ELASTIN LIKE PEPTIDE PROTEIN PRESERVES STROMAL CELL-DERIVED FACTOR 1 BIOACTIVITY IN PRESENCE OF ELASTASE IN VITRO

By

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And approved by

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ABSTRACT OF THE THESIS

Elastin Like Peptide Protein Preserves Stromal Cell-Derived Factor 1 Bioactivity in Presence of Elastase in Vitro

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During the normal wound healing process, there are four distinct but overlapping stages: hemostasis, inflammation, proliferation, and maturation. When the stages fail to proceed in an orderly and timely fashion, chronic skin wounds may happen. Chronic skin wounds are characterized by stalled revascularization, epithelialization, and excessive inflammation. Traditional chronic wound therapies require a prolonged healing process and are costly. This situation could be remediated by application of exogenous growth factors. One is stromal cell-derived growth factor 1 (SDF1), which plays an important role in revascularization together with its receptor, CXCR4. Activation of CXCR4 by SDF1 requires interaction of the amino-terminal domains of both molecules. Together, they regulate the revascularization process through the

SDF1-CXCR4 pathway. In a previous study, SDF1 was shown to accelerate mouse skin wound closure.

However, free SDF1 in vivo stability is poor because of proteases. Elastase is a type of protease released from neutrophils degranulated during inflammation that can cleave amino-terminal residues *Lysine - Proline-Valine* from SDF1. The proteolyzed chemokine fails to bind CXCR4 or induce angiogenesis functions. Therefore, repeated high doses of topical SDF1 were required to achieve a therapeutic effect. Together with costly producing and purifying processes, this made SDF1 treatment impractical. Therefore, our lab developed the novel fusion protein SDF1-ELP. This fusion protein consists of SDF1 and elastin-like peptide (ELP) repeats. A unique property of SDF1-ELP monomers is that they can reversibly self-assemble into nanoparticles, thus protecting SDF1 from proteases.

In this study, we set up a controlled release Transwell system to evaluate the effects of SDF1-ELP nanoparticles and monomers on HUVEC proliferation in the presence of elastase. This system mimics the topical application of drug on the skin wound. The monomers are gradually released from the nanoparticle drug depot and diffuse to the target cells through the wound fluid, which contains proteases.

In this study, we found that compared with free SDF1, SDF1-ELP had similar dose-dependent bioactivity as measured by HUVEC proliferation. However, SDF1-ELP nanoparticles were far more stable in presence of 100nM elastase. Previous work has indicated that SDF1-ELP

monomers can be gradually released from its nanoparticles. Compared with free SDF1, monomers themselves are more elastase resistant as well. During a 48-hr incubation in the presence of 100nM elastase, ~20% SDF1-ELP was degraded. Nevertheless, SDF1-ELP still preserved its bioactivity as evaluated by HUVEC proliferation.

In conclusion, SDF1-ELP nanoparticles may serve as a drug depot that can gradually release monomers with enhanced stability within elastase. Besides, SDF1-ELP can promote endothelial cell proliferation, which plays an essential role in revascularization during the wound healing process. Therefore, SDF1-ELP is a promising recombined growth factor for the treatment of chronic skin wounds.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I Introduction and Motivation

1.1. Introduction

During the normal wound healing process, there are four distinct but overlapping stages: hemostasis, inflammation, proliferation, and maturation [1]. When the stages fail to process in an orderly and timely fashion, chronic skin wounds may happen. Chronic skin wounds are characterized by stalled revascularization, epithelialization, and excessive inflammation [2][3]. These wounds are rarely seen in healthy individuals; however, they are often combined with other pathology conditions such as diabetes, hypertension, and immunosuppression [3]. In the US, over 6.5 million patients are affected by chronic wounds [4], and more than $50 billion are spent on chronic wound treatment annually [5].

There are several methods used for chronic skin wound treatment, such as debridement, negative pressure wound therapy, hyperbaric oxygen therapy, and engineered skin substitutes [6]. However, the prolonged healing process and high cost significantly affect patients' life quality. This situation could be remediated by applying an exogenous growth factor. The therapeutic efficacy of topical growth factors on chronic wounds [7] has been studied and proven in animal models.

One growth factor, stromal cell-derived growth factor 1 (SDF1), has been shown to accelerate mouse skin wound closure. [7]. SDF1 plays an important role in revascularization together with its receptor, CXCR4. Activation of CXCR4 by SDF1 requires interaction of the amino-terminal domains of both molecules [23]. Together,

they regulate the revascularization process through the SDF1-CXCR4 pathway. In this pathway, SDF1-CXCR4 recruits endothelial progenitor cells and promotes their proliferation and differentiation into mature vascular endothelium that contributes to revascularization [8-10].

Although SDF1 treatment can achieve therapeutic efficacy, due to the poor in vivo stability caused by proteases, repeated high doses of topical SDF1 were required. Elastase is a type of protease released from neutrophils degranulated by inflammatory mediators that can cleave amino-terminal residues *Lysine - Proline-Valine* from SDF1. The proteolyzed chemokine fails to bind CXCR4 or induce angiogenesis functions [24]. Together with costly production and purification processes, this made SDF1 treatment impractical [7]. Therefore, our lab designed a novel fusion protein that combined the SDF1 molecule with elastin-like peptide repeats (ELPs) [11]. ELPs are nonimmunogenic, non-pyrogenic, and biologically compatible [12] derivatives of tropoelastin with pentapeptide repeats of *Valine-Proline-Glycine-(Xaa)-Glycine*, where *Xaa* can be any natural amino acid except proline [11]. The unique property of ELPs and ELP fusion proteins allows a temperature-dependent reversible phase change from soluble monomer to insoluble aggregate. Inverse Transition Cycling (ITC) uses this property in combination with centrifugation steps as an inexpensive and simple method to purify recombined SDF1-ELP proteins [12].

In previous works done by our lab [11][13], the SDF1-ELP was recognized to have similar in vitro bioactivity as native SDF1 but enhanced stability in the presence of

proteases and better in vivo efficacy. However, the effect of proteases on SDF1-ELP bioactivity, especially the SDF1-ELP monomer, has not been evaluated yet. We hypothesize that SDF1-ELP nanoparticles, as well as the monomer, can retain their bioactivity in proteases. To explore this question, an in vitro controlled release system was set up to study human umbilical vein endothelial cell (HUVEC) proliferation in the presence of SDF1-ELP and elastase. In addition, an SDF1-ELP in vitro release model that targets HUVECs was constructed.

1.2. Motivation

Previous work done by Yeboah et al. demonstrated that the recombinant fusion protein SDF1-ELP had similar binding activity to the CXCR4 receptor as free SDF1 [11]. Also, the biological activity of SDF1-ELP measured by intracellular calcium release in HL60 cells was also similar to free SDF1 and was dose dependent [11]. However, compared to free SDF1, SDF1-ELP had improved stability in elastase. Elastase is a type of protease in wound fluid [19] that can quickly degrade free SDF1. Experiments indicated that SDF1-ELP could remain intact over 12 days in presence of ~1 μM elastase. However, SDF1 was degraded on Day 0 [11]. Another remarkable property of SDF1-ELP is its in vivo biological activity and stability that is greater than that of free SDF1. When applied to excision wounds in diabetic mice, SDF1-ELP treated wounds fully

epithelialized by Day 28, while SDF1-treated wounds took another 14 days to fully close [11].

One important property of SDF1-ELP is that it can self-assemble into nanoparticles when the temperature is over its inversion temperature (~35 °C). However, even above its inversion temperature, SDF1-ELP nanoparticles can gradually release monomers. Therefore, nanoparticles can act as a drug depot [12]. By modeling the chemotactic potential of SDF1-ELP with HL-60 cells, which express the SDF1 receptor (CXCR4), Yeboah et al. found that SDF1-ELP nanoparticles (250nM or more) were needed to achieve the same migration effect as 10nM SDF1 because mainly SDF1-ELP monomers caused the HL60 migration [13]. During the migration experiment, 8% of SDF1-ELP was released from SDF1-ELP nanoparticles in 4 hours [13]. Thus, they concluded that SDF1-ELP monomers may play a major role during the wound healing process.

However, the SDF1-ELP monomer biological activity and stability in presence of proteases have not been carefully examined. In addition, although HL60 were convenient responder cells that have the SDF1 receptor and can interact with SDF1, they are not specifically relevant to wound healing. Therefore, we used another cell type which not only has SDF1 receptors but also takes part in the wound healing process.

When SDF1-ELP is applied to the skin wound, the monomers can serve as drug gradually released from the nanoparticle drug depot and diffuse to the target cells through the wound fluid, which contains proteases (**Fig.1**). In this research, we set up a controlled

release Transwell system **(Fig.2**) to attempt to mimic the topical application of a drug on a skin wound, as well as to evaluate the biological activity and stability of the SDF1-ELP monomers. The SDF1-ELP nanoparticles were put onto a culture insert with a permeable membrane (serving as drug depot) whose pore size is nominally smaller than the SDF1-ELP nanoparticles. Theoretically, only SDF1-ELP monomers can freely pass the membrane to enter the outer well. Cultured on the bottom of the outer well, there was an endothelial cell (EC) layer targeted by the SDF1-ELP monomer. In addition, elastase was added to the medium in the outer well to mimic the presence of wound fluid. Since theoretically only the SDF1-ELP monomer can enter the outer well, their biology activity towards targeted ECs and their stability in the presence of elastase could be evaluated.

In the human body, EC forms the interface between tissue and blood or lymph. During the wound healing process, ECs perform a vital role in revascularization and express the SDF1 receptor CXCR4. Several studies have proven that SDF1 can target endothelial progenitor cells and endothelial cells through the SDF-CXCR4 pathway to induce vascularization [8][15-18]. Furthermore, ECs can be cultured as a flat monolayer, thus making it suitable to incorporate as the target cell while keeping a simple geometry for studying cellular responses in the system previously described in **Fig. 2**. In this thesis, the scope was limited to studying proliferative response of HUVECs to SDF1-ELP and SDF1, because the relevant assays are relatively simple, as a preliminary step to establish the system.

The following chapters describe the development of this system, beginning with investigating the dose-effect of SDF1-ELP on EC proliferation, optimizing the concentration of elastase in the system (since high elastase concentration may cause detachment and death of ECs), and finally studying the proliferative response of the ECs to SDF1-ELP introduced into the Transwell.

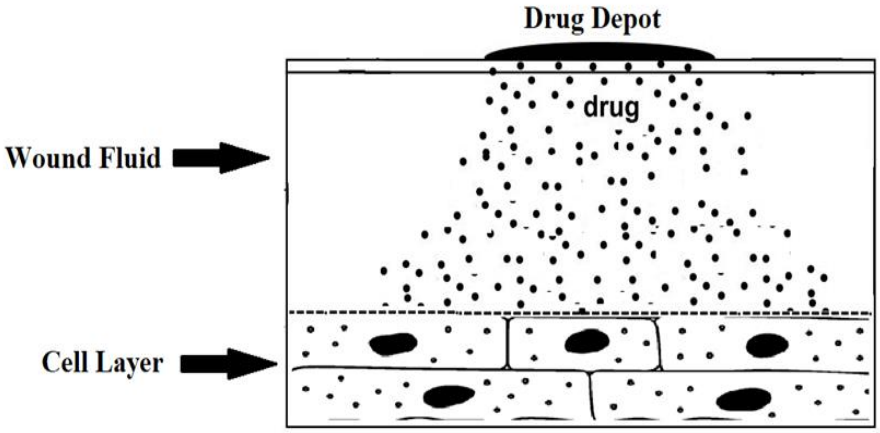


Figure 1. Topical application of drug on skin wound. The drug depot releases drug molecules that diffuse to the surface of target cells in the wound. During this process, the drug molecules are exposed to the action of proteases, which can affect the amount reaching the cells.

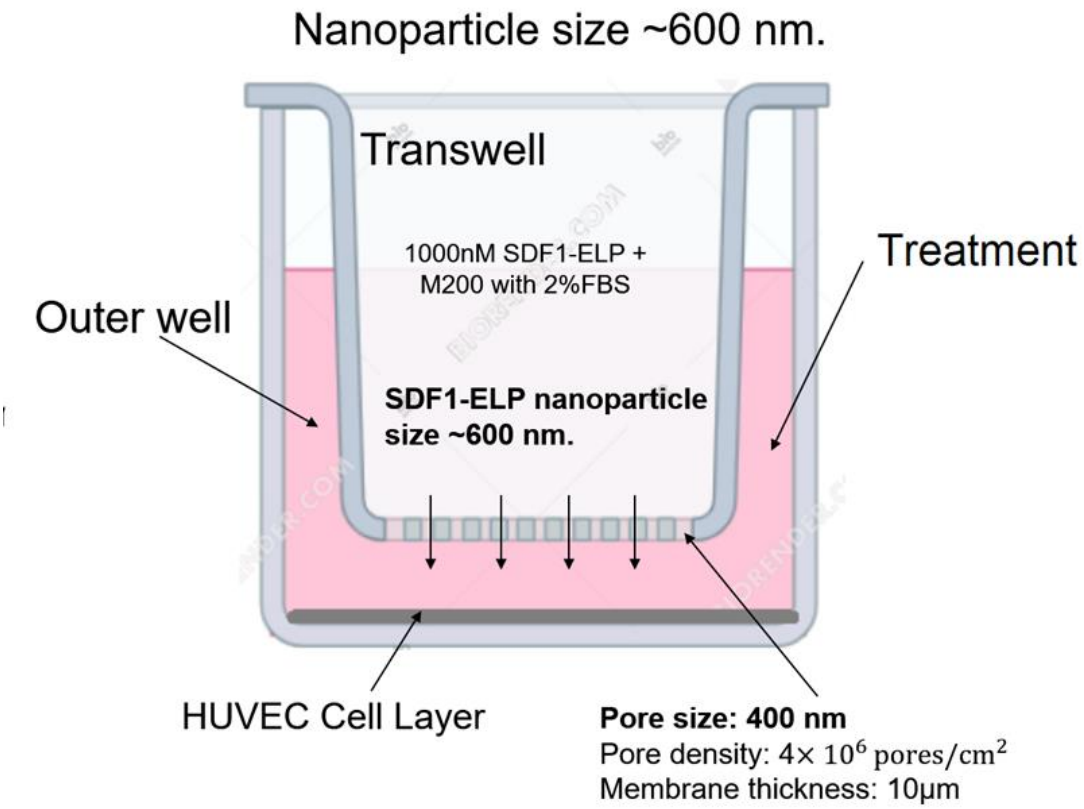


Figure 2. SDF1-ELP monomer Transwell® release system. The drug depot consists of SDF1-ELP nanoparticles that are retained above the porous Transwell membrane. SDF1-ELP monomers (and possibly small multimers) released from nanoparticles can however diffuse through the pores to reach the outer well and eventually the target cell layer (here HUVECs). The medium in the outer well is supplemented with elastase to mimic the proteolytic activity of wound fluid.

