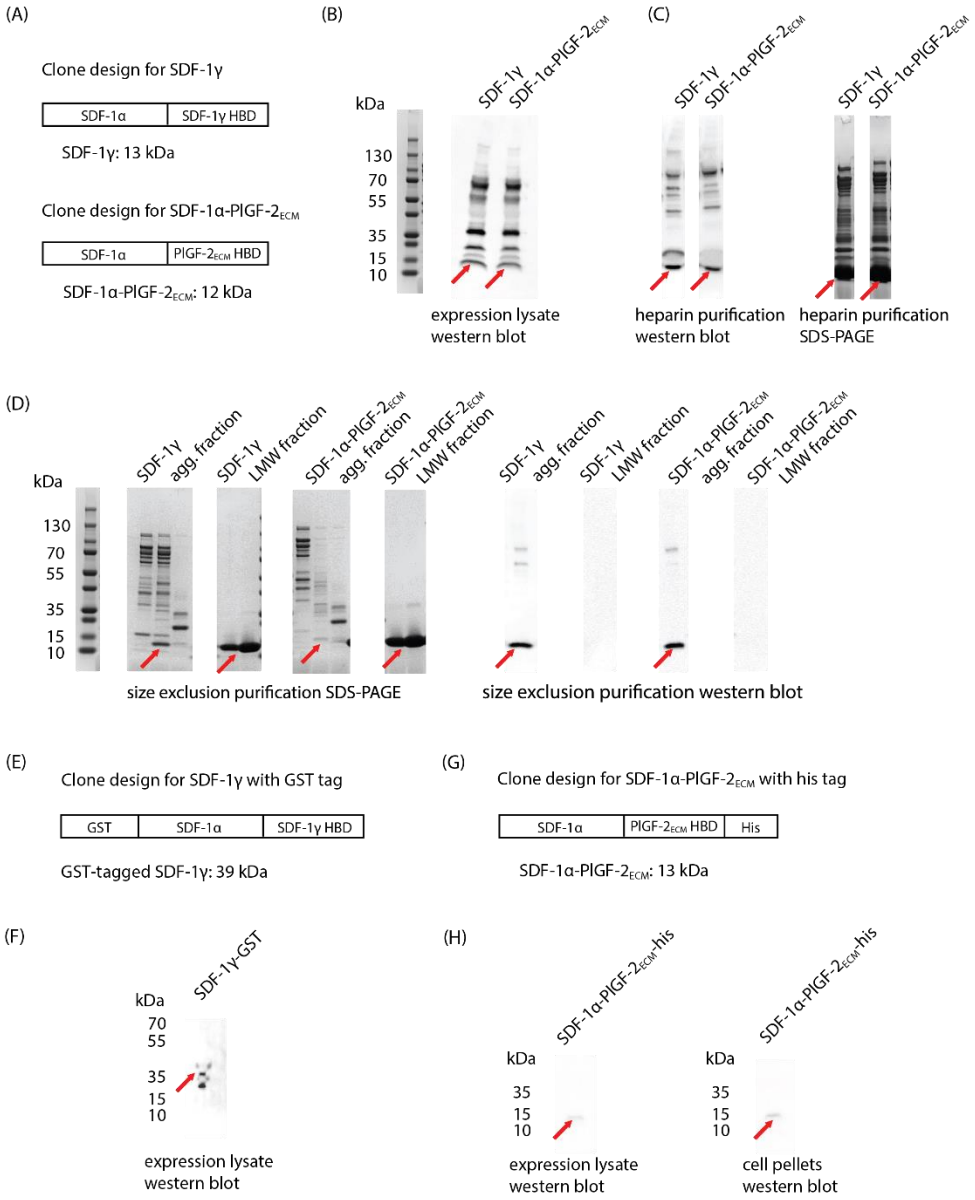

Fig 14. **Expression of SDF-1α-PlGF-2$_{ECM}$ and SDF-1γ in bacterial cells. (A)** Design of the SDF-1α-PlGF-2$_{ECM}$ and SDF-1γ (HBD = heparin-binding domain). **(B)** Anti-SDF-1 western blot of SDF-1α-PlGF-2$_{ECM}$ and SDF-1γ expressed in E.coli bacteria. **(C)** Anti-SDF-1 western blot and SDS-PAGE analysis of SDF-1α-PlGF-2$_{ECM}$ and SDF-1γ proteins after heparin-affinity mediated purification. Bands around 13 kDa (shown by red arrows) correspond to the theoretical size of SDF-1γ (13 kDa) and SDF-1α-PlGF-2$_{ECM}$ (12 kDa). **(D)** Anti-SDF-1 western blot and SDS-PAGE analysis after size exclusion purification of SDF-1γ and SDF-1α-PlGF-2$_{ECM}$, following heparin purification (agg. = aggregates, LMW = low molecular weight). **(E)** Design of the GST-tagged SDF-1γ. **(F)** Anti-GST western blot and SDS-PAGE analysis of GST-SDF-1γ expressed in bacteria. **(G)** Design of his-tagged SDF-1α-PlGF-2$_{ECM}$. **(G)** Anti-his western blot analysis of the lysate supernatant and bacteria pellet after expression of the his-tagged SDF-1α-PlGF-2$_{ECM}$.

