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P12, a novel fibronectin peptide, promotes cell survival by augmenting PDGF-BB survival signals

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Abstract of the Dissertation

P12, a novel fibronectin peptide, promotes cell survival by augmenting PDGF-BB survival signals

Jia Zhu
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Intensive research has demonstrated that extracellular matrix (ECM) and growth factor (GF) signaling collaborate at many different levels. Through integrins, ECM generates signals that enhance or suppress GF signaling. Some ECM activates GF receptor in the absence of GF. Furthermore, ECM contains growth factor binding-domains (GFBD) that can either sequester or present GF in ways that modify cell response. The ability of ECM to modulate GF signals has important implications in tissue engineering/regenerative medicine, and in devising therapies to treat acute and chronic wounds.

Previously in our lab, a novel fibronectin peptide, P12, was shown to bind platelet-derived growth factor BB (PDGF-BB). In vivo experiments showed that P12 can limit burn injury progression, suggesting an active role in cell survival. In my research, I explored the molecular mechanism of P12 effect in adult human dermal fibroblasts (ADHF) under nutrient deprivation, a stress condition that mimics burn injury. In my system, I found that P12 acted as a cell penetrating peptide and redirected ligand-bound PDGF receptors from the clathrin-mediated endocytosis (CME) to a slower, clathrin and dynamin-independent pathway. As a consequence, P12 slowed the internalization and degradation of PDGF-BB/PDGFR-β, augmented its survival signal, and promoted cell survival under nutrient deprivation. My findings demonstrate a new role for extracellular matrix proteins in modulating growth factor signaling and cell survival.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AHDF, adult human dermal fibroblast
GF, growth factor
CPP, cell penetrating peptide
PDGF, platelet-derived growth factor
PDGFR, platelet-derived growth factor receptor
GFBD, growth factor binding domain
FN, fibronectin
ECM, extracellular matrix
EGF, epidermal growth factor
VEGF, vascular endothelial growth factor
BSA, bovine serum albumin
EGFP, enhanced green fluorescent protein
SP12, scrambled P12
CME, clathrin-mediated endocytosis
HRP, horseradish peroxidase
PBS, phosphate-buffered saline
SPR, surface plasmon resonance
TBS, tris-buffered saline
HBSS, Hank's balanced salt buffer
CHC, clathrin heavy chain
Dyn, dynasore
KD, knockdown

Acknowledgments

It has been a very fulfilling five years studying for my Ph.D. in Stony Brook University under the mentoring of Dr. Clark. Dr. Clark has always been very supportive along my learning process. He has inspired me and filled me with positive energy. I will remember the great help and support from the current and previous Clark lab members: Fubao, Monica, Atulya, Laurie, Lauren and Qiong. Thank you for being great companions and coworkers, especially Fubao, who has taught me a lot of things during my rotation and the beginning years.

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The journey of a PhD student can be lonely and boring. It is full of frustrations and depressing results. I was very fortunate to have a group of amazing classmates: Lingyin, Maha, Nikki, Krithika, Narmatha, Miriban, Yi, Angad, Mingxuan and Donghyuk to share and to vent. They are always there for me whenever I need help with protocols, reagents or instruments. The time we spend together lights up my PhD life and I hope we can maintain this special bond even after graduation.

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Chapter 1

Background and Significance

1.1 Clinical relevance

Burn injuries claim the lives of 3400 United States civilians each year and send another 450,000 to seek medical attention (2012.burn incidence and treatment in the US: 2012 fact sheet. American Burn Association). Approximately 8% of those 450,000 burn victims require hospitalization, of which 30,000 are admitted to the 127 specialized U.S. burn centers. This accounts for over 900,000 hospital days per year and more than $1 billion per year in associated costs, including loss of productivity. In addition, 25% of people with burns greater than 75% of total body surface area result in death. Although burn wound care has advanced over the years, there is currently no approved therapy to treat burn injury progression and promote robust burn wound healing.

1.2 Burn injury progression

Burn injury progression is the progression of tissue death that expands from the initial injured area (zone of coagulation) to the area around it (zone of stasis and zone of hyperemia) in as early as 24h following the initial burn. Zone of stasis is characterized by decreased blood flow (ischemia) that can potentially be saved. However, if left untreated, a partial thickness burn can progress into a full thickness burn. In a full thickness burn all the dermis and subcutaneous tissues are destroyed (**Figure 1.1**). At this point, there is no treatment except for grafting. This process results in increased hospitalization, infection rate, mortality, and scarring. Thus, it is crucial to limit burn injury progression in the zone of stasis (ischemia) at early hours post burn.