CHAPTER II Materials and Methods

2.1. Materials

2.1.1. Human Umbilical Vein Endothelial Cell (HUVEC) Culture

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from human umbilical veins and cryopreserved at the end of the primary culture. They were purchased at this stage from Thermo Fisher (Invitrogen, cat. no. C-003-5C). Endothelial

cells express CXCR4 receptors on their surface that can be targeted by SDF1. During wound healing, SDF1 can induce vascularization by endothelial cells [8][14-17].

2.1.2. Medium

Medium 200 (GIBCO, cat. no. M-200-500) supplemented with Low Serum Growth Supplement (LSGS) (GIBCO, cat. no. S-003-10) was used to maintain the HUVEC line. However, during the experiments when HUVECs were exposed to the SDF1 conditions, we found that LSGS masked the effect of the supplied growth factor. Therefore, we switched the cells to an experiment medium made with Medium 200 + 2% Fetal Bovine Serum (FBS). Comparing to Medium 200 +LSGS, HUVEC growth rate was lower in the experiment medium; however, cells would respond to the supplied growth factors.

2.2. Methods

2.2.1. Endothelial Cell Preparation

$2x10^5$ HUVECs were seeded in a T75 flask containing 15 mL growth medium (M200+LSGS). When cells reached ~70% confluence, they were either used in an experiment or passaged. To harvest cells from the flask, 3 mL Trypsin-EDTA (GIBCO) were added to the flask after aspirating the medium, followed by gently shaking the flask for 3 minutes and then mixing in 6 mL Trypsin Neutralizer (GIBCO, cat.no. R-002-100). All liquid was transferred to a 150mL tube. The tube was centrifuged at 400 relative

centrifugal field (RCF)for 7 minutes, the supernatant was carefully discarded and the cell pellet preserved. The cell pellet was resuspended in 1 ml fresh growth medium and cell number was counted in a hemocytometer. The cell suspension was diluted to a suitable concentration to be used or passaged. In this research, passage 2-5 (P2-P5) HUVECs were used.

2.2.2. Expression and Purification of SDF1-ELP Fusion Protein

The SDF1-ELP fusion protein expression and purification were modified from the method designed by Yeboah et al. [11]. To express SDF1-ELP, a pET25B+ vector (Life Technologies) with SDF1-ELP was transformed into *Escherichia coli* BL21 Star DE3 (Invitrogen). In the plasmid, SDF1 was previously fused to ELP repeats via NdeI and XbaI restriction sites [11].

On Day 1, transformed *E.coli* was cultured on an agar plate overnight at 37°C. On Day 2, a single bacterial colony was picked for an overnight culture in 10 mL of LB medium containing 1:1000 (v/v) carbenicillin. On Day 3, all 10 mL liquid was transferred to 1 L terrific broth (Thermo Fisher), supplemented with 4% glycerol, and with 1:1000 (v/v) carbenicillin. The culture was left overnight on a shaker at 225 rpm and 37°C.

On Day 4, the culture was centrifuged at 3000 ×g. The supernatant was discarded, and the pellet saved. Then the pellet was resuspended in 25 mL of PBS and sonicated on ice for 3 minutes in 5s on/25s off cycles. Poly(ethyleneimine) solution (Sigma Aldrich) was added to the lysate at 0.5% w/v to precipitate the nucleic acid contamination. After

centrifuging, SDF1-ELP nanoparticle formation was induced by the addition of 0.3M of Sodium Citrate and incubation at 40 °C. Then inverse transition cycling (ITC) was performed to purify the SDF1-ELP product.

The inverse transition cycling (ITC) method was used to purify the SDF1-ELP product (**Fig.16** in Appendix). In previous research, the inversion temperature of SDF1-ELP was determined to be ~35°C. Therefore, SDF1-ELP was purified by warming the protein to 40°C to induce aggregation and centrifuged at that same temperature, followed by resuspending the pellet in ice-cold PBS to dissolve the particles, which were then centrifuged at 4°C to removed insoluble impurities. Three rounds of temperature cycling were used to obtain SDF1-ELP nanoparticles with the desired purity. For control studies, the ELP protein alone was expressed and purified in a similar method.

SDS-PAGE was run to check the purity of the SDF1-ELP product. All reagents and materials for this assay were obtained from Bio-Rad Laboratories. The SDF1-ELP product in 1X PBS buffer was diluted with loading buffer and run on an 8–16% Mini-PROTEAN® TGX™ 10 well, 50 µl Gel in a Bio-Rad Mini Protean Tetracell. Precision Plus Protein™ Kaleidoscope™ standards were used to show the approximate molecular weight.

Then, the SDF1-ELP product concentration was measured with a protein quantitation assay (Thermo Fisher, Pierce™ BCA-RAC Assay, cat.no.15045).

2.2.3. SDF1-ELP Dose Optimization – Proliferation Assay

A typical dose of SDF1 used in prior research was 10nM [22], and in previous research, 1000nM SDF1-ELP was used to test the bioactivity towards HL60 cells as the target [11]. However, the optimal SDF1-ELP dose for HUVECs has not been tested yet. In order to determine the optimal dose for HUVEC proliferation, this experiment was performed.

The HUVECs were seeded in 24 well plates at $2.5x10^4$ cells/well in 500 µL/well of growth medium (M200+LSGS). Cells were allowed to attach to the plate for 24 hours in a 5% CO_2 atmosphere at 37˚C. On Day 2, the medium was then switched to 500µL experiment medium (M200+2%FBS) supplied with 0 nM, 10 nM, 100 nM, or 500 nM SDF1-ELP nanoparticles.

Table 1. SDF1-ELP Dose Optimization: Medium, Supplement, and Treatment

Group	Medium	Supplement	Treatment
Control	M200	2% FBS	NA
E1	M200	2% FBS	10 nM SDF1-ELP
E2	M200	2% FBS	100 nM SDF1-ELP
E3	M200	2% FBS	500 nM SDF1-ELP

After a 48-hr culture in a 5% CO_2 atmosphere at 37˚C, medium was removed, and cells were washed twice with warm experiment medium. Experiment medium was then added with 10% Alamar Blue® in a total volume of 500 µL/well, and the fluorescence (540 nm excitation/590 nm emission) was measured after 2 hours of incubation at 37˚C. Alamar Blue® is a fluorescence-based method to noninvasively measure cell number.

The fluorescence intensity was converted to cell number using a standard curve. The cell numbers were normalized to the cell number of the control group (0nM SDF1-ELP group) and expressed as cell number fold change.

2.2.4. SDF1-ELP Biological Activity Targeting HUVECs - Proliferation Assay

The HUVECs were seeded in 24 well plates at $2.5x10^4$ cells/well in 500 µL/well of growth medium (M200+LSGS). Cells were allowed to attach to the plate for 24 hours in a 5% CO_2 atmosphere at 37˚C. On Day 2, the medium was then switched to 500µL experiment medium (M200+2%FBS) supplied with 0 nM, 10 nM, and 100 nM of SDF1-ELP, free SDF1 (R&D, ***E.coli***-derived CXCL12/SDF1 alpha protein, cat.no.350-NS-010) or ELP alone.

Table 2. SDF1-ELP Biological Activity Targeting HUVECs: Medium, Supplement, and Treatment

Group	Medium	Supplement	Treatment
Control	M200	2% FBS	NA (not applicable)
E1	M200	2% FBS	10 nM SDF1-ELP
E2	M200	2% FBS	100 nM SDF1-ELP
E3	M200	2% FBS	10 nM SDF1
E4	M200	2% FBS	100 nM SDF1
E5	M200	2% FBS	10 nM ELP
E6	M200	2% FBS	100 nM ELP

After a 48-hr culture in a 5% CO_2 atmosphere at 37˚C, the same procedure as described in section **2.2.3** was performed to determine the cell number fold change.

2.2.5. Elastase Concentration Optimization – Proliferation Assay

Previously, the stability of SDF1-ELP was tested and demonstrated within 1,000 nM of elastase [11]. However, for HUVECs, that concentration is too high for cells to survive. Elastase concentration higher than 300 nM can cause the detachment of HUVECs from its growth surface [20]. In addition, in wound fluid, the elastase concentration is >60 nM [19][21]. We hypothesized that an elastase dose between 60 nM and 300 nM could be used in our study. Therefore, this experiment was performed to optimize the elastase concentration used in later experiments.

This experiment was conducted using the same protocol as described in **2.2.3** except on Day 2, when the medium was switched to 500µL experiment medium (M200+2%FBS) supplied with 0 nM, 10 nM, 100 nM, and 500 nM human leukocytes elastase (Sigma-Aldrich, Elastase from human leukocytes, cat.no. E8140).

Table 3. Elastase Concentration Optimization: Medium, Supplement, and Treatment

Group	Media	Supplement	Treatment
Control	M200	2% FBS	NA
E1	M200	2% FBS	10 nM Elastase
E2	M200	2% FBS	100 nM Elastase
E3	M200	2% FBS	500 nM Elastase

2.2.6. SDF1-ELP Nanoparticle Stability and Bioactivity Targeting HUVECs in Presence of Elastase - Proliferation Assay

The proliferation assay was conducted using the same protocol as described in **2.2.3**. Group specification is shown in the table below.

Table 4. SDF1-ELP Nanoparticle Stability and Bioactivity: Medium, Supplement, and Treatment

Group		Media	Supplement	Treatment
Control	C1	M200	2% FBS	NA
	C2	M200	2% FBS	100nM Elastase
Blank	B1	M200	2% FBS	10 nM SDF1
	B2	M200	2% FBS	100 nM SDF1
	B3	M200	2% FBS	10 nM SDF1-ELP
	B4	M200	2% FBS	100 nM SDF1-ELP
Elastase	E1	M200	2% FBS	10 nM SDF1+100nM Elastase
	E2	M200	2% FBS	100 nM SDF1+100nM Elastase
	E3	M200	2% FBS	10 nM SDF1-ELP+100nM Elastase
	E4	M200	2% FBS	100 nM SDF1-ELP+100nM Elastase

2.2.7. SDF1-ELP Monomer Controlled Release System Validation

Under normal circumstances, SDF1-ELP nanoparticles coexist with their monomers at equilibrium. The system can be considered as a reversible first-order reaction with SDF1-ELP nanoparticles as the reactant and SDF1-ELP monomers as the product [13]. In order to evaluate the properties of the SDF1-ELP monomer, we designed a controlled release Transwell® system to separate nanoparticles and monomers based on their size. The pore size of the Transwell® insert (Corning, cat.no. CLS3470) permeable membrane is 400 nm, which is smaller than the SDF1-ELP nanoparticle size (>600 nm [11]) but greater than the monomer size (<10 nm [13]). Theoretically, only the monomer can freely pass the member and enter the outer well. Therefore, the SDF1-ELP

concentration in the outer well would gradually increase until it reaches an equilibrium. To validate the idea, we performed the experiment described in **Fig.1**. The treatment conditions are shown in the **Table 5**.

We prepared eight 24-well plates. The HUVECs were seeded in 24-well plates at $2.5x10^4$ cells/well in 500 µL/well of growth medium (M200+LSGS). Cells were allowed to attach to the plate for 24 hours in a 5% CO_2 atmosphere at 37˚C. On Day 2, the medium was switched to 600µL experiment medium (M200+2%FBS), and Transwell® inserts were added to the plates. Then, 200µL of 1000nM SDF1-ELP (total 200pmol) were added to each Transwell® insert. Theoretically, ~8% of SDF1-ELP can coexist with its nanoparticle [13]. If the system can only let the monomers pass to the outer well, the SDF1-ELP dose applied to HUVECs would be around ~20nM, which is in the optimal dose range (10-100nM). However, the HUVECs are expected to consume SDF1-ELP thus shifting the equilibrium to the monomer side. Therefore, the SDF1-ELP concentration in the vicinity of the cells could be somewhat lower than 20nM.