Expression of SDF-1γ in mammalian cells

We then investigate expression of the SDF-1 variants in HEK293 mammalian cells. We focused first on the expression on SDF-1γ, and designed two different clones using either its native secretion signal peptide of SDF1 or the optimized IgGκ signal peptide (Fig. 15A). In addition, a histidine tag was added to the C-terminus of both variants via a Gly_3Ser short linker. We cloned these constructed by PCR (Fig. 15B) and subcloned them into pSeqTag-A expression vector. We then transfected HEK-293F cells and cultured them in suspension for 7 days, after which we collected the supernatant and cell pellets for analysis. SDF-1γ could not be detected in the supernatant at day 7, as shown by SDS-PAGE and western blot (Fig. 15C). However, SDF-1γ could only be detected in the cell pellets as a single band at the expected molecular weight (Fig. 15D), but the yield of expression was again too low to pursue further experimentations. Based on this result, we did not proceed with the production of SDF-1α-PlGF-2_{ECM} in HEK293 cells.

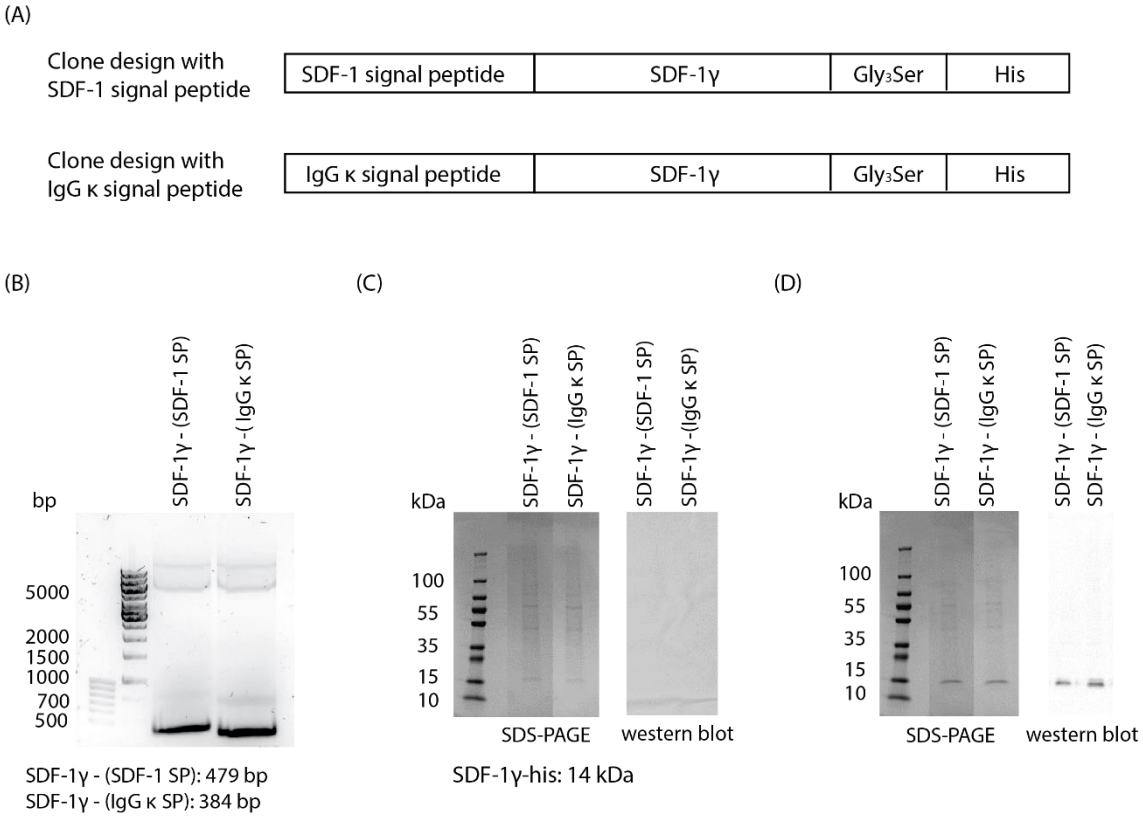

Fig. 15. **Expression of SDF-1γ in HEK293 mammalian cells. (A)** Protein designs of the two SDF-1γ variants of interest, containing either SDF-1 native signal peptide or IgGκ signal peptide. A his-tag was added at the C-terminus of both clones via a Gly_3Ser linker. **(B)** Agarose gel electrophoresis of the cloned DNA sequences for the constructs designed in (A) (SP = signal peptide). **(C)** SDS-PAGE and anti-his-tag western blot analysis of the SDF-1γ variants expressed in the supernatant of HEK293-F cells, 7 days after transfection. No expression was detected (SP = signal peptide). **(D)** SDS-PAGE and anti-his-tag western blot analysis of the SDF-1γ variants in the HEK293 cell pellets 7 days after transfection. Both SDF-1γ constructs were detected as a single band at ~14 kDa, which corresponds to their theoretical size (SP = signal peptide).

Delivery of SDF-1γ was not sufficient to improve healing in diabetic mice

Considering the challenges in producing SDF-1α-PlGF-2_{ECM}, we decided to assess the efficacy of the commercially available SDF-1γ in diabetic wound healing, reasoning that a lack of increase in therapeutic benefits as compare to SDF-1α would suggest that the approach of engineering an ECM-binding variant of SDF-1α is limited.