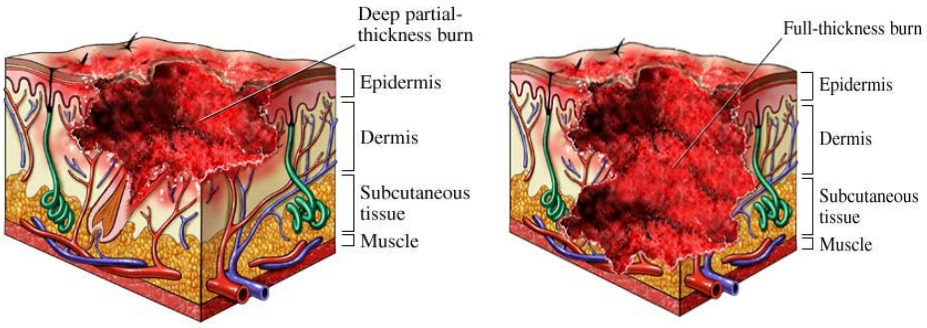


Figure 1.1 Comparison of a partial thickness burn and a full thickness burn. If a partial thickness burn is deeper in the dermis, it is likely to progress into a full thickness burn in the first 24h to 48h after burn. (http://www.azburncenter.com/BurnTips/PartialThicknessBurns_)

1.3 FN-derived growth factor binding peptide limits burn injury progression

In venous thrombus resolution, proteolytic fragments of FN that contain a VEGF binding site are expressed in a distinct spatial and temporal pattern[1]. Thus, FN may influence capillary morphogenesis by generation of fragments that modulate VEGF stimulated proliferation, migration, and protease activation. An analysis of FN fragments (Fnf) generated by proteolytic processing from matrix metalloprotease-2 (MMP-2) showed that Fnf of 30-kD and 120-kDa size positively affected proliferation of microvascular cells but not macrovascular cells [2]. A 45-kDa gelatin binding fragment of FN inhibited HREC proliferation but stimulated pericyte and smooth muscle cell proliferation.

P12, a peptide derived from fibronectin, has recently been discovered and characterized by the Clark laboratory and shows significant promise in the treatment of burns. This peptide provided protection against oxidative and cytokine stress for adult human dermal fibroblasts and reduced burn injury progression in a hot comb burn model. The competitive advantage that this product has over those commercially available and in-development is the limitation of burn injury progression, which is currently not addressed by any FDA-approved therapy.

As shown in **Figure 1.2**, P12 is derived from the first fibronectin type III repeat. It has a strong binding affinity for PDGF-BB and several other growth factors (vascular endothelial growth factor, transforming growth factor beta 1, fibroblast growth factor 2) as shown by Biacore (**Figure 1.3**) and competitive binding experiments. The K_d for P12-PDGF-BB binding is 200nM. This means the binding between P12 and PDGF-BB is stronger than epinephrine binding to beta-adrenergic receptor. The strong binding between PDGF-BB and P12 suggest that P12 may work together with PDGF-BB in physiological conditions.

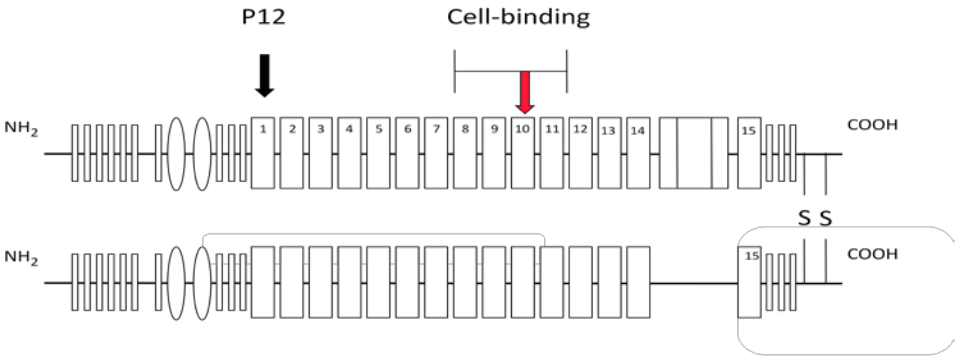


Figure 1.2 The structure of human plasma fibronectin and the origin of P12. Fibronectin is an important ECM protein that is critical for both tissue formation and wound healing. P12 is derived from the first type III repeat (anastellin) of fibronectin [3] based on its growth factor binding activity.

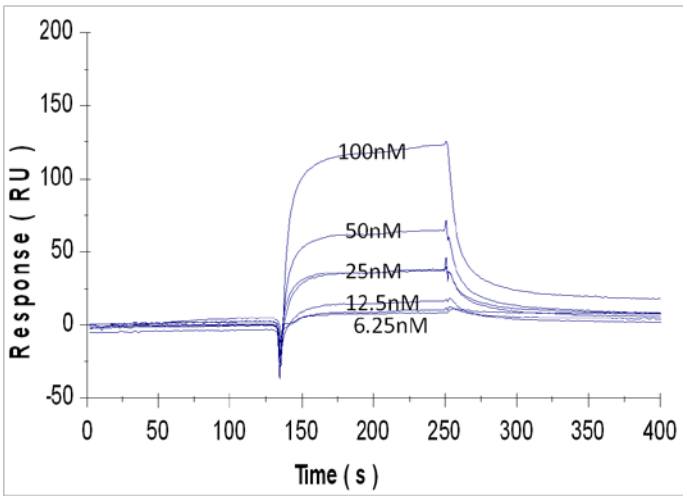


Figure 1.3 P12 binds PDGF-BB with high affinity. P12 was fixed to a CM5 chip through amine coupling. Real-time interaction of PDGF-BB with P12 was determined by plasmon surface resonance (BiaCore 2000) with or without 10 μM P12. The chip cell without PDGFR-β was used as a reference. Sensorgrams are representative of three different experiments. Dissociation constants (K_d) were derived from the ratio of kinetic dissociation constants divided by kinetic association constants (Lin et al. submitted 2013).