We also prepared a blank group which has the same setup but without cells on the bottom of the outer wells, and a control group with cultured HUVECs in plain experiment medium (no SDF1-ELP added). Group specifications are shown in the table below.

At 0hr, 1hr, 2hr, 4hr, 6hr, 10hr, 24hr, and 48hr time points, we took out one plate and collected the entire supernatant in the Transwell® inserts and the outer walls. Then, the cells were washed twice with warm experiment medium, and fresh experiment

medium with 10% Alamar Blue® was added in a total volume of 500 µL/well. The fluorescence (540 nm excitation/590 nm emission) was measured after 2 hours of incubation at 37˚C. The fluorescence intensity was converted to cell number by standard curve. The cell numbers were normalized to the cell number at 0hr and expressed as cell number fold change.

Table 5. SDF1-ELP Monomer Release System Validation: Medium, Supplement, and Treatment

Group	Media	Supplement	Treatment	Cell
Blank	M200	2% FBS	SDF1-ELP 1000nM	N
Control	M200	2%FBS	NA	Y
Experiment	M200	2% FBS	SDF1-ELP 1000nM	Y

The collected supernatants were analyzed with SDF1-alpha ELISA (R&D) to measure the SDF1-ELP concentration profile in both the Transwell® and outer well.

2.2.8. SDF1-ELP Monomer Stability and Bioactivity Targeting HUVECs in Presence of Elastase

This experiment was conducted using the same protocol as described in section **2.2.7**, but several new experiment groups were examined. Group information is shown in the table below.

Table 6. SDF1-ELP Monomer Release in Presence of Elastase: Medium, Supplement, and Treatment

Group		Media	Supplement	Treatment	Cell
Control	C1	M200	2% FBS	NA	Y
	C2	M200	2% FBS	SDF1-ELP 1000nM	Y
Blank	B1	M200	2% FBS	SDF1-ELP 1000nM	N
	B2	M200	2% FBS	SDF1-ELP 1000nM+ Elastase 100nM	N
Experiment		M200	2% FBS	SDF1-ELP 1000nM+ Elastase 100nM	Y

The same experiment was conducted again with 400 nM free SDF1 for comparison, **Table 7**. The overall SDF1 concentration in the entire system was ~100 nM, which is the optimized dose for HUVEC proliferation.

Table 7. SDF1 Release in Presence of Elastase: Medium, Supplement, and Treatment

Group		Media	Supplement	Treatment	Cell
Control	C1	M200	2% FBS	NA	Y
	C2	M200	2% FBS	SDF1 400nM	Y
Blank	B1	M200	2% FBS	SDF1 400nM	N
	B2	M200	2% FBS	SDF1 400nM+ Elastase 100nM	N
Experiment		M200	2% FBS	SDF1 400nM+ Elastase 100nM	Y

2.2.9. Statistical Analysis

After performing a one-way ANOVA, the Fisher Least Significant Difference test was used to analyze the data from two independent groups. A p-value <0.05 is considered statistically significant. A p-value of <0.05 is represented by a star (*) on the graphs.

CHAPTER III Results

3.1. Expression and Purification of SDF1-ELP

We used the ELP self-assembling property to purify the SDF1-ELP product; after three rounds of ITC above and below the inversion temperature of 35°C, a single band on SDS-PAGE was observed **(Fig.3)**. The band was at ~31 kDa, consistent with the predicted molecular mass of SDF1-ELP [11]. The BCA assay indicated that SDF1-ELP product concentrations ranged from 10μM to 60μM and were different in each batch.

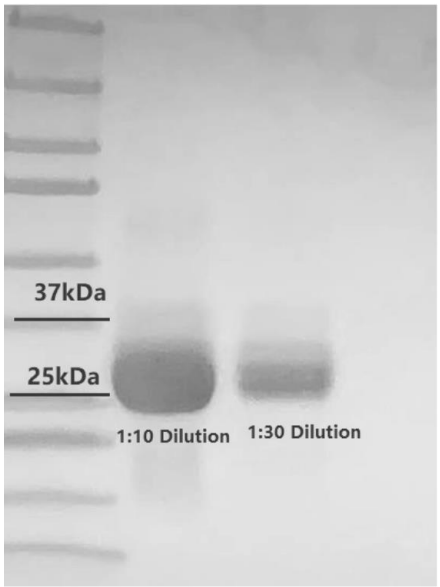


Figure 3. SDF1-ELP assessed by SDS-PAGE. SDF1-ELP generated a single band around 31kDa. The MW ladder was generated by Tetracell Precision Plus Protein™ Kaleidoscope™ standards.

3.2. SDF1-ELP Dose Optimization

To optimize the SDF1-ELP dose used in this research, we evaluated its effect on cell proliferation in HUVECs. Different concentrations of SDF1-ELP in the range of 0 to 500 nM were tested on HUVECs cultured in M200 media supplemented with 2% FBS. 0 nM served as control group. After a two-day culture, we noticed that SDF1-ELP at 100 nM caused the highest cell proliferation **(Fig. 4)**. Furthermore, SDF1-ELP at 500 nM resulted in lower cell proliferation compared to control.

10nM, 100nM, and 500nM groups were compared with each other and with the control group using a Fisher's LSD test. We found that the 10nM and 100nM groups were significantly higher than the control group ($p < 0.05$). However, 500nM was

significantly lower than the control group. Moreover, we found that the proliferation caused by 100nM SDF1-ELP was significantly higher than the 10nM group. Therefore, in the 0-100nM range, increasing SDF1-ELP enhanced HUVEC proliferation. Thus, SDF1-ELP in the range of 0-100nM was used in subsequent studies.

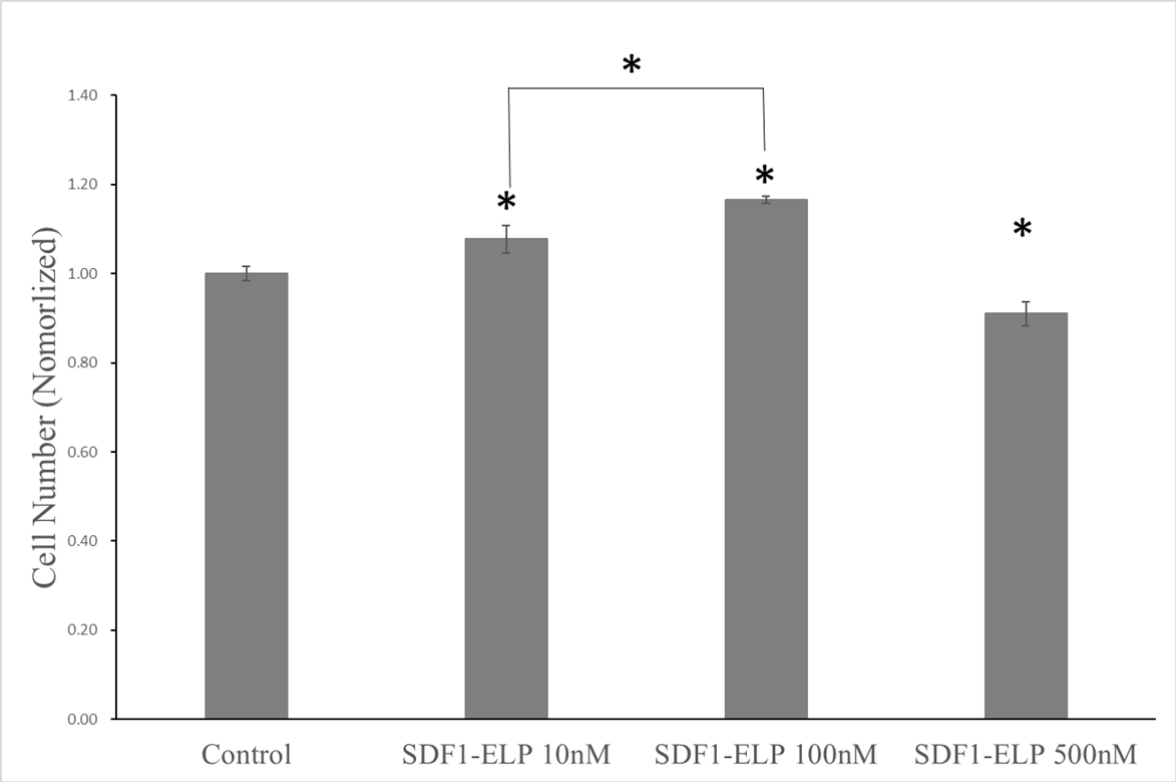


Figure 4. Effect of SDF1-ELP on HUVEC proliferation measured by cell number. Cell number was estimated by the Alamar Blue assay after culturing for 2 days in the specified conditions. Data shown are normalized cell number by the cell number of control group (M200+LSGS) expressed as mean ±standard error of the mean (SEM). N=3. (*: $p < 0.05$, one way ANOVA, Fisher's LSD post-test).

3.3. SDF1-ELP Nanoparticle Bioactivity Targeting HUVECs

Next we wanted to verify that the biological activity of SDF1-ELP is comparable to that of native SDF1. For this purpose, we use a proliferation assay to measure their effects on HUVEC number change over two days of culture. The effect of SDF1-ELP nanoparticles, free SDF1, and ELP (empty ELPs lacking SDF1) with a dose in the range of 0 to 100nM for each were examined on HUVECs. After a two-day culture with the supplied growth factors, we found that SDF1-ELP nanoparticles and free SDF1 at 100nM caused the highest HUVEC proliferation (**Fig. 5**). We then directly compared SDF1-ELP nanoparticles with free SDF1 at the same dose. Neither the 10nM nor 100nM groups showed a significant difference. In addition, free SDF1 had the same dose-response as SDF1-ELP nanoparticles in the 0 to 100nM range, namely that increasing dose promoted further proliferation. At the 10nM dose, SDF1-ELP and free SDF1 increased HUVEC number by over 8%; at 100nM, HUVEC number increased by ~20%. Therefore, in the 0 to 100nM range, SDF1-ELP had a similar bioactivity to free SDF1 using the same doses when targeting HUVECs.

We also compared the effect of SDF1-ELP nanoparticles at two representative doses with plain medium (control) and ELP, and found that SDF1-ELP caused a significantly enhanced proliferation over the other two groups. In addition, as shown in **Fig.5**, ELP at two doses showed no significant difference compared to the control group. Thus, ELP alone had no contribution to enhanced HUVEC proliferation.

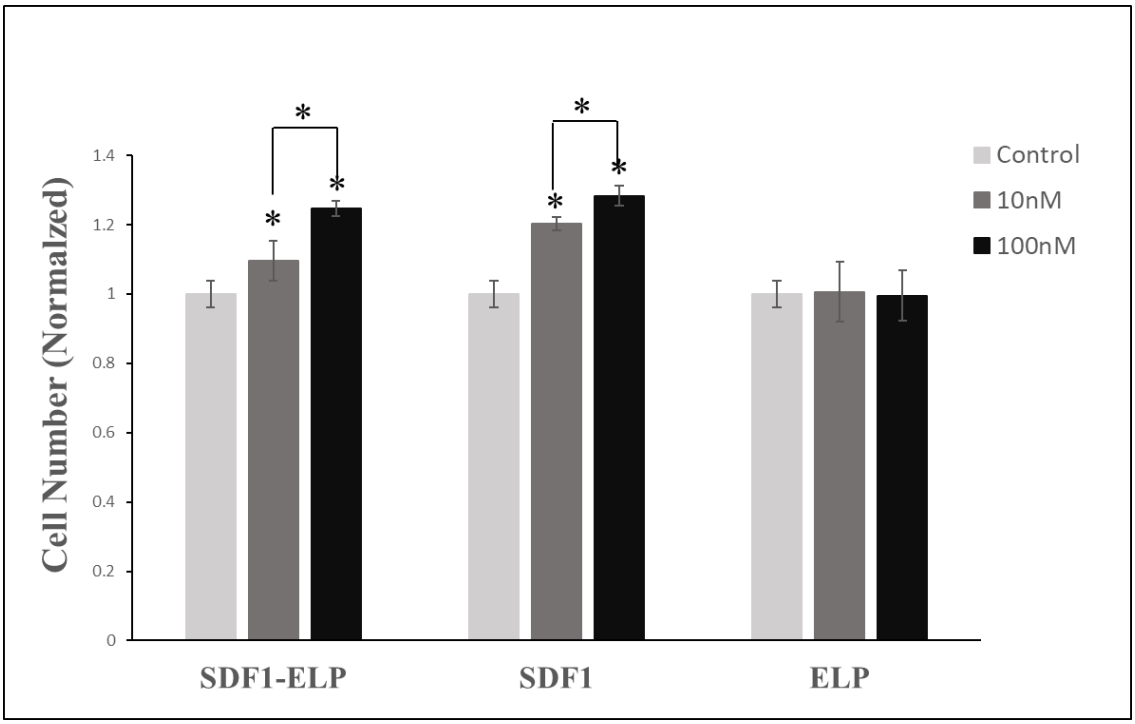


Figure 5. Dose response using SDF1-ELP, SDF1, and ELP on HUVEC proliferation measured by cell number. Cell number after 2 days of culture in the specified conditions was estimated by the Alamar Blue assay. Data shown are normalized cell number by the cell number of the control group (M200+LSGS) expressed as mean ±standard error of the mean (SEM). N=3. In each group, two representative doses were compared with the control group. (*: $p < 0.05$, one-way ANOVA, Fisher's LSD post-test)

3.3. Elastase Concentration Optimization

Previously, the stability of SDF1-ELP was tested and confirmed in 1,000 nM of elastase [11]. However, for HUVECs, that concentration is lethal, and elastase concentration higher than 300 nM can cause the detachment of HUVECs from its growth surface [20]. Several studies have reported that the elastase concentration in wound fluid is >60nM [19][21]. Therefore, the effect of elastase at 10nM, 100nM, and 500nM on HUVEC survival and proliferation was investigated.