We first tested the efficacy of SDF-1γ variants compared to SDF-1α in full-thickness wounds in the db/db mouse, which is a model for Type 2 diabetes. We started with a dose study in which we delivered between 200 ng and 5 μg per wound of SDF-1 variants in fibrin gels. The fibrin gels were directly polymerized on the wounds upon surgery, and the wounds were then analyzed at 10 days after treatment for closure and granulation tissue formation by histological staining. No significant difference could be observed between the groups for both wound closure and granulation tissue formation. For SDF-1α, application of high doses (2 μg/wound or 5 μg/wound) on wounds showed trends toward improved wound closure as compared to low doses (200 ng) but not as compared to fibrin (Fig. 16A, B). In addition, SDF-1γ did not perform better than SDF-1α. Noteworthy, the variability in this experiment was very high and might have masked small effects of SDF-1α and SDF-1γ.

Therefore, in our experimental setting, we could not show any therapeutic benefit of SDF-1α or SDF-1γ in improving wound closure or granulation tissue formation in Type 2 diabetes chronic wounds.

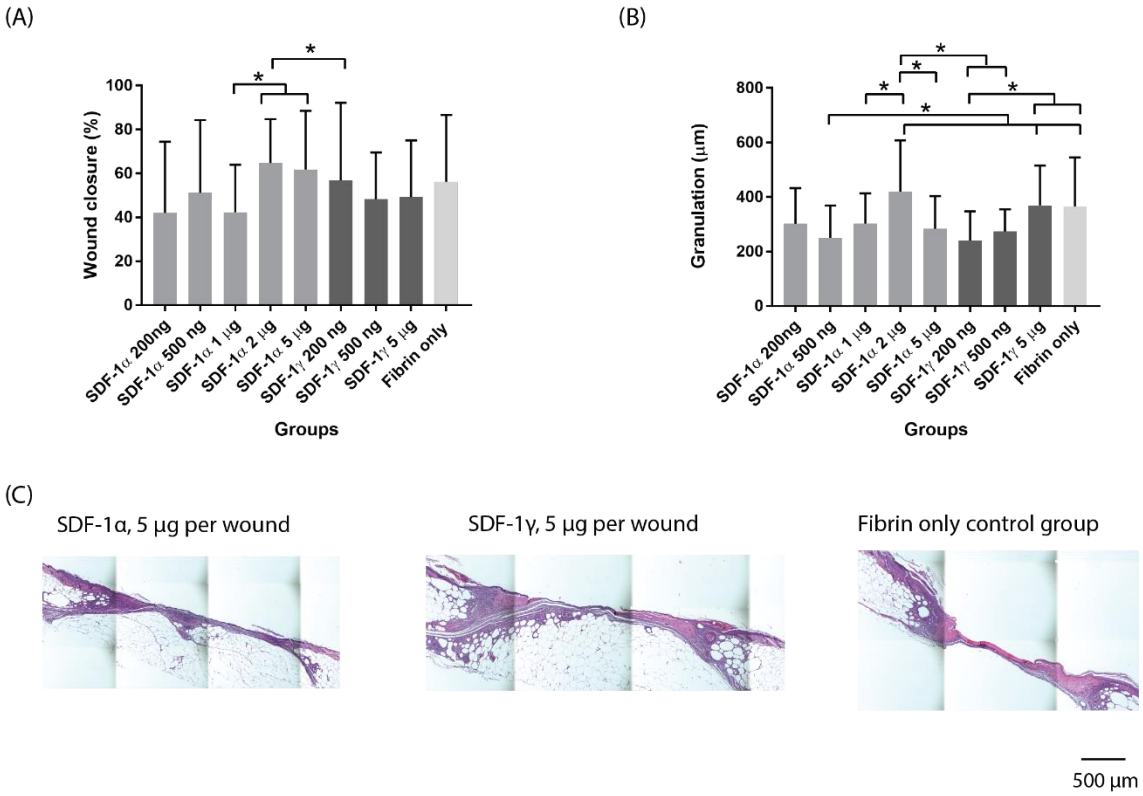

Fig 16. **Dose study of the SDF-1 variants for diabetic wound healing in db/db mice.** Different doses of SDF-1α or SDF-1γ in fibrin matrices were applied to 6 mm diameter skin wounds in db/db mice immediately after wounding. **(A)** Wound closure and **(B)** granulation tissue formation was quantified 10 days after the surgery by histomorphometric analysis (n=10 for each group, mean±SD, *p<0.05, one-way ANOVA test). No difference was observed between the groups. **(C)** Representative histology of the wounds treated with 5 µg SDF-1α, 5 µg SDF-1γ or fibrin only at day 10 after wounding (scale bar = 500 µm).

SDF1 modulates angiogenesis in diabetic wounds

Though SDF-1 variants were not able to accelerate chronic wound healing, we further analyzed their effects on wound angiogenesis, vasculogenesis and endothelial progenitor cell recruitment, as these mechanisms have been reported to be underlying the therapeutic benefits of SDF-1[15].

We first analyzed wounds of db/db mice treated with high dose of SDF-1α and SDF-1γ (1 μg, 2 μg and 5 μg per wound) via flow cytometry, as high doses previously showed trends toward improved wound healing. Particularly, the wounds were digested into single cell suspension and stained for $CD31^+$ endothelial cells (ECs), SMA^+ smooth muscle cells (SMCs), $CD34^+$ endothelial progenitor cells (EPCs) and $CXCR4^+$ cells. ECs and SMCs are the main cell types involved in angiogenesis and the formation of a stable neovasculature. In addition, EPCs cells have been shown to be recruited by gradients of SDF-1[15] and are involved in blood vessels formation via vasculogenesis. Finally, $CXCR4^+$ cells include mesenchymal stem cells, EPCs, and muscle satellite cells, which are stem/progenitor cells with central roles in wound healing[2].