To test if P12 can work together with PDGF-BB to promote cell survival, Dr. Fubao Lin from the lab carried out several pilot in vitro studies. Indeed, these studies in the lab showed P12, in the presence of PDGF-BB, can promote adult human dermal fibroblast (AHDF) metabolism and proliferation (**Figure 1.4**).

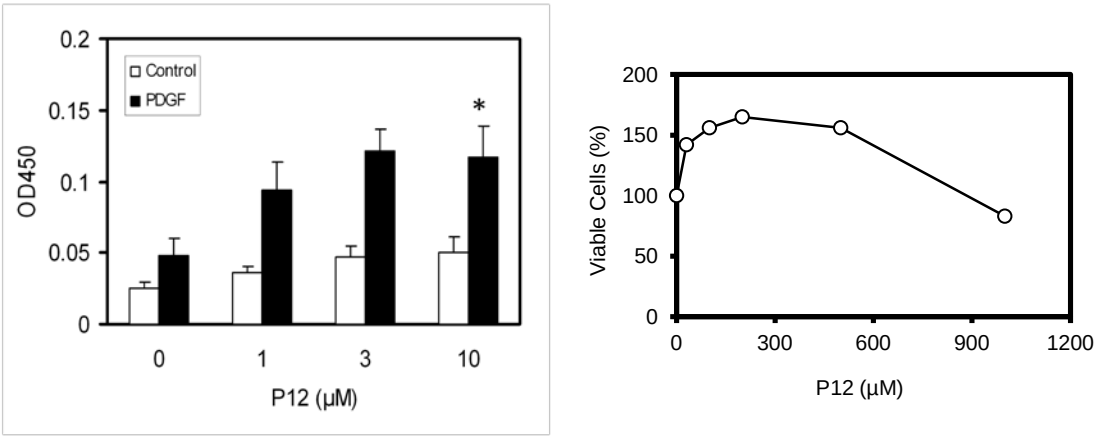


Figure 1.4 P12 promote cell metabolism and proliferation in the presence of PDGF-BB. Left panel: AHDF were cultured for 5 days in DMEM without FBS, 200 cells/well, P12 or PDGF-BB in 5% BSA added 4 hrs after seeding. Cell metabolism was measured by XTT assay which measures mitochondria dehydrogenase activity (asterisk, $P<0.05$ compared to control, n=3). Error bar indicates SD. Right panel: cell number for AHDF plated at 50% confluence in 5% FBS for 72h. (Data from Dr. Fubao Lin)

PDGF-BB is one of the most potent pro-survival growth factors for mesenchymal cells. It is shown to activate PI3K/ Akt pathway and promote cell survival under various stress conditions [4-6]. It is also shown to activate the MAPKs and lead to cell proliferation and migration [7]. In vivo experiments have suggested the application of PDGF-BB to treat various ischemic diseases including myocardial infarction, stroke, and ischemic ulcer [7, 8].

Inspired by the pro-survival activity of P12, the lab investigated P12 IV infusion in a pig hot comb burn model. The rationale is that if P12 can promote the survival of human dermal fibroblast in the zone of ischemia, it may limit burn injury progression. In this study, P12 showed a significant protective effect for the interspace tissue (zone of ischemia) in between burned areas (**Figure 1.5**). After 7 days, the interspaces in the untreated control group are all necrotic while in the P12 treated group viable interspaces were observed between the burned areas.

The dose curve in Figure 5 showed 1mg/kg is the optimal dose to save interspatial tissue. That concentration, calculated based on the total volume of blood, is equal to 10µM. The amino acid sequence scrambled peptide (SP12) showed no protective effect for the interspace tissue (zone of ischemia) in the hot comb model at various doses.