As shown in **Fig. 6**, compared with the medium only group (control), the HUVEC number and shape exhibited no obvious change in the presence of 10nM elastase (**Fig. 6b**). When the elastase dose was increased to 100nM, the cell number did not change significantly; however, the cell shape became more spherical **(Fig. 6c),** which indicated a slight detachment of HUVECs. At the 500nM dose, over 90% of HUVECs were gone, and only some debris (**Fig. 6d**) were left on the surface.

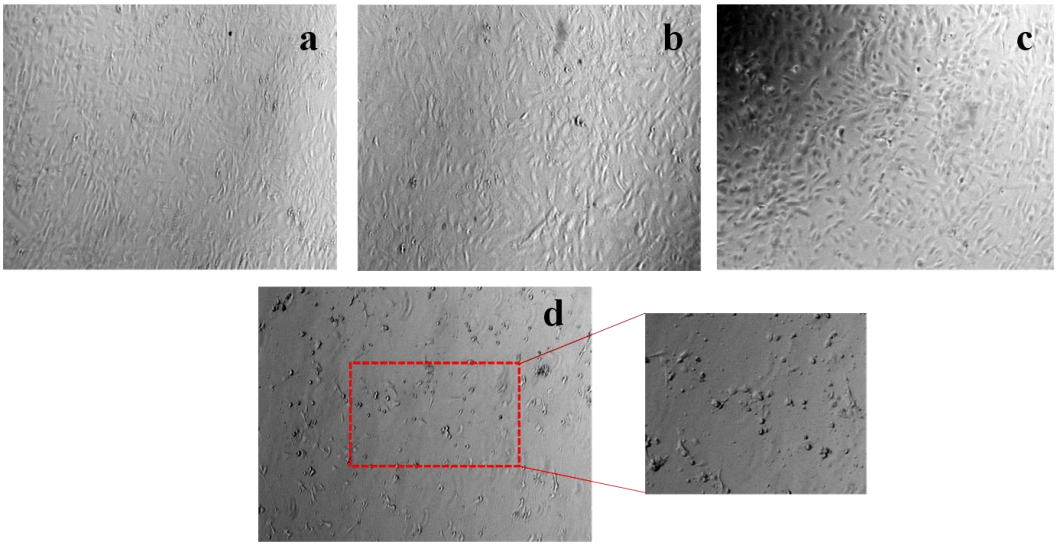


Figure 6. HUVECs cultured in presence of elastase. (a) HUVECs cultured in experiment medium (M200+2%FBS). Cells were in their normal shape. (b) HUVECs cultured in presence of 10nM elastase. Cells could still retain their normal shape. The cell number did not obviously change. (c) HUVECs cultured within 100nM elastase. Cells became more spherical; however, cell number appeared to be maintained. (d) HUVECs cultured in 500nM elastase. Over 90% cells were gone and only some debris were left.

Quantification of cell number using an AlamarBlue® proliferation assay confirmed these results. As shown in **Fig. 7**, 10nM and 100nM elastase groups were not significantly different from the control group; however, the 500nM elastase group was

significantly lower than the control. As mentioned earlier, elastase concentration in wound fluid is reported to be >60nM [19][21]; thus, 100nM elastase was chosen in subsequent experiments.

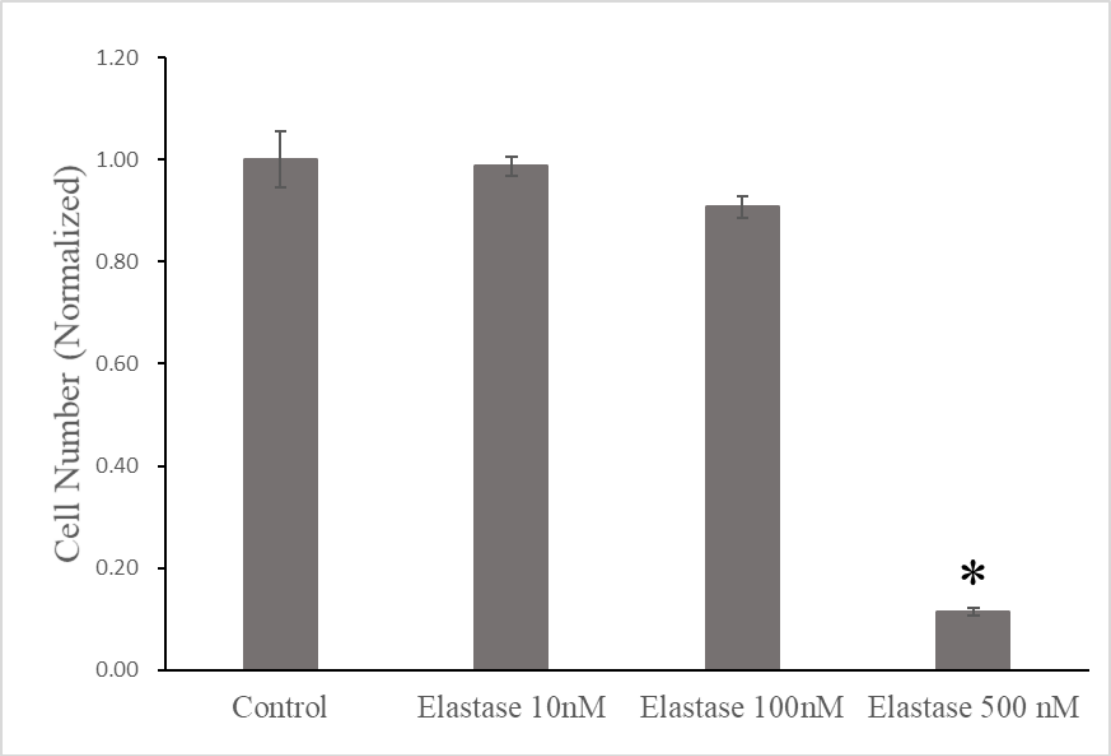


Figure 7. Effect of elastase on HUVEC proliferation measured by cell number. Cell number was estimated by the Alamar Blue assay after 2 days of culture in the specified conditions. Data shown are normalized cell number by the cell number of control group (M200+LSGS) expressed as mean ±standard error of the mean (SEM). N=3. Each group was compared with control. (*: $p < 0.05$, one-way ANOVA, Fisher's LSD post-test).

3.4. SDF1-ELP Nanoparticle Stability and Bioactivity Targeting HUVECs in Presence of Elastase

To characterize the SDF1-ELP nanoparticle stability and bioactivity targeting HUVECs, two representative doses (10nM and 100nM) of SDF1-ELP nanoparticles and free SDF1 were tested on HUVECs. In addition, we set up two growth conditions; one in

presence of 100nM elastase and one without elastase. that the purpose of this experiment was therefore to evaluate the impact of elastase on SDF1-ELP nanoparticle and free SDF1 bioactivity. The bioactivity was measured based on the HUVEC proliferative response using an Alamar Blue assay.

In each condition, four experiment groups were compared to the control group separately, and SDF1-ELP nanoparticle groups were compared to the free SDF1 groups at the same dose. In addition, we compared the SDF1-ELP nanoparticle groups and free SDF1 groups in two different growth conditions (with or without elastase).

As shown in **Fig. 8**, when there was no elastase, HUVEC proliferation was equally promoted by both SDF1-ELP nanoparticles and free SDF1. At the 10nM dose, cell number was increased by ~10%; at 100nM, cell number increased by ~18%. There was no significant difference in proliferation between the SDF1-ELP nanoparticle and free SDF1 groups at the same dose.

However, in the presence of 100nM elastase, the proliferation in response to SDF1-ELP nanoparticles only was preserved. In contrast, the proliferative response was lost in the free SDF1 groups. Moreover, we found that the proliferation rate in SDF1-ELP groups was significantly higher than in the free SDF1 groups at the same dose.Finally, when comparing the response to SDF1-ELP nanoparticles at the same dose with and without elastase, the difference was <6% and not statistically significant.

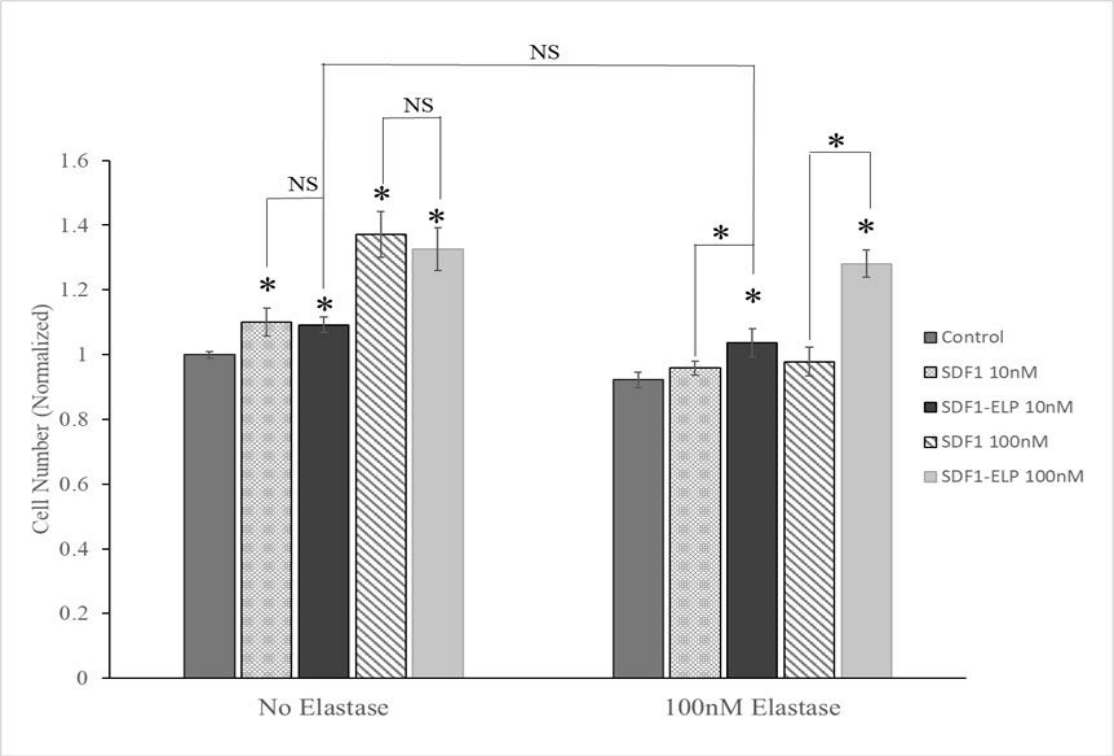


Figure 8. SDF1-ELP nanoparticle stability and bioactivity targeting HUVECs in presence of elastase. Cell number was estimated by the Alamar Blue assay after 2 days of culture in the indicated conditions. Data shown are normalized cell number by the cell number of control group (M200+LSGS) expressed as mean ±standard error of the mean (SEM). N=3. In each growth condition, four experiment groups were compared with control group. (*: $p < 0.05$, one-way ANOVA, Fisher's LSD post-test). Also, SDF1-ELP nanoparticle groups were compared with free SDF1 group at same dose. (*: $p < 0.05$; NS: $p \geq 0.05$, one-way ANOVA, Fisher's LSD post-test).

In summary, SDF1-ELP retained its bioactivity targeting HUVECs when evaluated based on the proliferative response in presence of 100nM elastase. In contrast, free SDF1 lost its bioactivity. Thus, SDF1-ELP nanoparticles had greater stability and potency than free SDF1 in the presence of elastase.

3.5. SDF1-ELP Monomer Release System Validation

SDF1-ELP nanoparticles coexist in thermodynamic equilibrium with the

monomer form, as well as small multimers. When target cells (here HUVECs) are incubated in direct contact with the SDF1-ELP, it is not possible to differentiate the bioactivity of the nanoparticles vs. that of the monomers and/or smaller multimers that are released from the nanoparticles. This is important because in an in vivo use of the SDF1-ELP, we anticipate that monomers and small multimers may diffuse more rapidly to the target than the larger nanoparticles. Thus, the purpose of these studies is to assess more specifically the bioactivity of the monomers and small multimers that are released from the nanoparticles in a simulated in vivo environment.

In order to evaluate the properties of the SDF1-ELP monomers and small multimers, we designed a controlled release Transwell® system to separate nanoparticles from monomers and small multimers based on their size. The pore size of the Transwell® insert permeable membrane is nominally 400 nm, which is smaller than the SDF1-ELP nanoparticle size and greater than the size of monomers and multimers. Theoretically, only the monomers and small multimers should freely pass the membrane and enter the outer well to reach the target cells. If the SDF1-ELP monomers and small multimers can successfully diffuse to the outer well, their concentration in the outer well would gradually increase until it reaches equilibrium. Furthermore, HUVEC proliferation would be enhanced compared with the control group (without SDF1-ELP). Since it is not possible to distinguish monomers from multimers in this assay, to keep terminology in the description of the results below, we use the term “monomers” to describe both.