For the flow cytometry analysis, we gated on single live cells and measured the proportion of cells positive for each of the markers (Fig. 17A). We observed that SDF-1α at doses of 1 μg or 2 μg per wound was able to increase angiogenesis in diabetic wounds, as shown increased population of $CD31^+$ ECs (Fig. 17B). Indeed, the $CD31^+$ ECs population was respectively 4-fold and 2-fold higher in the SDF-1α groups at a dose of 1 μg and 2 μg than in the fibrin group. In addition, the delivery of 1 μg of SDF-1α also recruited twice the amount of $CXCR4^+$ cells as compared to fibrin. Surprisingly, these results were not seen at a dose of 5 μg of SDF-1α or SDF-1γ, in which no difference was detected as compared to fibrin only. However, SDF-1 did not show any effects in promoting mature

and stable vasculature or recruiting EPCs, as suggested by the lack of increase of SMA^+ or $CD34^+$ cells respectively (Fig. 17B).

Besides flow cytometry, we also stained the wounds for $CD31^+$ ECs and SMA^+ SMCs. Overall, we did not observed significant difference in the amount of blood vessels present in the wounds between the different groups, but we noticed that vessels might be enlarged in some conditions. Therefore, we specifically measured the cross-section area of the 10 largest blood vessel in the sections and average them in each group. We found that SDF-1γ showed a trend toward increased blood vessel size (Fig. 17C). Representative images are presented in Figure 17D.

Interestingly, in a very similar experiment in the NOD mouse (a mouse model for type I diabetes), we observed that this effect was significant upon delivery of 200 ng of SDF-1α at day 7 post-surgery (Fig. 17E, F). In this experiment, the average blood vessel section area for the 10 largest blood vessels increased from 500 μm^2 to around 1500 μm^2 (Fig. 17E).

Therefore, SDF-1α at appropriate doses was able to show trend towards improved angiogenesis and enlarged blood vessels in diabetic wounds, particularly in the Type I diabetes model in NOD mice. Thus, we concluded that SDF-1 did modulate angiogenesis in the wound granulation tissue, but this was not sufficient to accelerate wound healing in db/db mice. Additional experimentations in the NOD model would be valuable to understand how the different models of diabetic wound healing influenced the outcomes upon SDF-1 treatments, and if therapeutic benefits could be highlighted in wounds of NOD mice.