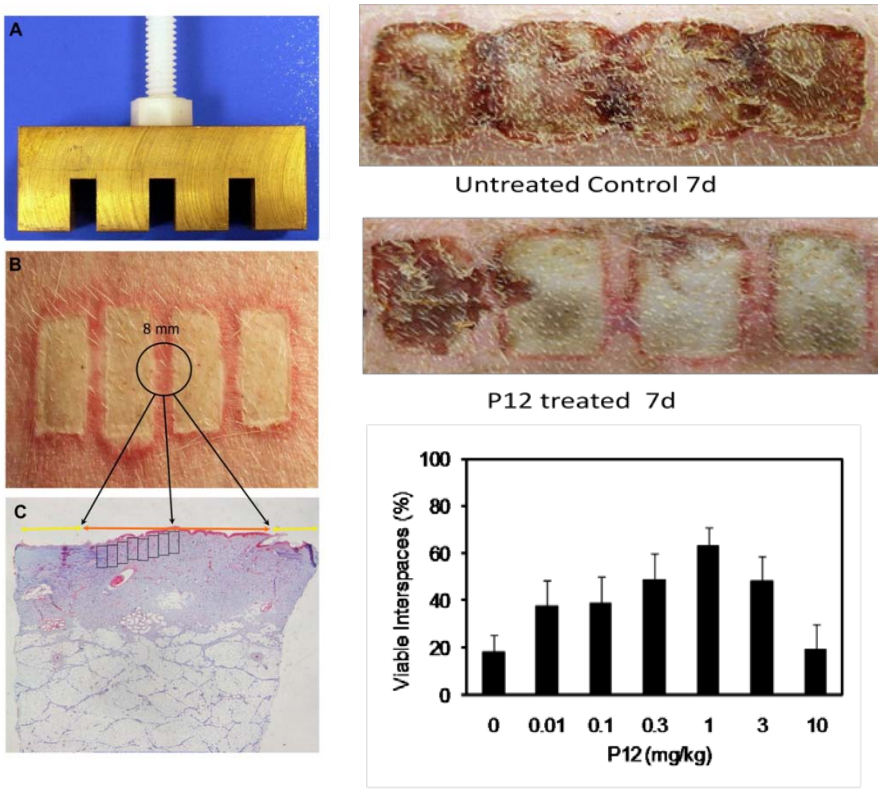


Figure 1.5 P12 limited burn injury progression in a pig hot comb burn model. Left panel, Burns are created with a brass comb with four 10 x 25 mm prongs separated by 5mm interspaces. The viability of the interspace tissue was measured as indicator for burn injury progression.

Right panel, Comparison of viable interspaces between the control and P12 treated pigs (Data from Dr. Fubao Lin) [9].

However, the mechanisms by which P12 can rescue the interspace tissue are still unclear. In order to answer that question, we must first understand the underlying mechanism behind burn injury progression (which is currently very poorly understood). Biopsies from the interspaces at various stages of the burn are stained with hematoxylin and an antibody against cleaved caspase 3, a marker for apoptosis. As shown in Figure 1.6, Biopsies from 1h and 4h after the burn showed only scattered cleaved caspase3. But, a region of cleaved caspase3 positive stain (apoptosis band) in the deep dermis appears 24h after the initial burn. This indicates appropriate therapy earlier might reduce burn injury progression. The apoptosis band is very similar to that observed in myocardial infarction and stroke, and likewise investigators believe therapy prior to this time might attenuate the injury progression.

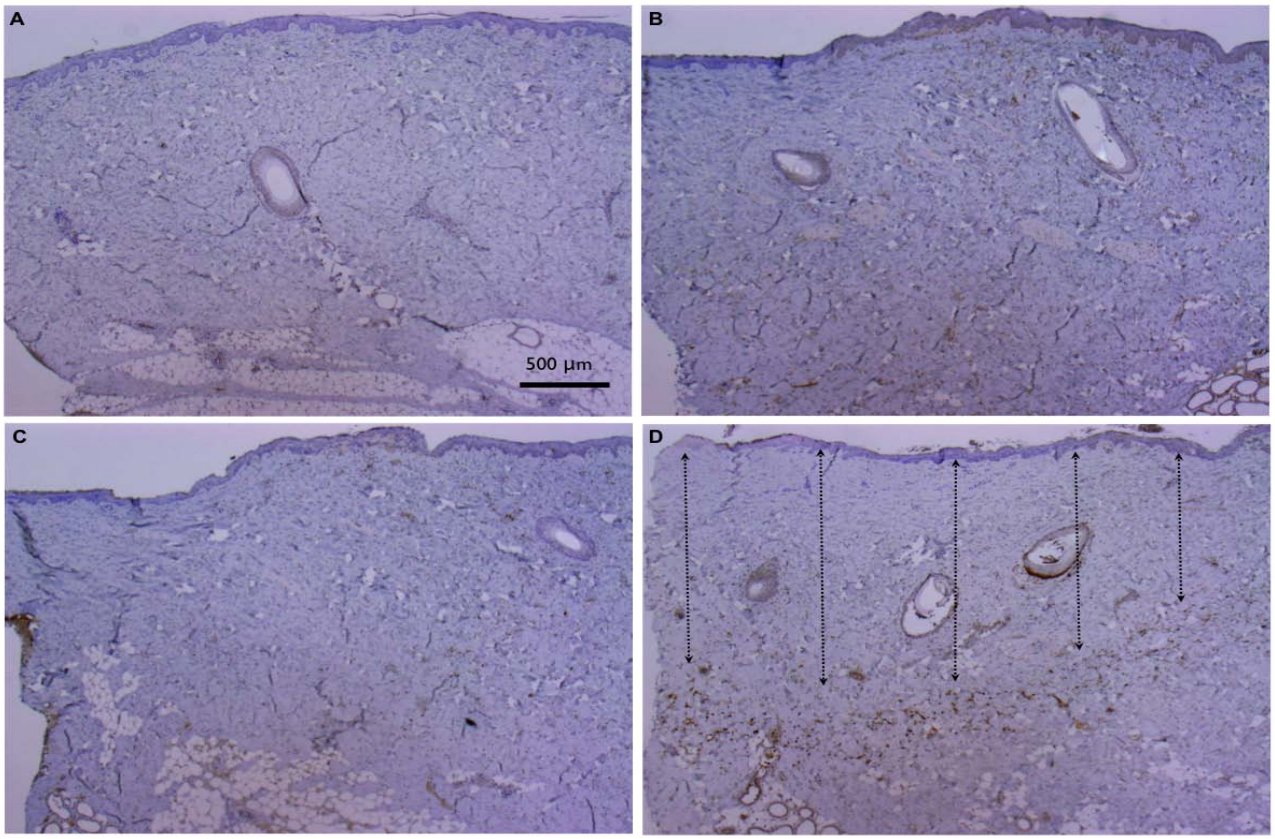


Figure 1.6 Cleaved caspase 3 staining at 1, 4, and 24 hours post-burn. Biopsies encompass the 5 mm interspace (zone of ischemia) were stained with antibody against Cleaved caspase 3 and counterstained with hematoxylin. A) Unburned control; B) Interspace tissue 1h post burn; C) Interspace tissue 4h post burn; D) Interspace tissue 24h post burn. A band of apoptotic cells were observed in the deep dermis in interspace tissue 24h post burn [9].