The SDF1-ELP concentration profiles of the experiment group (with SDF1-ELP

and HUVECs) and blank group (with SDF1-ELP; without HUVECs) are shown in **Fig.9**. The mass distribution of SDF1-ELP is shown in **Fig.11** in the Appendix to give a better illustration. The mass distribution was calculated with the volume of liquid in both compartments. A constant evaporation rate of medium was taken into the calculation. The HUVEC number increase of the experiment group (with SDF1-ELP) and control group (without SDF1-ELP) is shown in **Fig.12**.

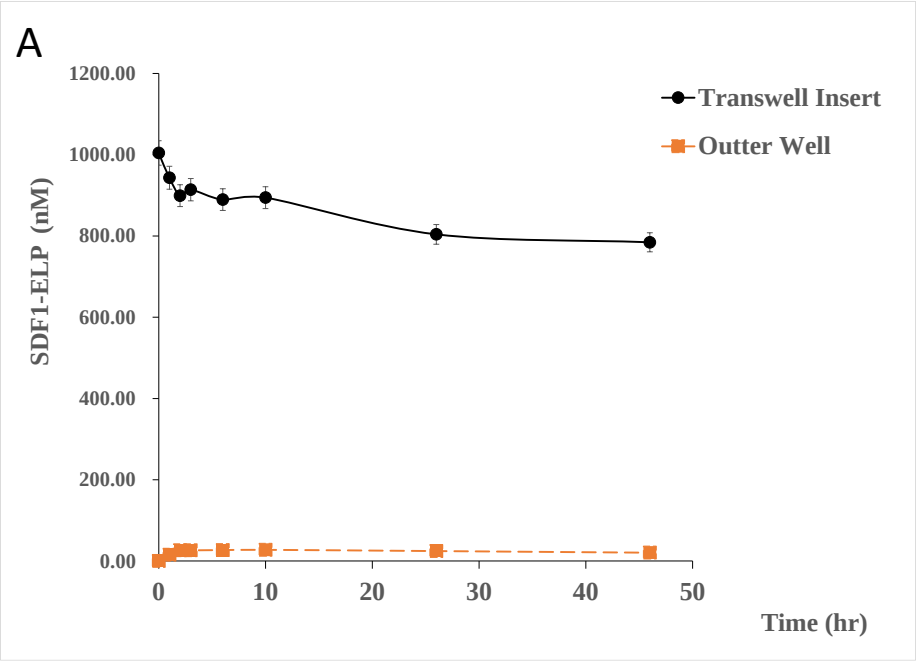
As shown in **Fig.10A** and **Fig**.1**1,** in the blank group ~10% of SDF1-ELP monomer was lost in the first 10-hr. After 48-hr, the number increased to ~20%. Among the SDF1-ELP lost in the insert over 48-hr, over 80% was diffuse to the outer well; the rest 20% SDF1-ELP might stacked in the membrane or stick on the well of insert. As we posited, the membrane maintained an SDF1-ELP concentration gap between the insert and outer well. Therefore, the membrane could control the SDF1-ELP monomer release to the bottom well.

As shown in **Fig.10B** and **Fig**.**11**, in the experiment group (with HUVECs), SDF1-ELP monomer rapidly diffused (~50% of the overall released amount) to the outer well in the first 6 hours, especially in the first 3 hours. After 10 hours, it approached an equilibrium so that SDF1-ELP concentration varied slowly thereafter. In addition, at the end of the experiment, the SDF1-ELP concentration in the insert and in the outer well of the blank group were higher than the experiment groups. **Fig.16** in the Appendix gives a better illustration of that. The reason for this phenomenon was that HUVECs most likely scavenged SDF1-ELP, thus decreasing their concentration in both compartments.

Consequently, when at equilibrium, the overall amount of SDF1-ELP in the experiment groups was lower, and the amount of lost SDF1-ELP (presumably consumed by the HUVECs) was higher than the blank group with no cells.

As shown in **Fig.11**, compared with the control group (without SDF1-ELP), the proliferation of the experiment group (with SDF1-ELP) was enhanced. At 24-hr, the experiment group had ~5% more cells; at 48-hr, that difference increased to ~10 %. Therefore, SDF1-ELP successfully diffused into the outer well to cause HUVEC proliferation enhancement.

In conclusion, this system successfully separated the SDF1-ELP nanoparticle and its monomer; and the monomers were utilized by HUVECs to enhance proliferation. Therefore, this system could be used to test SDF1-ELP monomer stability and bioactivity targeting HUVECs in the following experiment.



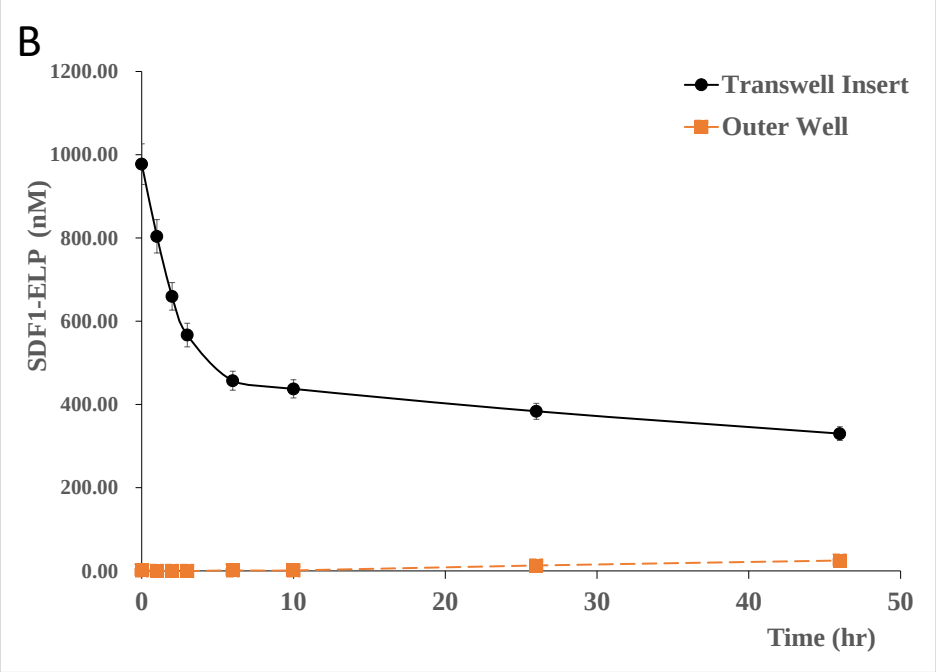


Figure 9. Release of SDF1-ELP monomers from Transwell insert to outer well. The Transwell release system was incubated at 37˚C for up to 48 h. Samples were collected at 0-hr, 1-hr, 2-hr, 3-hr, 6-hr, 10-hr, 24-hr, and 48-hr and total SDF-1-ELP content in Transwell and bottom well measured. (A) Blank group: SDF1-ELP(+); HUVECs(-). (B) Experiment group : SDF1-ELP(+); HUVECs(+).

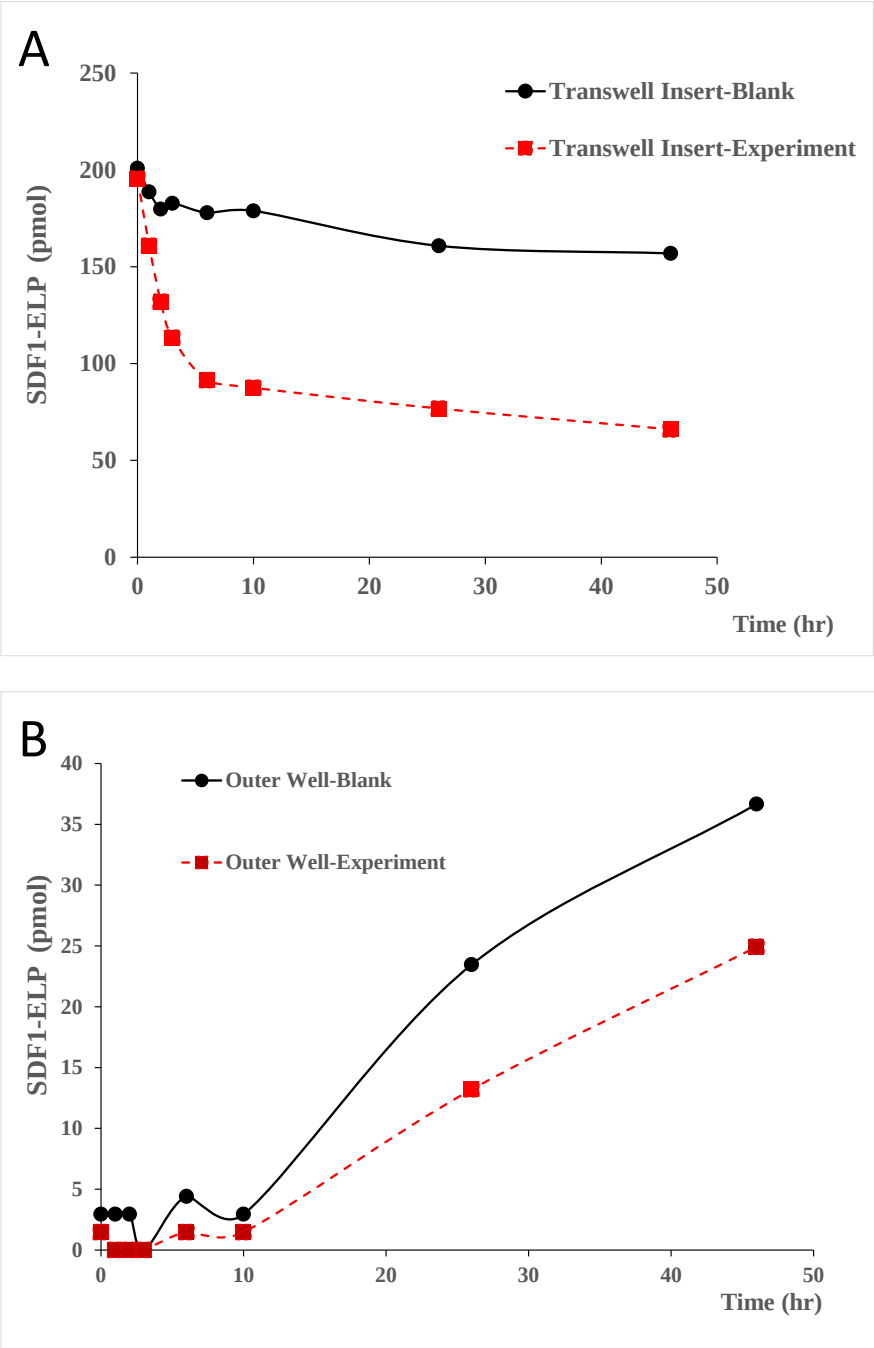


Figure 10. Release of SDF1-ELP monomers from Transwell insert to outer well. The Transwell release system was incubated at 37˚C for up to 48 h. Samples were collected at 0-hr, 1-hr, 2-hr, 3-hr, 6-hr, 10-hr, 24-hr, and 48-hr. The amount of SDF1-ELP was calculated by mass balance. The initial volume of liquid in the Transwell insert was 200µ; in the outer well was 600µL. A linear volume loss due evaporation was accounted for in the calculation. (A) Amount of SDF1-ELP in the insert. (B) Amount of SDF1-ELP in the outer well.

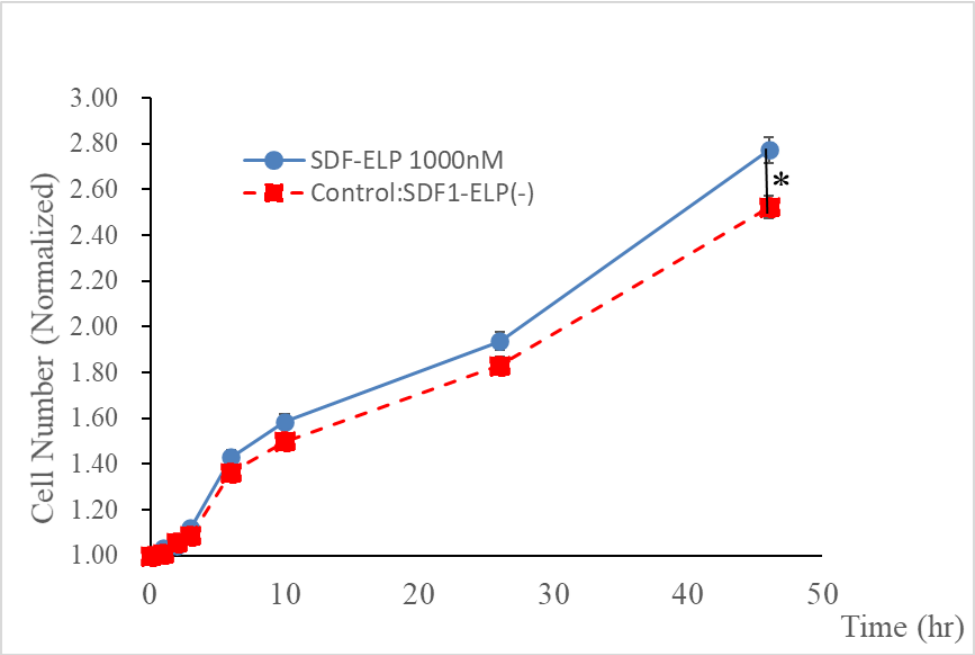


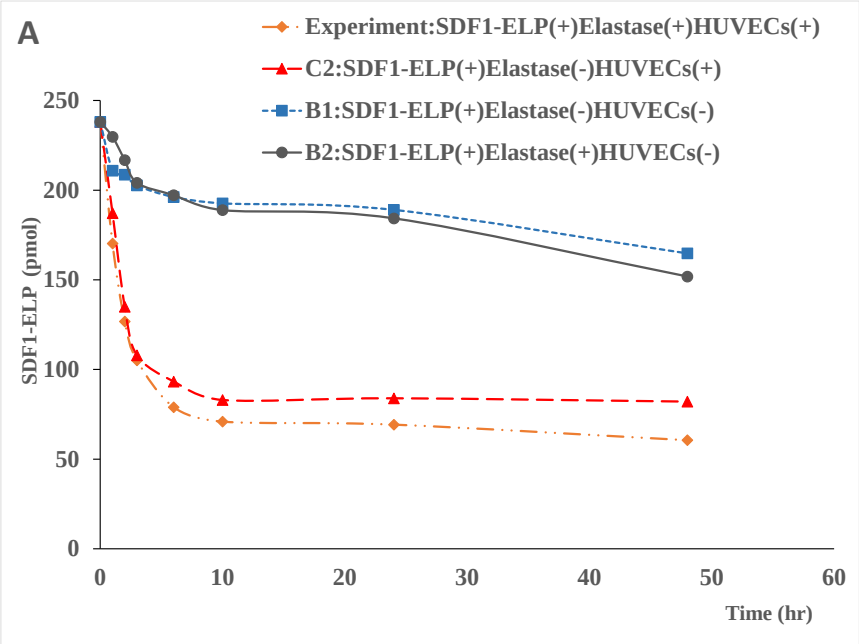
Figure 11. Effect of SDF1-ELP monomer on HUVEC proliferation measured by cell number. Cell number was estimated by the Alamar Blue assay at the time points indicated. Data shown are normalized cell number by the cell number of 0-hr. In the control group HUVEC was cultured in plain medium. In experiment group 1000nM SDF1-ELP was added to Transwell insert. (*: $p < 0.05$).