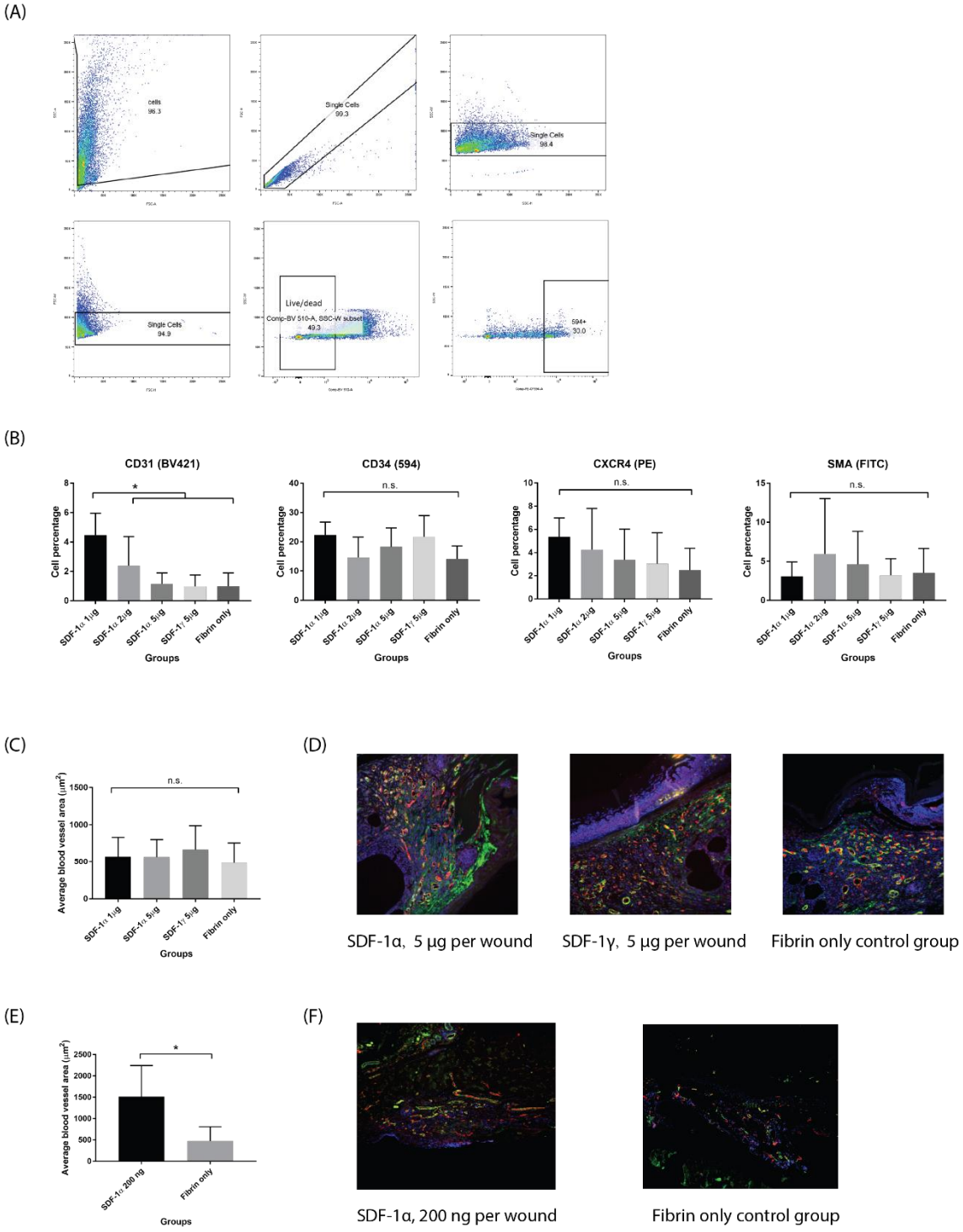

Fig 17. **Wound angiogenesis upon SDF-1α and SDF-1γ delivery in diabetic wounds.** Skin wounds in diabetic mice were treated with different doses of SDF-1γ or SDF-1α. Angiogenesis was assessed via quantification of ECs ($CD31^+$, red), and SMCs (α-SMA^+, green). Bioactivity of the SDF-1 variants was also assessed via the recruitement of EPCs ($CD34^+$) and CXCR4+ cells. **(A)** Gating strategy of the flow cytometry analysis of the db/db mice wounds. **(B)** Quantification of ECs, SMCs, EPCs and $CXCR4^+$ cells in db/db mice wounds 10 days after surgery via flow analysis (n=8 for each group, mean±SD, *$p<0.05$, one-way ANOVA test). **(C)** Quantification of

Figure 17, continued. blood vessel cross-section areas by averaging the 10 largest vessels per wound using immunohistochemistry, in the db/db mice at day 10 after surgery ($n \geq 5$ wounds for each group, mean±SD, $^*p<0.05$, one-way ANOVA test). **(D)** Representative images of angiogenesis and blood vessels in immunohistochemical stained wounds of db/db mice (red: $CD31^+$ ECs, green: α-SMA^+ SMCs, blue: DAPI). **(E)** Quantification of blood vessel cross-section areas in the NOD mice 7 days after surgery ($n \geq 5$ wounds for each group, mean±SD, $^*p<0.05$, Student's t test). **(F)** Representative immunohistochemistry images of the NOD wounds.

SDF-1γ can be digested by plasmin

Because SDF-1γ variants were not able to improve wound healing in our experimental settings, we hypothesized that it could have been prematurely degraded in the diabetic wounds, which are known to have excessive proteolytic activities, particularly with elevated levels of plasmin.

To examine if that could be the case, we incubated 4 μg of SDF-1γ with plasmin for 2 h at 37°C. Using SDS-PAGE, we observed that SDF-1γ was completely digested as highlighted by the absence of the SDF-1γ band (~13 kDa) after digestion (Fig. 18A). Cleavage site analysis showed that there are 16 cleavage sites in SDF-1γ, spreading through the whole sequence, with 6 of them could be cleaved within 5 min of incubation with plasmin (Fig. 18B).

This observation supported that SDF1 variants can be efficiently digested by plasmin in diabetic wounds, which could impair its activity upon delivery. Hence, strategies to improve SDF-1γ resistance to plasmin, either by sequence mutation to prevent plasmin-cleavage sites, or by biomaterial-mediated protection of SDF1 might be important to achieve therapeutic effects.