Based on the knowledge presented in the literature about blood vessel plugging in periburn tissue, we hypothesize that this band of apoptosis may result from the ischemia condition after blood vessel (BV) occlusion. Histological studies from the lab have confirmed blood vessel occlusions by either red blood cells (RBCs) or Fibrin/fibrinogen as early as 4h after the burn in both superficial and deep dermal plexus of the pigs (**Figure 7**). This will cause an ischemic condition for the dermal fibroblast cells in the zone of stasis (ischemia). Ischemic condition is shown to induce apoptosis in a variety of cells[10]. Taken together, the data suggest that the apoptotic band is a consequence of the ischemia created by earlier blood vessel occlusion.

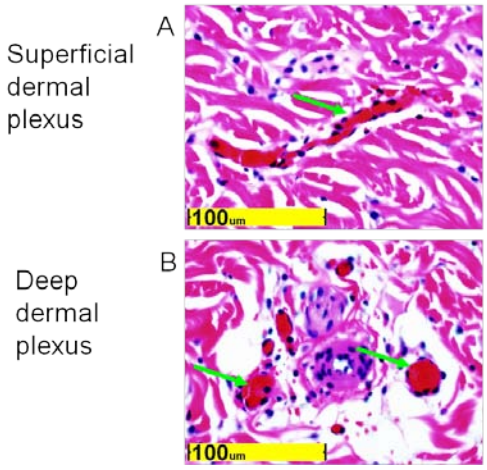


Figure 1.7 Blood vessel occlusion in the periburn tissue 4h post burn. H&E stain depicts significant occlusion in blood vessels in both superficial and deep dermal plexus in peri-burn tissue (Data from Dr. Wunan Zhou).

1.4 ECM regulation of growth factor signaling

ECM is composed of secreted molecules (glycoproteins, collagens, glycosaminoglycans and proteoglycans) that constitute the cell microenvironment [11]. ECM provides structural support and segregation for different tissues in vivo. In the skin, it is essential to the tensile strength and flexibility in the dermis and basement membrane [12]. The basement membrane also acts as a mechanical barrier, preventing malignant cells from invading the deeper tissues.

ECM provides much more than just mechanical and structural support. Multiple and specific domains in ECM can bind multiple interacting partners such as other ECM molecules, GFs, signal receptors and cell adhesion molecules. For example, FNIII 8-9 (RGD-containing central cell-binding domain of fibronectin) mediates FN-binding to cells and generates important survival signals either through α5β1 integrin [13] or by activating growth factor receptor (GFR) [14]. Through these domains, ECM regulates the nature, intensity, and duration of GF signaling. This in turn determines cell behavior, polarity, migration, differentiation, proliferation, and survival [15, 16].

Intensive research has demonstrated that ECM and GF signaling collaborate at many different levels. Through integrin, ECM signaling often synergize with GF signaling activating the same pathways. Adhesion to ECM is prerequisite for GF signaling. The type and stiffness of ECM condition the cell and regulate the outcome of GF signaling. Some ECM can activate GFR in the absence of GF. The expression level of GFR is regulated by ECM signaling (while GF regulates ECM deposition).

Furthermore, many GFBD have been discovered in ECM introducing a new layer of interaction between ECM and GF. First, ECM acts as a reservoir of GF and thereby forms GF gradients. Proteolytic processing of ECM can release GF deposited in ECM while at the same time generate bioactive GFBD peptides. ECM tethered GFs are presented as solid phase ligand that forbids GFR internalization which controls duration and outcome of GF signaling. Also, juxtaposition of GFBD and integrin binding site in ECM facilitates formation of adhesion complex, structural basis of ECM-GF synergistic signaling. Cells exert tension force on ECM that can expose cryptic sites that activate GFR or bind GF. Recently our lab demonstrated ECM peptide can act as cell penetrating peptide and regulate GF/GFR trafficking thus affecting GF signaling.

Many clinical trials with recombinant GF have shown that GF alone have minimal activity toward improving wound repair. Interestingly, the trials that have shown benefit all contain a material carrier, suggesting that spatiotemporal control over GF bioactivity is crucial to achieve tangible therapeutic effect [17]. Difficult to heal or chronic wounds exhibit ECM deficits and GF abnormalities that likely contribute to their stalled progression. Wound healing strategies that incorporate both ECM and GF may be beneficial for these wound types and, indeed, therapies of only one type or the other have generally proved disappointing [18]. Thus understanding interactions between ECM and GF in normal wound healing process may provide important insights and new therapeutic targets [19].