3.6. SDF1-ELP Monomer Stability and Bioactivity Targeting HUVECs in Presence of Elastase

In this experiment, five groups of treatments were tested and compared. The detailed specification of each group was previously shown in Table 6.

As shown in **Fig.12**, from 6-hr, there was a difference between the amount of SDF1-ELP consumed in the groups with elastase (B1, Experiment) and the groups without elastase; and this gap increased with time. At 48-hr, the difference between B1 and B2 was ~20%, and was ~15% between the experiment and C2 group (SDF1-ELP+HUVECs). Interestingly, SDF1-ELP consumption in the experiment group, which

had ~10% lower cell number **(Fig.13)** than the C2 group, was ~15% higher than in C2. Taking the B1 (SDF1-ELP only) and B2 ((SDF1-ELP+Elastase) results into consideration; the groups with elastase consumed ~20% more SDF1-ELP than the groups without elastase. The reason could be due to SDF1-ELP monomer degradation. In the first 6-hr, as shown in **Fig.12, Fig.18&19** in the Appendix, the SDF1-ELP monomers were rapidly released to the outer well and consumed by HUVECs. After this initial 6-hr period, the consumption of SDF-ELP by HUVECs was relatively slow. The amount of SDF1-ELP in the outer well gradually increased and degradation was induced. Since the mass difference between elastase groups and no elastase groups started to show up between 3-hr and 6-hr time points and grew further apart in the next sampling interval, we estimated that SDF1-ELP monomers could stably exist in 100nM elastase for 3-5 hours. After that, the SDF1-ELP monomers were likely partially degraded.



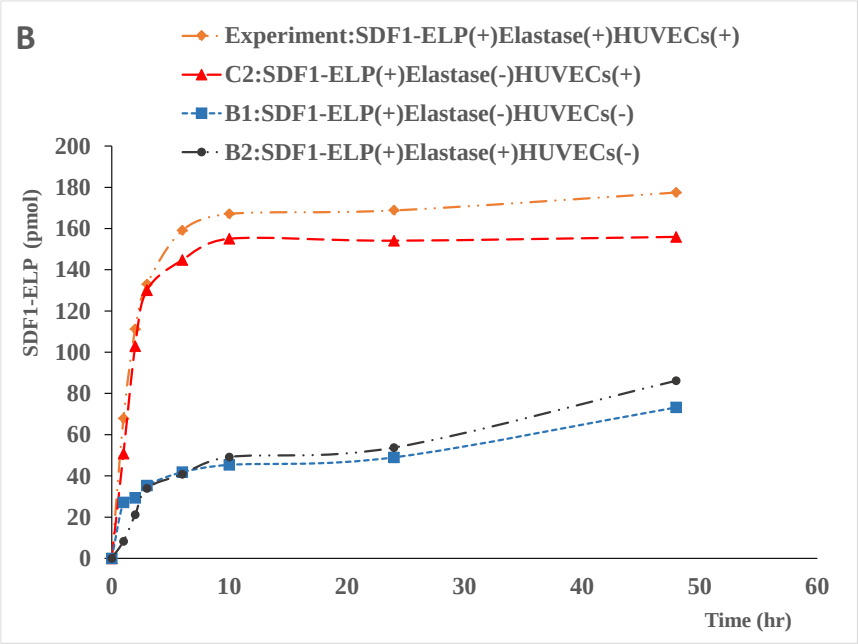


Figure 12. Total amount remaining and consumed amount of SDF1-ELP in Transwell insert and outer well. The Transwell release system was incubated at 37˚C for up to 48 h. Samples were collected at 0-hr, 1-hr, 2-hr, 3-hr, 6-hr, 10-hr, 24-hr, and 48-hr. The measured concentration of SDF1-ELP was multiplied by the volume in each compartment and summed up to calculate the total mass of SDF1-ELP in the system. (A) Average total SDF1-ELP in the entire system. (B) Consumed SDF1-ELP in the entire system, taken as the initial mass of SDF1-ELP minus that remaining at each time point.

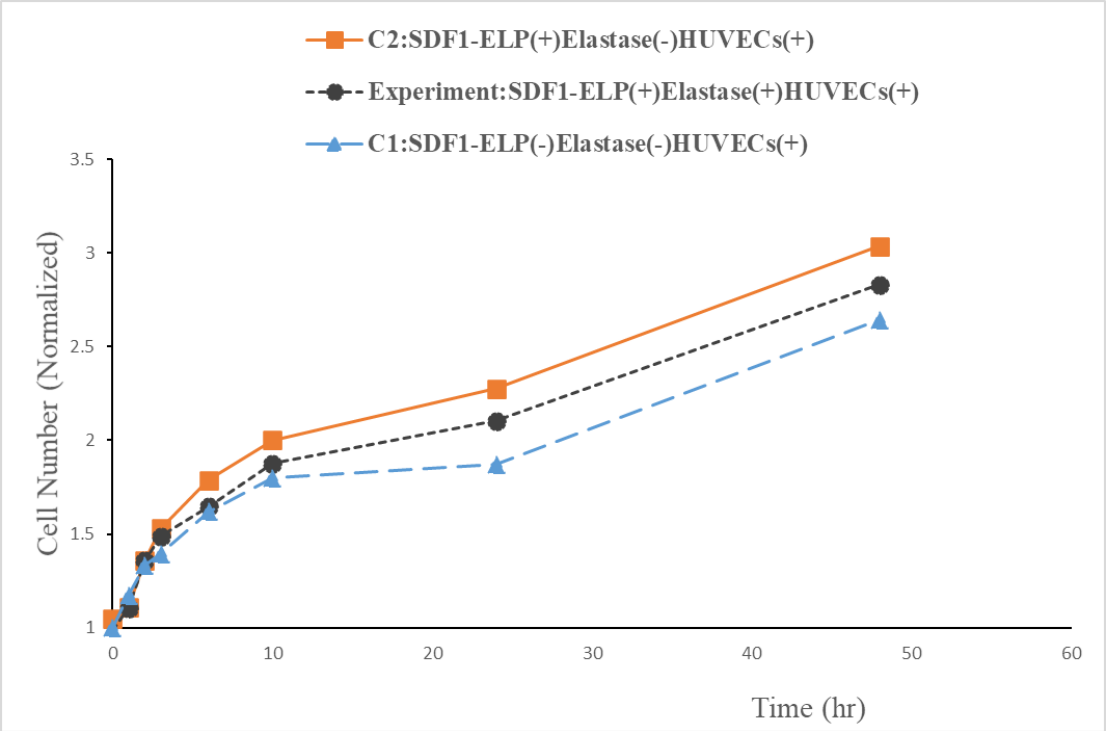


Figure 13. HUVEC proliferation measured by cell number change. Cell number was estimated by the Alamar Blue assay at the time indicated. Data shown are normalized cell number by the cell number at 0-hr. In the control group HUVECs were cultured in plain medium.

The other aim of this experiment was to evaluate the SDF1-ELP monomer bioactivity targeting HUVECs. As shown in **Fig.13**, compared with the C2 group (SDF1-ELP+HUVECs), HUVEC proliferation was lower in the experiment group. That was due to the negative effect of elastase on HUVEC proliferation. However, HUVEC proliferation in the experiment group (SDF1-ELP+Elastase+HUVECs) was ~12% higher than in the C1 group (plain medium). Therefore, although SDF1-ELP monomers were partially degraded, they could still retain significant bioactivity targeting HUVECs, based on an evaluation of the proliferative response in the presence of 100nM elastase.

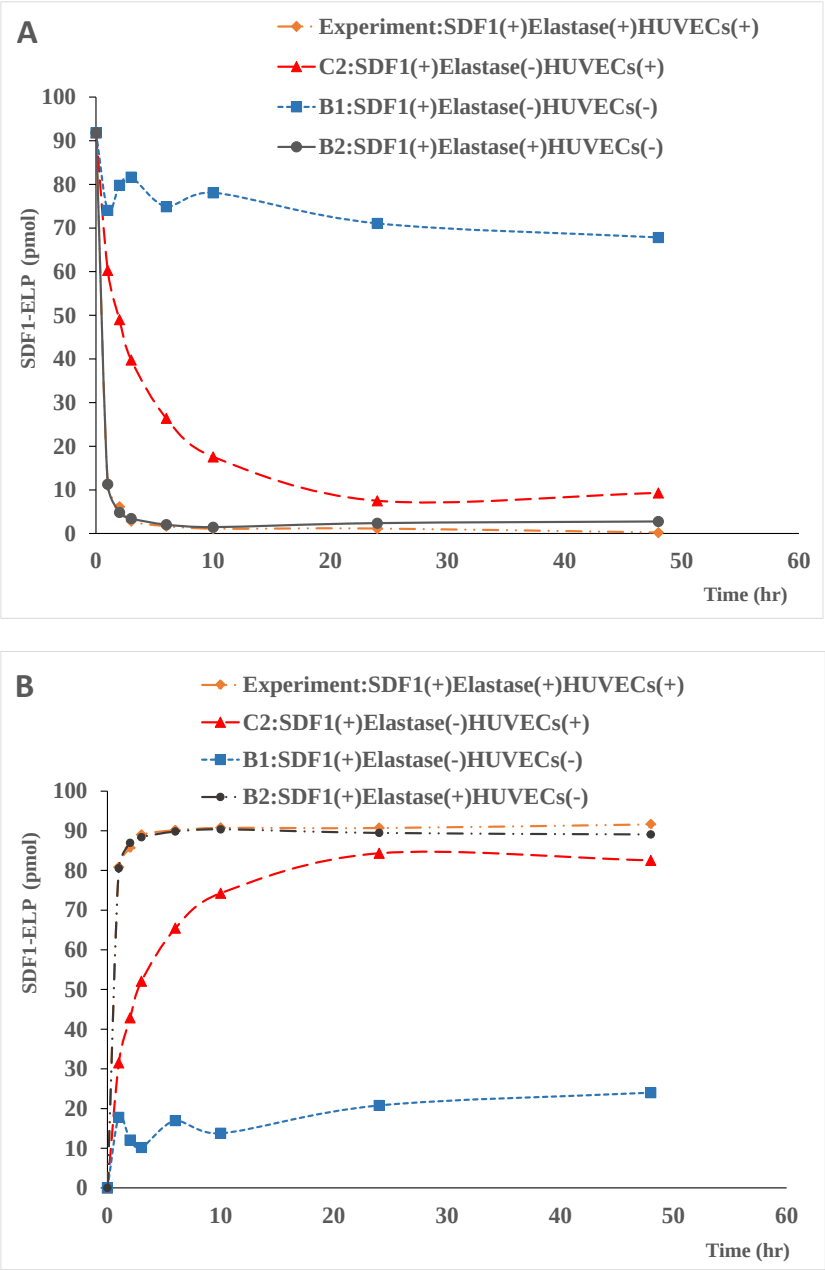


Figure 14. The total amount remaining and consumed amount of SDF1 in Transwell insert and outer well. (A) Average total SDF1 in the entire system. (B) Consumed SDF1 in the entire system, taken as the initial mass of SDF1-ELP minus that remaining at each time point.

In addition, compared with free SDF1, SDF1-ELP monomers show a superior

stability. As shown in **Fig.14**, >95% of free SDF1 was degraded in the first hour. However, 80% of SDF1-ELP monomers were preserved over 48-hr. Also, free SDF1 completely lost its bioactivity as measured by HUVEC proliferation. As shown in **Fig.15**, HUVEC proliferation in the experiment group (SDF1-ELP+Elastase+HUVECs) was ~15% lower than in the C1 group (plain medium).