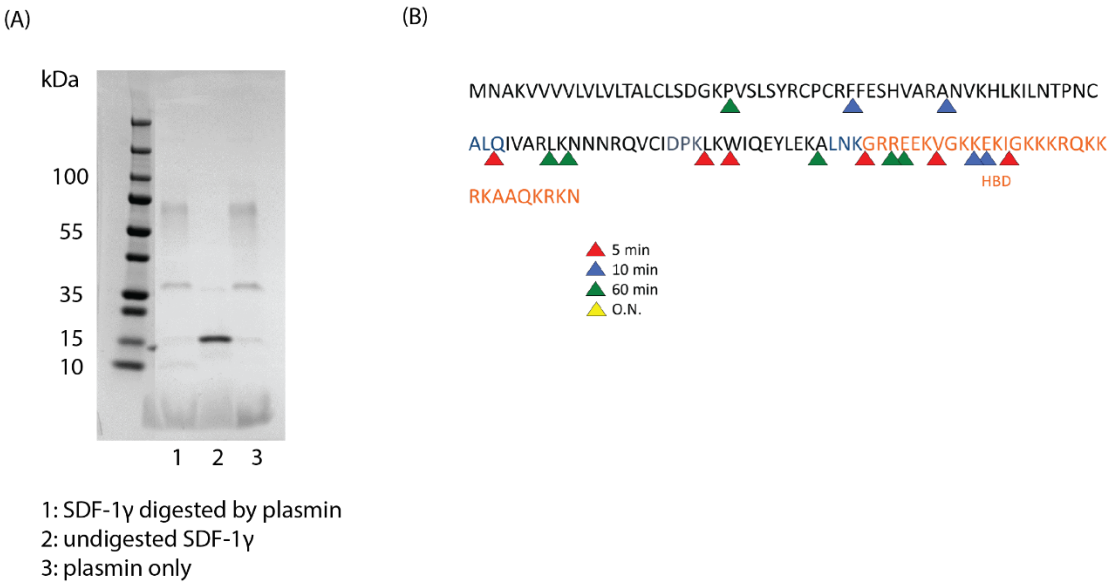

Fig 18. **SDF-1 can be degraded by plasmin.** (A) SDS-PAGE of SDF-1γ upon degradation by plasmin. Only SDF-1γ in absence of plasmin can be seen as a band at ~13 kDa, while incubation with plasmin resulted in full digestion of the SDF-1γ. (B) Plasmin cleavage sites of SDF-1γ at different time points of plasmin incubation. There are 16 cleavage sites in SDF-1γ (HBD = heparin binding domain; cleavage assay performed by proteomics core facility at the Ecole Polytechnique Fédérale Lausanne (EPFL); Analyzed with Dr. Marc Moniatte)

V. 5. Discussion

In this study, we investigated the effects of SDF-1 in diabetic wound healing. While it is known that SDF-1α has positive effects in skin regeneration, due to its important role in recruiting and regulating EPCs and MSCs, among other progenitors cells, its therapeutic use as been limited due to poor efficacy upon delivery[2]. Following an engineering approach already established by our group to improve the therapeutic efficacy of growth factors[16], we here explored the engineering of SDF-1α for super-affinity to the ECM, to increase its local sequestration in wounds and thus its on-site efficacy.

To do so, SDF-1α was fused to the heparin-binding domain of the PlGF-2, which has high-affinity of multiple ECM proteins. Interestingly, SDF-1γ, a natural splice variant of SDF-1α, contains a heparin-binding domain at its C-terminus, which we hypothesized could act similarly to the PlGF-2_{ECM} domain (Fig. 14A). Therefore, we aimed to compare the efficacy of the engineered SDF-1α-PlGF-2_{ECM} and SDF-1γ, both expected to be superior to SDF-1α, in mouse models of diabetic wound healing.

We first found that protein expressions of SDF-1α-PlGF-2_{ECM} and SDF-1γ were challenging. Indeed, expression of these proteins was tested in BL21DE3 E.coli, in which we found that the variants could be expressed, although they were forming multimers that were not efficiently purified by size exclusion (Fig. 14B-D). Moreover, addition of GST- or his-tags on the SDF-1 variants did not provide solutions to facilitate the purification processes. Indeed, the GST-tag was mostly cleaved during the expression, and the his-tag fused protein expression level was very low (Fig. 14E-H). Similarly, production of SDF-1γ in HEK293-F mammalian cells yielded to low amount of proteins that remained localized into the cell pellets, despite the use of the native or the IgGκ secretion signal sequence (Fig. 15). Retention of SDF-1γ in the cell pellets might be due to a poor

secretion or to a strong binding of SDF-1γ to the cell-surface heparan sulfates, which would prevent its release into the supernatant upon production. Indeed, it has been showed that SDF-1γ can bind to heparan-sulfates via its heparin-binding domain[19]. Overall, we found that the SDF-1 variants were hard to express and purify using common protein production strategies, thus requiring more customized methods for achieving higher yield and acceptable purity of both SDF-1γ and SDF-1α-PlGF-2_{ECM}.

Consequently, before investing additional efforts on protein expression optimization, we wanted to assess the therapeutic potential of SDF-1 on diabetic wounds using the commercially available SDF-1α and SDF-1γ. We mostly tested the delivery of the SDF-1 variants in the type 2 diabetic db/db mouse model of wound healing. We tried delivery of different doses of SDF-1α and SDF-1γ ranging from 200 ng to 5 μg in fibrin, and evaluated wound healing primarily through wound closure and granulation tissue formation. We found that neither SDF-1α nor SDF-1γ was able to significantly improve diabetic wound healing as compared to fibrin alone, although the high variability of this model might have masked some small therapeutic effects of the SDF-1 variants (Fig. 16). Nevertheless, more detailed analyses on wound angiogenesis showed that the delivery of 1-2 μg of SDF-1α increased $CD31^+$ ECs in the granulation tissue, and give trends toward increased amount of EPCs. In addition, we found that delivery of SDF-1γ showed trends toward enlargement of blood vessels in the wound granulation tissue (Fig. 17). Interestingly, this effect was more pronounced upon delivery of SDF-1α in the type 1 diabetic NOD mouse model, thus highlighting the importance of the model choice, and questioning whether the comparison between SDF-1γ and SDF-1α would have not been more conclusive in the NOD model. Besides that, the time point for wound analysis at day 10 day post-surgery in the db/db model was chosen based on the readouts for wound closure and granulation tissue formation, but might not have been the most appropriate

one to highlight differences in angiogenesis and vasculogenesis. Indeed, EPCs are generally recruited from the bone marrow to wound sites shortly upon injury to differentiate into mature endothelial cells (ECs)[20]. At 10 days, the level of EPCs might have already decreased due to the differentiation. Therefore, it seems that further model optimization would be important before continuing the research on SDF-1 efficacy.