1.5 Significance of the study

As of 2012, there are 450,000 cases of burn injuries receiving medical treatment in the US. This makes it the 4th leading cause of injuries after motor vehicle collisions, falls, and violence, higher than the combined incidence of tuberculosis and HIV infections [20]. Stress-mediated cell death plays an important role in tissue injury such as stroke, heart attack, and burn injury progression. The novel activity of P12 to promote cell survival led us to believe that it may also salvage the ischemia zone of injured tissue. To test this hypothesis, the effects of P12 infusion on burn injury progression were studied in both rat and swine hot comb models. Burn injury progression is a kinetic process in depth and size over the course of several days leading to excess mortality and morbidity including wound contractures and poor quality of life. During burn injury, the initial burn results in a central zone of irreversible cell necrosis surrounded by a potentially reversible zone of ischemia with viable cells. Over the subsequent 24-72 hours, burn injury will progress extensively in size and depth resulting in the conversion of reversible 2nd degree burns to irreversible full-thickness 3rd degree burns accompanied by cell necrosis and apoptosis mediated by various stresses [21]. Hence, preventing cell necrosis and apoptosis in burn injury is the major goal of the therapy for limiting burn injury progression. Although the commercial market is saturated with cutaneous wound healing products, therapy to limit burn injury progression is an unmet need. In vivo studies using a pig burn injury model

showed exciting data supporting P12 efficacy in preventing burn injury progression and P12 has received orphan drug designation from FDA.The overall goal of my PhD research is to study the molecular mechanism underlying the exciting in vivo P12 effect we observed. It is important to understand the molecular mechanism of P12 if it is to enter clinical trial stage. Furthermore, we can modify the molecule or delivery methods based on insights into its molecular mechanism.

Intensive research has demonstrated that ECM and GF signaling collaborate at many different levels. At wound site, fibronectin is prone to proteolysis during burn injury due to the large amount of proteases released upon injury [22]. The FN fragments generated from proteolysis has been proposed to play an important role in would healing [23]. So it is likely that P12 effect is a naturally occurring mechanism underlying the process of wound healing and insights into this phenomenon will add another layer of ECM regulation on GF signaling.

Chapter 2

Fibronectin Peptide P12 Promotes AHDF Survival by Augmenting PDGF-BB Survival Signals

2.1 Introduction

Tissue injury after stroke, heart attack, burns or other trauma is a dynamic process that extends in size and severity over the course of several days, leading to excess mortality and morbidity [21, 24, 25]. After injury in such situations, the central necrotic tissue is surrounded by ischemic, less severely affected tissue that potentially remains salvageable. However, since the ischemic tissue is exposed to various stresses such as nutrition depletion, reactive oxygen species (ROS), cytokines, and hypoxia, tissue cells in this region often die, by either apoptosis or necroptosis [9, 26, 27], unless effective post-injury therapy is given [28]. Hence, preventing cell death in potentially salvageable tissue of an acutely damaged organ is a major goal for therapies of these injuries.

Many growth factor-binding sites have been identified in ECM [29]. They may act as reservoirs and serve to sequester and protect growth factors from degradation, and/or enhance their activity [19]. Recently, our lab identified a novel fibronectin peptide, P12, with significant binding affinity to PDGF-BB. P12 sequence is cryptic in fibronectin, but it could get exposed by the tension force applied by AHDF [30] or by proteolytic digestion in cutaneous wound or burns [31]. Initial studies demonstrated that P12 can work as a co-factor of PDGF-BB to promote AHDF survival [32].

Platelet-derived growth factors (PDGFs) are major mitogens and survival factors for most mesenchymal cell types including fibroblasts [33]. PDGF occurs as five isoforms, classic PDGF-AA, AB, and BB isoforms, and the more recently described PDGF-CC and -DD isoforms [34]. The most potent PDGF isoforms, -AB and –BB, are secreted either by platelets (PDGFAB), macrophages (PDGF-BB), or epidermal cells (PDGF-BB) in response to injury. PDGFB knockout in mice are invariably embryonic lethal while knockouts of other isoforms are not [35].

Much of the tissue damage in burn patients results from injury progression, a dynamic process of tissue death around the burned area occurring in the first 24 to 48h after burn [36]. Previously our lab has shown evidence of significant blood vessel occlusion, which would cause hypoxia and nutrient deprivation in peri-burn tissue in the first few hours after burn injury [37], and a band of apoptosis in deep dermis by 24h after burn injury [36]. This suggests that progressive tissue death characteristic of burn injury progression is related to nutrient deprivation/hypoxia in peri-burn tissue. PDGF-BB has been shown to promote AHDF survival under various stress conditions including nutrient deprivation [38]. Thus, P12 was investigated as a therapy to limit burn injury progression in a porcine animal model [39]. The burn injury progression was effectively limited for the swine infused with one dose P12 1 hour after burn. The effective doses of P12 to limit burn injury are from 0.01 to 3 mg/kg body weight and optimal dose occurs at 1 mg/kg. In contrast, peptide with same amino acid composition as P12 but with scrambled sequence showed no effects on burn injury progression from dose of 0.1 to

10 mg/kg, indicating that the effect of P12 on burn injury progression is sequence specific. These results demonstrated that P12 is a novel peptide that supports cell survival not only in vitro but also limits tissue injury in both small and large animal models. IV injection of P12 significantly enhanced re-epithelialization at 7days post-burn, a FDA assigned primary outcome for efficacy in wounds. However, the molecular mechanism of P12 remains unclear.