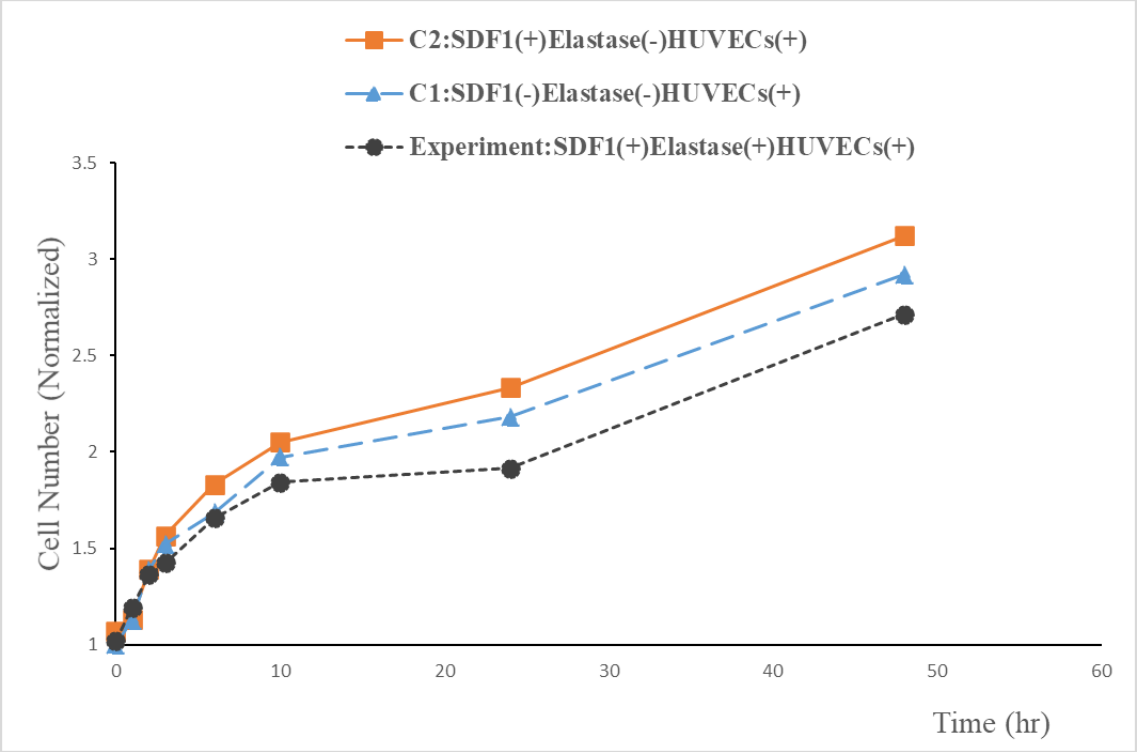


Figure 15. HUVEC proliferation measured by cell number change. Cell number was estimated by the Alamar Blue assay at the time indicated. Data shown are normalized cell number by the cell number at 0-hr. In the control group HUVECs were cultured in plain medium.

CHAPTER IV Discussion and Conclusion

In this study, we used a proliferation assay to evaluate the HUVEC responses to SDF1-ELP and free SDF1. We investigated the SDF1-ELP nanoparticle stability and bioactivity in presence of 100nM elastase and compared it with that of free SDF1. Moreover, we also designed a Transwell release system to separate the SDF1-ELP monomers from its nanoparticles and tested monomer stability and bioactivity targeting HUVECs in the presence of elastase.

In vitro, SDF1-ELP enhanced HUVEC proliferation in the 0-100nM range. We found that when there was 10nM SDF1-ELP in the culture media, after two-day culture, HUVEC cell number was ~8% higher than in the plain medium group. When 100nM SDF1-ELP were added to the medium, this number increased to ~18%. However, when the SDF1-ELP dose reached 500nM, HUVEC proliferation was hampered by ~13% compared to the plain medium group. In addition, free SDF1 was also applied to HUVECs to serve as a control group against which comparisons in the efficacy of SDF1-ELP could be made. The proliferation assay result indicated that, at the same dose, there was no significant difference between the HUVEC responses to SDF1-ELP and free SDF1. Therefore, we concluded that SDF1-ELP had similar bioactivity as free SDF1 when targeting HUVECs.

However, despite the similarity in in vitro bioactivity of SDF1-ELP to SDF1, we observed SDF1-ELP nanoparticles to be more stable than free SDF1 in presence of 100nM of elastase. In 100nM elastase, SDF1-ELP nanoparticles could still promote

HUVEC proliferation to the same level as the control group with no elastase. On the contrary, free SDF1 completely lost its bioactivity. The proliferation assay result indicated that, compared with the enhanced HUVEC proliferation in the no elastase group, it dropped back to the same level as the plain medium group. This result replicated the conclusion of a previous study [12].

The most important part of this study was to develop a system to study the release of monomers from nanoparticles and monomer stability. We designed a controlled release Transwell system with a porous filter with a nominal pore size of 400 nm, which is smaller than SDF1-ELP nanoparticle average size. Theoretically, only the monomer can freely pass the member and enter the outer well. This system mimics the topical application of a drug on a skin wound. The SDF1-ELP nanoparticles were put onto a culture insert with a permeable membrane (serving as drug depot). Cultured on the bottom of the outer well, there was a HVEC layer targeted by the SDF1-ELP monomer. The monomers are gradually released from the nanoparticle drug depot and diffuse to the target cells. In addition, elastase was added to the medium in the outer well to mimic the presence of wound fluid. Theoretically only the SDF1-ELP monomer can enter the outer well, their biology activity towards targeted HUVEC and their stability in the presence of elastase could be evaluated.

In the blank groups without HUVEC, ~10% of SDF1-ELP monomer was lost in the first 10-hr. After 48-hr, the number increased to ~20%. Among the SDF1-ELP lost in the insert over 48-hr, over 80% was diffuse to the outer well; the rest 20% SDF1-ELP

might stacked in the membrane or stick on the well of insert. When HUVEC introduced, SDF1-ELP monomer rapidly diffused (~50% of the overall released amount) to the outer well in the first 6 hours, especially in the first 3 hours. After 10 hours, it approached an equilibrium so that SDF1-ELP concentration varied slowly thereafter. In addition, at the end of the experiment, the SDF1-ELP concentration in the insert and in the outer well of the blank group were higher than the experiment groups. The reason for this phenomenon was that HUVECs most likely scavenged SDF1-ELP, thus decreasing their concentration in both compartments. In this system, HUVEC proliferation responded positively to the addition of the SDF-ELP nanoparticles to the Transwell membrane, which confirms that the SDF-ELP released was biologically active.

When 100 nM of elastase added to the system, a difference between the amount of SDF1-ELP consumed in the groups with elastase and the groups without elastase appeared. For example, in presence of 100nM elastase over 3 hours, SDF1-ELP monomer was partially lost, presumably due to degradation. During the 48-hr monomer releasing experiment, ~20% of monomers were degraded. However, its bioactivity, as evaluated by HUVEC proliferative response, was retained. Even though the elastase could negatively impact HUVEC proliferation, with SDF1-ELP monomer applied, the proliferation could still be promoted to a higher level than the control group with plain medium.

We then replaced SDF1-ELP with free SDF1 and repeated the same experiment. Compared with SDF1-ELP monomer, the degradation rate of free SDF1 was much more

rapid. In the presence of the same dose of elastase, >95% free SDF1 was degraded in first hour and completely lost bioactivity. However, ~80% of SDF1-ELP monomers were preserved over 48-hr. Therefore, SDF1-ELP monomer's stability was superior.

In the SDF1-ELP and free SDF1 controlled release experiment, the HUVEC proliferation data also seemed to correlate with the amount of SDF1 or SDF1-ELP available in the system, with elastase decreasing the proliferative response. However, the comparison no elastase vs. with elastase is not necessarily an effect of the degradation of the SDF1 or SDF1-ELP only, because elastase by itself could impair HUVEC proliferation.

In conclusion, the unique property of ELPs makes SDF1-ELP nanoparticles suitable to form a drug depot that can not only gradually release its monomers, but also can enhance their stability towards elastase. Moreover, SDF1-ELP can promote endothelial cell proliferation, which plays an important role in revascularization during the wound healing process. Therefore, SDF1-ELP is a promising recombinant growth factor for the treatment of chronic skin wounds.

CHAPTER V Future Work

To provide more information about the effect of SDF1-ELP monomer bioactivity and stability, future work is necessary. Firstly, it would be helpful to further investigate the effect of SDF1-ELP monomer on endothelial cells. During wound healing, ECs play

an important role in revascularization through the SDF-CXCR4 pathway to induce vascularization [8][15]. In this study, we mainly evaluated the proliferative response; however, EC migration is another important process during angiogenesis in the wound healing process. Therefore, an in vitro wound healing “scratch” assay or tube forming assay could be used in future studies to evaluate other relevant responses. With a similar controlled release system, the SDF1-ELP monomer effect on EC migration could be investigated.

Since a large portion of chronic wounds consists of chronic diabetic wounds, another factor that may play a role in prolonging the wound healing process is glucose level. The blood sugar level of diabetes patients is higher than healthy individuals; therefore, we should study the SDF1-ELP monomer bioactivity in a high glucose environment.

In this study, elastase was used to mimic the wound fluid environment. Thus, in future studies, it would be worthwhile to set up an ex vivo experiment to test SDF1-ELP monomer stability and bioactivity in wound fluid. However, the protease level in wound fluid may be much higher (>300nM) than the dose ECs can tolerate [19-21]. Therefore, it may be necessary to use a cell line that can tolerate higher protease levels.

Another deficiency of this study was that the SDF1-ELP release rate was too high. The majority of the release was observed in the first 6-hr. This fast release was due to the relatively large pore size (400nm) of the permeable membrane compared to SDF1-ELP nanoparticle size (600nm), although it is the smallest size commercially available. The

relatively large pore size is not guaranteed that all nanoparticles are bigger than 400 nm; there could be some smaller ones that come through Thus, it would be a good idea to customize a controlled release system with a permeable membrane. Ideally, the pore size of the membrane should be ~10nm [13].

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Appendix:Inverse Transition Cycling Purification

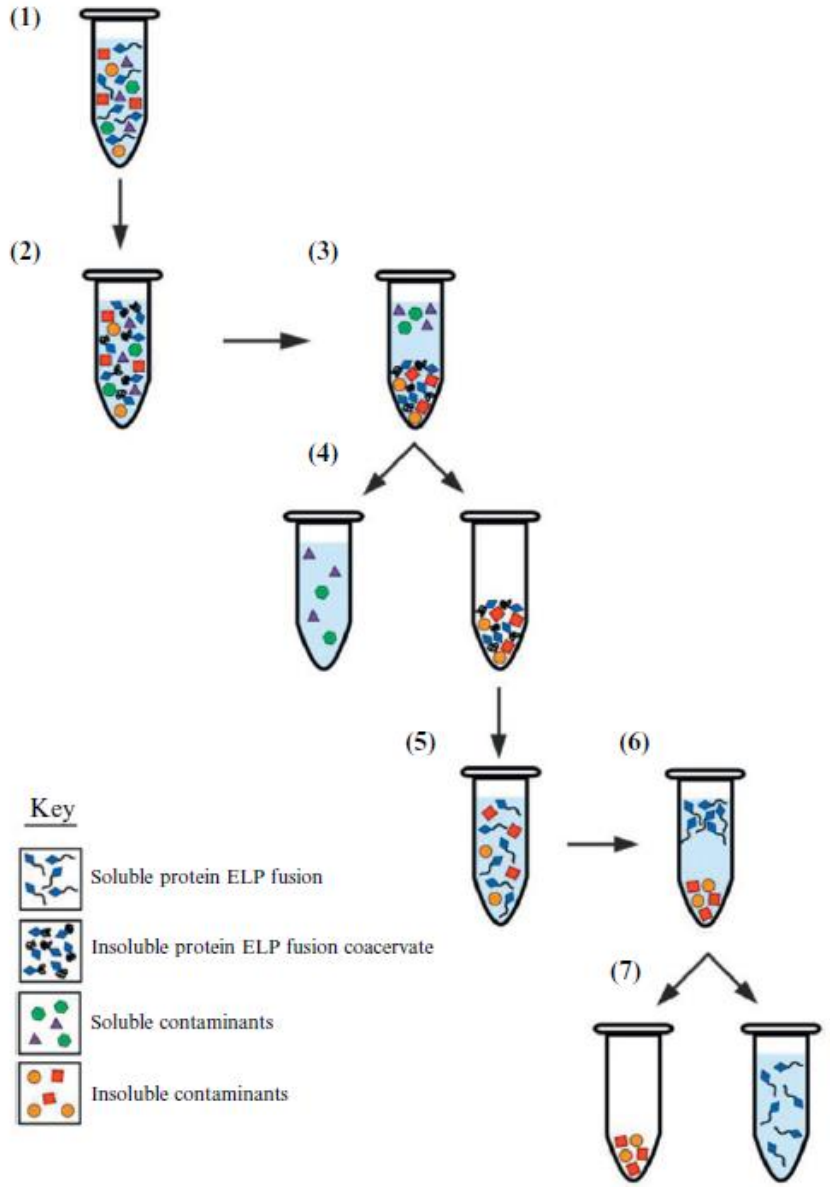
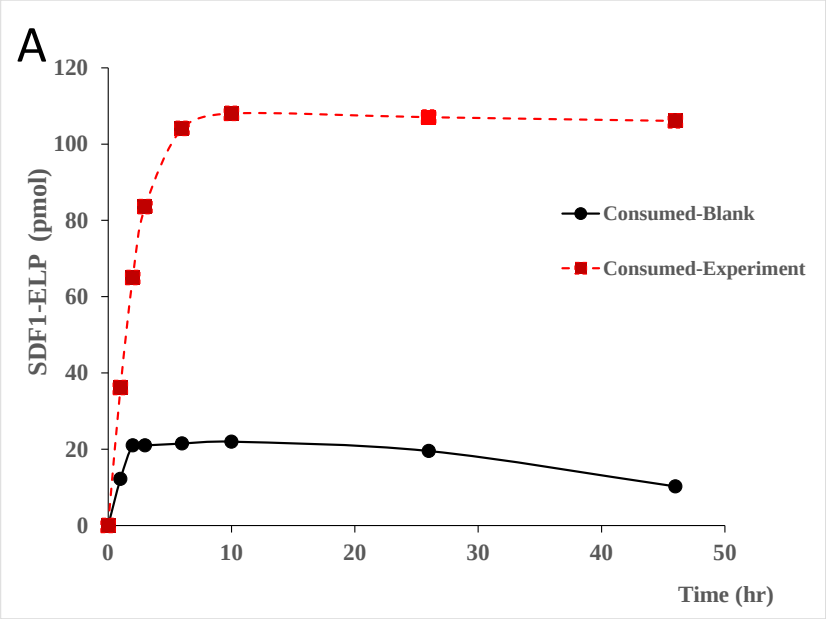