Because SDF-1α is known to be sensitive to proteases, we suspected that the poor efficiency of SDF-1γ could also be caused by proteolytic degradation. Although fibrin could protect SDF-1γ from excessive protease cleavage upon delivery in wounds, the release of the SDF-1 from fibrin is expected to be mediated by plasmin degradation[16]. We here demonstrated that plasmin can also very efficiently degrade SDF-1γ, thus likely releasing non-active fragments of the growth factor rather than a bioactive form (Fig. 18). While single amino acid substitutions are sometimes use to develop protease-resistant variants of growth factors[21], protease cleavage prediction algorithms detected multiple cleavage sites in the sequence of the SDF-1γ, particularly in its heparin-binding domain, making amino acid substitutions very difficult without impairing the ECM-binding properties of SDF-1γ.

As compared to other published studies that demonstrated some therapeutic efficacy of SDF-1, we did not overall obtain data supporting relevant therapeutic potential this growth factor for healing of diabetic wounds in the db/db mouse model. While the environment of diabetic wounds is particularly difficult to heal, due to the chronic inflammation, high proteolytic content, damaged ECM and impaired growth factor signaling, efficacy of SDF-1 in acute wound healing has been shown to be more promising[22][23].

That being said, a few studies showed positive outcomes of SDF-1α in diabetic wounds. Such studies mostly delivered SDF-1α via expression of plasmid or lentiviral gene

transfer[12] in the wound, rather than as a protein formulation, which provided a sustained production of SDF-1α[10][12]. Compared to them, we applied a single dose of SDF-1 protein in fibrin, which was not sufficient to achieve sustained release of bioactive SDF-1. Interestingly, Yeboah et al. showed improved wound vascularization and closure in the db/db mouse model by delivering an engineered SDF-1 protein in fibrin[24]. Particularly, the authors engineered SDF-1α with an elastin-like peptide to increase its stability in wound fluid, as well as to provide a non-conventional way to efficiently purify the recombinant protein via temperature cycling, thus providing solutions to two challenges that we faced during our study. They found an overall increase of healing of about 20% using the engineered SDF-1α as compared to the wild-type SDF-1α, and about 40% as compared to fibrin only. This suggests that further improvement of our delivery formulations and model would be necessary to detect the positive outcomes of SDF-1 variants[11][24].

Finally, because SDF-1α showed less promises than other growth factors we previously engineered in our laboratory, such as VEGF-A or PDGF-BB, we believed that focusing on a different growth factor candidate or studying SDF-1 in combination with other drugs might be more valuable than the optimization of our current approach.

V. 6. References

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VI. CONCLUSION AND FUTURE DIRECTIONS

VI.1. General Discussion and Significance of the Thesis

In this thesis, we aimed to enhance fibrin biomaterials that are widely used in the clinic, yet that have some limitations. We particularly focus our efforts on two complementary aspects: 1) the engineering of fibrin with the protease inhibitor α2PI to extend longevity, and 2) the enhancement of fibrin regenerative properties via incorporation of signaling factors to improve tissue healing, including growth factors and chemokines. Ultimately, both strategies can be combined to developed optimal fibrin formulations with controlled stability and controlled release of pro-regenerative drugs.

First, in Chapter 1, we engineered exogenous fibrin biomaterials with the human-derived protease inhibitor α2PI to extend their longevity and explored potential applications. We importantly compared α2PI to the current clinical fibrinolysis inhibitor aprotinin and demonstrated a much higher efficacy in protecting fibrin *in vitro* and *in vivo*. This was partly due to the natural ability of α2PI to crosslink into fibrin during polymerization, which prevents its release upon delivery and increase its *in situ* efficacy, in contrast to aprotinin that fast diffuses out of fibrin. Such efficacy results are particularly important to support the use of α2PI in fibrin sealants, as a substitute to aprotinin. Indeed, despite good efficacy, aprotinin has been shown to be associated with immunogenicity side-effects due to its bovine-origin[1], whereas α2PI would constitute a safer and more effective human-derived fibrinolysis inhibitor.

In addition, we showed that α2PI was able to effectively extend longevity of fibrin biomaterials made of low fibrinogen concentration, as low as 4 mg/mL, which is close to the physiological concentration of fibrinogen in human plasma. While this result could be expected considering the physiological role of α2PI in protection of the fibrin clot, it opens an interesting perspective toward dose reduction of fibrinogen in the clinical formulation of fibrin sealants. Indeed, the current clinical dosing of fibrinogen ranges between 40-100 mg/mL, such high concentrations being in part required to counteract the fast degradation rate of fibrin. Reducing fibrinogen concentrations would therefore allow to reduce the cost of fibrin materials and some manufacturing challenges. Particularly, clinical-grade fibrinogen is purified from human plasma which creates some limitations in terms of sourcing and risks of pathogen transmissions[2]. The use of recombinant fibrinogen has been so far hindered by relatively low expression yields but could potentially be re-considered if the fibrinogen amount needed for therapy is substantially reduced.

Then, we used low-dose fibrin gels supplemented with α2PI as a delivery carrier for growth factors in diabetic wounds, which are difficult to heal due to their particularly high proteolytic environment. We showed that the α2PI-engineered fibrin enhanced the regenerative effects of VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM}, which led to improved wound closure and granulation tissue formation in the type 2 diabetes db/db mouse model. In that context, α2PI tightly controlled the degradation rate of fibrin and slowed the release of the growth factors in the wounds, which allowed to sustain their regenerative effects over a longer period of time. A more detail kinetic study of the release of GFs in the wounds would be interesting to conduct to determine the optimal rate of fibrinolysis that leads to the best efficacy of the GFs. However, while the diabetic wound healing model in db/db mice is a gold-standard for the research and development of

diabetic wound therapies, human chronic wounds are known to have an even higher proteolytic environment, better mimicked in some other large animal models. Optimization of α2PI delivery in the db/db mouse model therefore bear some important limitations and might not be as insightful for clinical translation.