As a logical extension of previous work in the lab, the goal of this study is to examine P12 effect as a growth factor co-factor in promoting AHDF cell survival under stress conditions that mimic burn injury such as ER stress, hypoxia and nutrient deprivation.

2.2 Materials and methods

Materials: P12, scrambled P12 and C-terminal Cys-tagged P12 were purchased from American Peptide (Sunnyvale, CA). Antibody against p-PDGFR-β (Tyr751), p-Akt (Ser473), p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Tyr204), p-P38 (Thr180/Tyr182) and GAPDH are purchased from Cell signaling Technology (Denver, MA). AHDF (CF31) primary cells are isolated from dermis of a 31 year old female. Hank's Balanced Salt Buffer from Sigma (St. Louis, MO). Cell Proliferation kit II (XTT) and In Situ Cell Death Detection kit (TUNEL reaction mixture) was purchased from Roche Diagnostics (Indianapolis, IN). SiRNA for CHC KD (sc-35067) was purchased from Santa Cruz Biotechnology (Dallas, TX). Allstar negative control siRNA and Hiperfect transfection reagents were purchased from Qiagen (Valencia, CA). EZ-Link Sulfo NHS-SS Biotinylation Kit was purchased from Pierce (Rockford, IL).

Cell culture and Transfections: AHDFs were maintained in DMEM (Gibco) with 10% FBS at 37°C and 5% CO2 in a humidified atmosphere. Cells from passage 8-12 were used. SiRNA and mock was used at 10nM. Cells were transfected twice with Hiperfect Transfection Reagent according to manufacturer's recommendations at 36-h intervals.

TUNEL staining: AHDF treated according to figure legend was fixed and permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min at 4°C, stained according to kit protocol. Cell nuclei were stained with DAPI. Images were captured with a 10X, aperture 0.4 objective on an inverted Diaphot-TMD fluorescent microscope (Nikon) using a CCD camera (Molecular Devices).
ER stress: Tunicamycin stock solution was made in DMSO. AHDF were treated with various doses of Tunicamycin to induce ER stress.

Western blot: Cells were frozen and dissolved in lysis buffer from Cell Signaling (#9803) containing 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na3VO4, 1µg/ml leupeptin supplemented with 1mM PMSF, scraped and sonicated, then spun and boiled in running buffer. Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked by 2% BSA in TBST and probed with primary antibodies over night at 4°C. HRP conjugated secondary antibodies were used for detection. Images were taken with a Kodaq image station 440CF (Rochester, NY). Intensity of the chemi-luminescent signal was quantified by Kodaq molecular imaging software v4.0.3.

Separation of Internalized PDGFR-β: Surface proteins were labeled with EZ-Link Sulfo NHS-SS Biotinylation Kit (contained a cleavable S-S to allow removal) following kit instructions. The cells were treated with 1nM PDGF-BB +/- 10μM P12 for the indicated times. Cells were put on ice and remaining surface-exposed biotin was removed by 20mM sodium 2-mercaptoethanesulfonate in 50mM Tris followed by 20mM iodoacetic acid on ice. After cell lysis, biotinylated proteins were pulled down with streptavidin agarose beads.

Immunoprecipitation: For immunoprecipitation, cells were treated with PDGF+/-P12 for the indicated time in HBSS and cells were solubilized in lysis buffer. An equal amount of each protein lysate was incubated with anti-PDGFR-β (#3175) antibodies for 12 hours at 4°C, followed by incubation with 20μl of protein G-Sepharose beads for 1 hour. The immune complexes were collected and analyzed by Western blot.

2.3 Results

P12 worked synergistically with PDGF-BB to promote AHDF survival under ER stress

P12 has been shown to enhance cell survival under various stress conditions including serum starvation, hypoxia/nutrition deprivation, and oxidative stresses. These stresses play an important role in the progression of tissue injury.

To further understand the role of P12 in protecting tissue cells from stress-mediated death, I studied whether it protects cells from endoplasmic reticulum stress (ER stress)-induced cell death. ER stress is a condition which results in the accumulation and aggregation of unfolded proteins. Protein aggregation is toxic to cells and numerous pathophysiological conditions such as Alzheimer disease, Huntington disease, and diabetes have been associated with ER stress. To study P12 on ER stress-induced cell death, AHDF were treated with or without tunicamycin, a reagent which induces ER stress by blocking all N-glycosylation of proteins [40]. As shown in Figure 2.2, tunicamycin treatment resulted in dose-dependent fibroblast death. In the presence of P12, tunicamycin-induced cell death at 0.03μM and 0.1μM were inhibited. These results indicated that P12 also protected fibroblasts from ER-stress-mediated cytotoxicity.