Figure 16. SDF-ELP fusion protein purification by inverse transition cycling (ITC). The ***E. coli*** are sonicated to obtain lysate prior to ITC purification (1) SDF1-ELP nanoparticles was induced by the addition of Sodium Citrate(2) SDF-ELP aggregates are collected after centrifuge. (3) The supernatant with soluble impurity is discarded while the SDF1-ELP stays in the pellet (4) Dissolve the pellets in cold PBS buffer (5). The solution is again centrifuged (6) and the pellet containing insoluble contaminants is discarded. SDF1-ELP dissolved in supernatant (7). Steps 2 through 7 should be repeated 3 times to reach desired purity. *Image was taken from Hassouneh W, Christensen T, Chilkoti A. Elastin-like polypeptides as a purification tag for recombinant proteins. Curr Protoc Protein Sci. 2010 Chapter 6 Unit 6 11.*

Appendix: SDF1-ELP Monomer Release System Validation



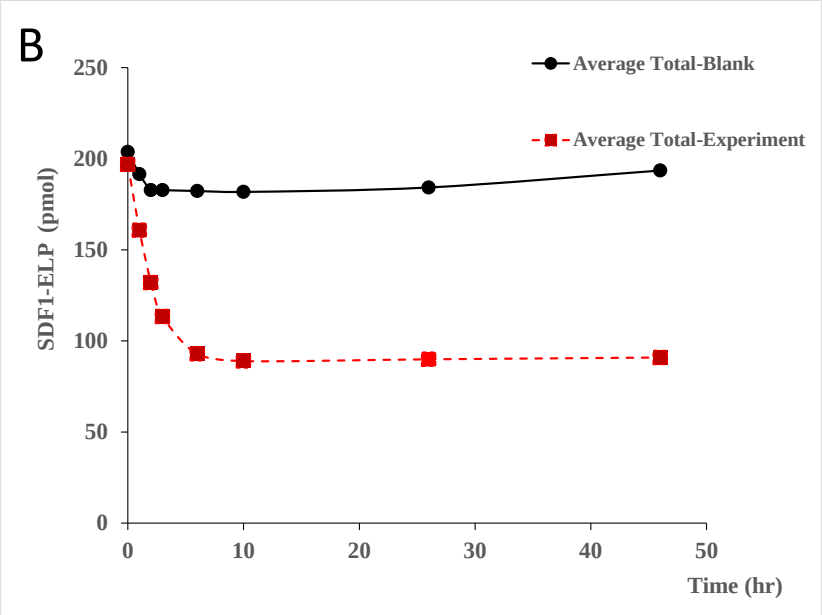
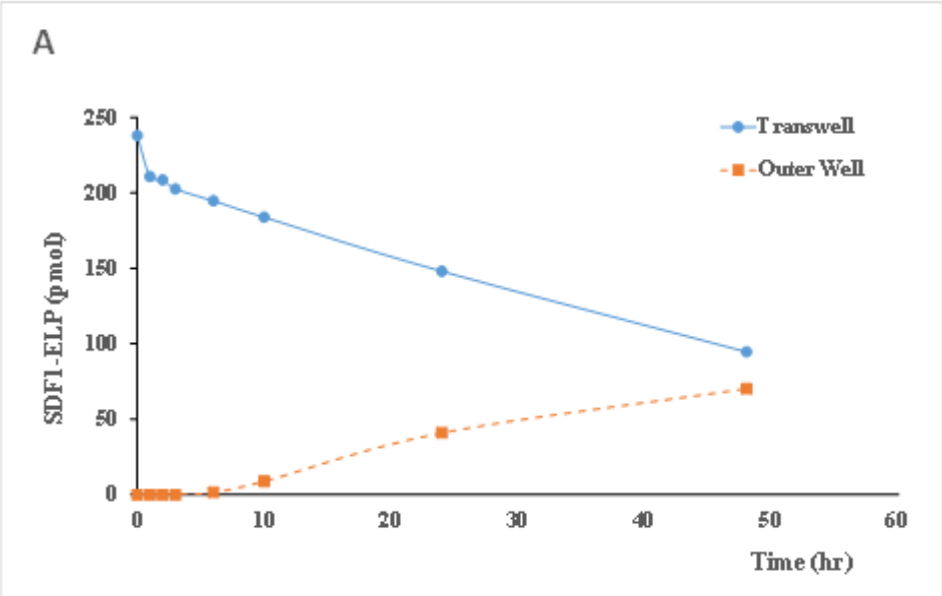


Figure 17. The total amount and consumed SDF1-ELP in Transwell insert and outer well. The Transwell release system was incubated at 37˚C for up to 48 h. Samples were collected at 0-hr, 1-hr, 2-hr, 3-hr, 6-hr, 10-hr, 24-hr, and 48-hr. The amount of SDF1-ELP was calculated by mass balance. The initial volume of liquid in the Transwell insert was 200µ; in the outer well was 600µL. A linear volume loss due evaporation was taken into calculation. (A) Consumed SDF1-ELP in the entire system. (B) Average total SDF1-ELP in the entire system.

Appendix: SDF1-ELP Monomer Stability and Bioactivity



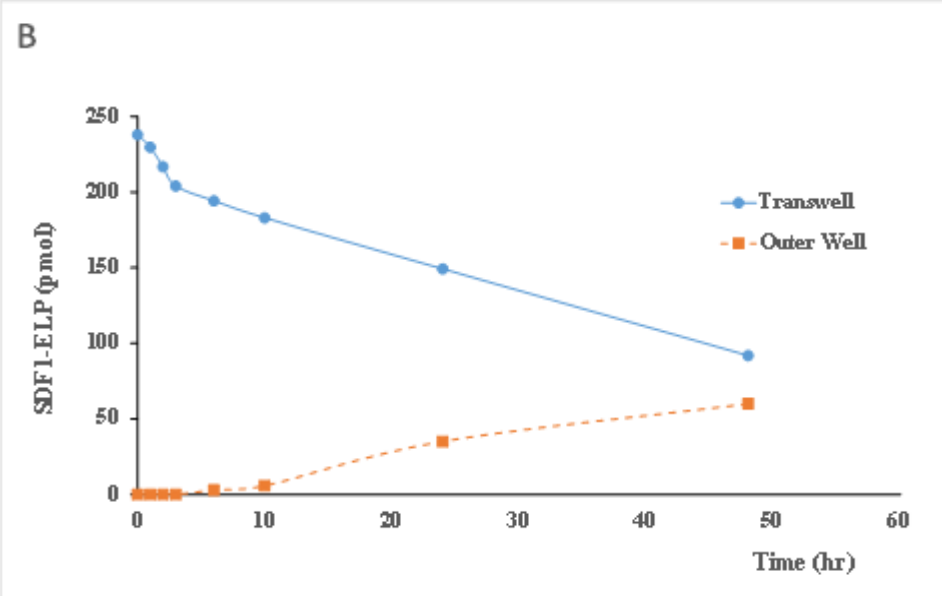
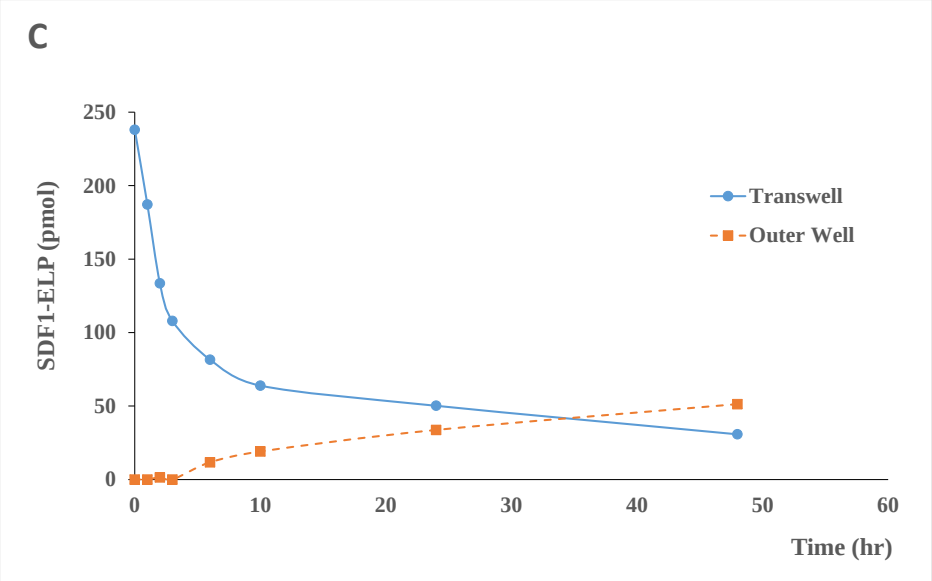


Figure 18. Release of SDF1-ELP monomers from Transwell insert to outer well. The Transwell release system was incubated at 37˚C for up to 48 h. Samples were collected at 0-hr, 1-hr, 2-hr, 3-hr, 6-hr, 10-hr, 24-hr, and 48-hr. The amount of SDF1-ELP was calculated by mass balance. The initial volume of liquid in the Transwell insert was 200µ; in the outer well was 600µL. A linear volume loss due evaporation was taken into calculation. (A) B1:SDF1-ELP(+)Elastase(-)HUVECs(-). (B) B2:SDF1-ELP(+)Elastase(+)HUVECs(+)



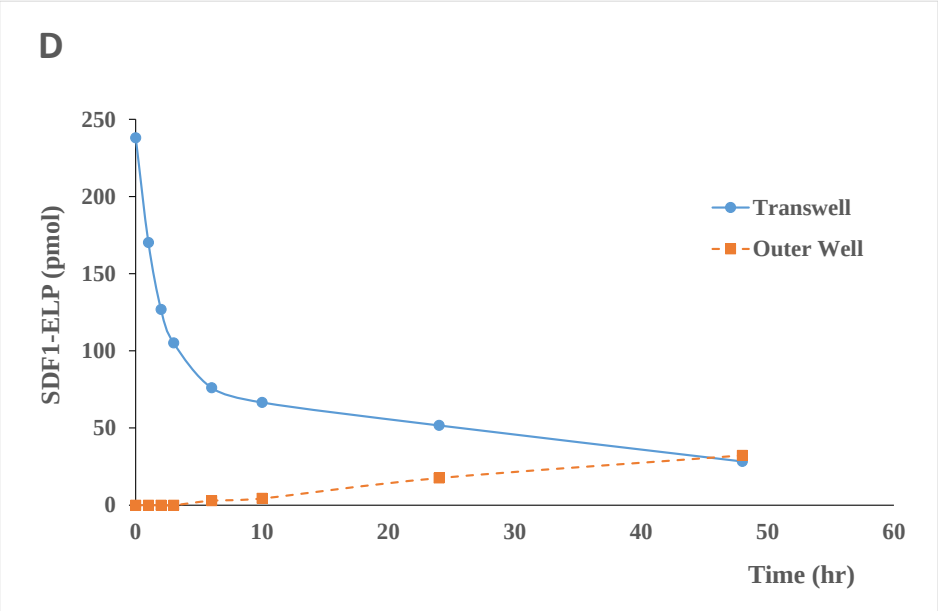


Figure 19. Release of SDF1-ELP monomers from Transwell insert to outer well. The Transwell release system was incubated at 37˚C for up to 48 h. Samples were collected at 0-hr, 1-hr, 2-hr, 3-hr, 6-hr, 10-hr, 24-hr, and 48-hr. The amount of SDF1-ELP was calculated by mass balance. The initial volume of liquid in the Transwell insert was 200µ; in the outer well was 600µL. A linear volume loss due evaporation was taken into calculation. (C) C2:SDF1-ELP(+)Elastase(-)HUVECs(+). (D) Experiment:SDF1-ELP(+)Elastase(+)HUVECs(+)