In Chapter 2, we focused on the effects of α2PI in protecting endogenous fibrin, for application in diabetic wound healing and as a hemostatic agent to reduce bleeding during surgery. We first demonstrated that α2PI can protect fibrin clots derived from healthy or diabetic unpurified plasma. In diabetic wounds, we this time delivered co-delivered α2PI with VEGF-A-PlGF-2_{ECM} and PDGF-BB-PlGF-2_{ECM}, in absence of carrier materials reasoning that they will get incorporated in the natural fibrin clots directly and delivered from there. While we found that efficacy on wound healing was reduced as compared to when using exogenous fibrin as a carrier materials, granulation tissue formation was significantly increased by the presence of α2PI. Further optimization of the delivery strategy in dosing and frequency would indicate whether a carrier-free delivery system could be fully effective. Engineering carrier-free delivery systems have the advantages of reducing cost and manufacturing processes, and to turn the treatment into a drug-based therapy rather than a combination material containing drugs and a matrix, which would limit the treatement complexity and increase its translatability. Nevertheless, it is common to think that the delivery of matrices support tissue regeneration by providing a scaffold for cell migration. Accordingly and based on this thesis results, it would be promising as a future step to evaluate the delivery of GFs from plasma-derived biomaterials supplemented with α2PI in diabetic wound healing. Specifically, the utilization of plasma from patients as a drug delivery biomaterials will be easy to operate,

and can completely avoid the pathogen transmission risk, as well as lower the costs related to fibrin biomaterial production, distribution, and preservation.

Lastly, we demonstrated that α2PI have a strong efficacy, comparable to aprotinin, as a hemostatic agent when delivered intravenously to reduce bleeding during surgery and thus the need of patient blood transfusion. While aprotinin has been approved for this purpose in the case of CABG surgeries, hemorrhage risks associated with excessive endogenous plasmin activity have additionally be reported in cesarean section (C-section) surgery and heart-stent placement[3][4][5][6]. In such applications where the protease inhibitors is to be delivered systemically, the safety of the non-immunogenic human-derived α2PI would represent a strong advantage as compared to aprotinin, to limit adverse effect upon re-exposure. However, further safety experiment should be carefully conducted on α2PI to determine whether an acute high dose injection intravenously could increase the risks of thrombosis by shifting the balance toward reduced systemic fibrinolysis.

Finally, in Chapter 3, we explored the effects of SDF-1α and SDF-1γ variants, as a promising chemokines to incorporate into fibrin materials to increase tissue regenerative outcomes. However, we found that engineering of SDF-1 variants were particularly challenging in terms of protein production and purification processes. In addition, our preliminary results indicated a limited efficacy of SDF-1α and SDF-1γ in accelerating diabetic wound healing, despite their ability to modulate angiogenesis in both type 1 and type 2 diabetic wounds. While further engineering and optimization efforts on these chemokines are certainly valuable to release its full potential, we believed that other GFs might be less challenging and as powerful for the engineering of regenerative fibrin biomaterials.

VI.2. Future perspectives on clinical translation of α2PI

The main finding of this thesis relies on the demonstration that α2PI is a very promising drug of high anti-fibrinolytic efficacy and an *a priori* better safety profile than the current clinical drug.

While α2PI can be purified from human plasma, we found that the protein is efficiently produced in recombinant mammalian expression, which is a strong advantage for future manufacturing. Future optimization of expression in Chinese hamster ovary (CHO) cells might be valuable as clinical proteins are often produced in this organism rather than in HEK cells. In addition, strategies to purify untagged proteins would need to be developed, although some already exist for the purification of α2PI from human plasma, using affinity-based chromatography or precipitation and zinc chromatography[7][8].

Recombinant α2PI could then be evaluated in safety studies for local delivery into fibrin, in which very high safety profiles are expected, due to its covalent local retention in the material, and its slow release upon degradation that should not significantly affect the baseline of the physiological concentration of α2PI in blood. In addition, human origin of α2PI will solve the immunogenicity issue related to the use of aprotinin. The outcomes of safety studies for intravenous injection of α2PI are more difficult to anticipate, and will depends on the therapeutic dose that will need to be injected. While there is a high chance that α2PI would be safe systemically at reasonable dose in healthy patients, considering its physiological presence in the bloodstream, it might have some limitations in patients with history of thrombosis or other cardiovascular preconditions. Patients undergoing CABG might be particularly at risk and so safety of α2PI for use during CABG surgery will have to be tested in this specific subset of patients.

On the other hand, we demonstrated high efficacy of α2PI in several applications of regenerative medicine, and having α2PI developed as a drug and approved for one clinical application might accelerate its use in the other applications, potentially leading to a broad impact of the drug.

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Overall, in this thesis, we showed the effects of α2PI and growth factors in regenerative medicine via engineering of fibrin biomaterials. Having proved effects of α2PI in several treatment models, we believe that α2PI can be implemented into clinical treatment as an alternative or improvement for current treatment. We hope that α2PI will improve the efficacy of many regenerative medicine treatments in the future with minimal risks and costs.

VI.3. References

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