P12 inhibits JNK activation under ER stress

To understand the mechanism of P12 on protecting cells from stress-mediated death, the effects of P12 on Jun N-terminal kinase (JNKs) activation were studied by determining the phosphorylation of JNK protein using Western blot method. JNKs play a critical role in cell apoptosis initiated by both extrinsic and intrinsic pathways [41]. To date, three JNKs, namely JNK1, JNK2, JNK3 encoded by three distinct genes have been identified [42]. In response to specific stimuli such as heat shock, reperfusion injury, ER and oxidative stress, JNKs were activated by phosphorylation at its Thr- or Tyr-residues of a TXY motif [43]. JNKs in turn activate apoptotic signaling either through the up regulation of pro-apoptotic genes via the transactivation of specific transcription factors including C-Jun or by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through phosphorylation.

To study the role of P12 on JNK activation, AHDF were exposed to tunicamycin in the presence or absence of PDGF-BB with or without P12 or scrambled P12. The level of p-JNK

was determined by western blot. As shown in Figure 2.1, p-JNK was induced significantly by tunicamycin at 1 hour and the induction lasted up to two hours. In the presence of P12, p-JNK induction was completely inhibited, while scrambled P12 (SP12) showed much weaker effect on inhibiting tunicamycin-induced p-JNK elevation indicating that the effects of P12 on JNK activation is sequence specific. JNK activation has been associated with numerous diseases including Alzheimer's disease and diabetes [44]. Inhibition of JNK activation by cell-permeable JNK-inhibitory peptide has been reported to improve insulin resistance and ameliorated glucose tolerance in diabetic mice [45]. The strong inhibitory effect of P12 on JNK activation and its cell penetrating sequence make it a possible candidate for the treatment of Alzheimer disease and diabetes.

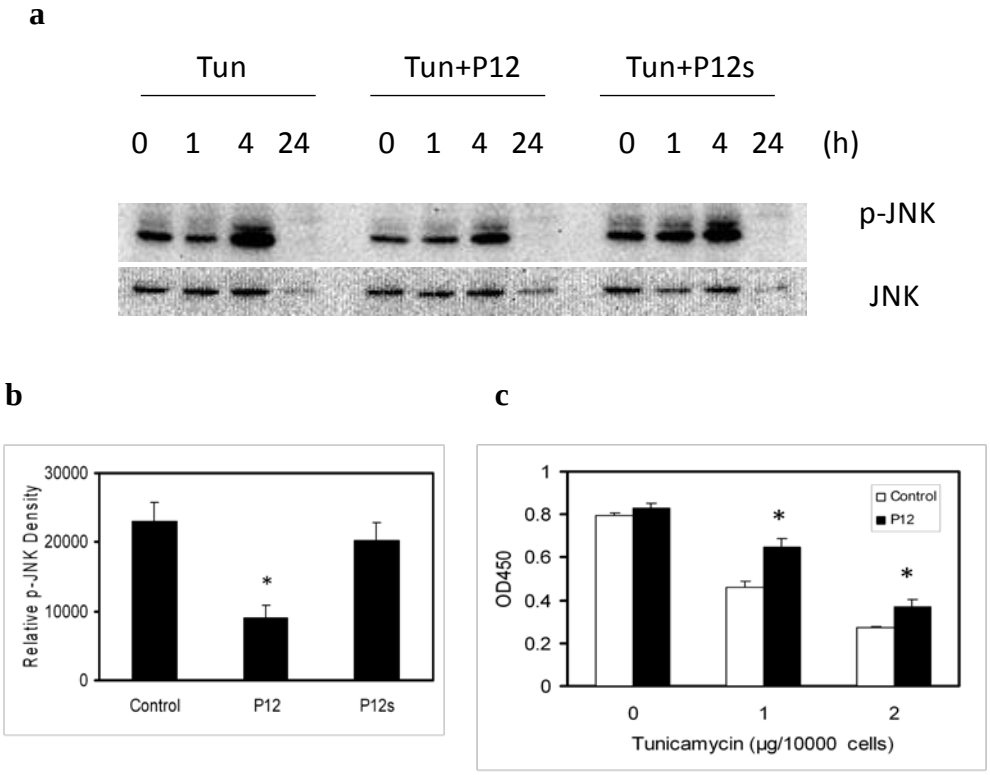


Figure 2.1 P12 inhibited tunicamycin (Tun)-induced JNK activation and enhanced adult human dermal fibroblast (AHDF) survival under Tun-induced stress. a) P12 inhibited Tun-induced JNK activation. AHDF were cultured in DMEM with 10% FBS and then exposed to 5 µg/ml Tun (1 µg/10^4 cells) with or without P12 or scrambled P12 (P12s) for the indicated time. The level of p-JNK was tested by western blot with samples containing equal amounts of protein. The results are representative of three independent experiments. b) Quantitative analysis of p-JNK band at 4 h. Bar graphs showed mean ± SD (asterisk, $P<0.05$ compared with control, n=3). **c**) P12 enhanced AHDF survival under Tun-induced endoplasmic reticulum (ER) stress. AHDF were cultured in serum-free DMEM at 1000 cells/well in 96-well plate overnight. The cells were challenged with 1, 2, 5 µg/10,000 cell Tun for 4 hours and cell viability was determined by XTT assay. Bar graphs showed mean ± SD, (asterisk, $P<0.05$ compared to control, n=4).

P12 enhanced PDGF-BB ability to support AHDF survival under nutrient deprivation.

Since P12 worked synergistically with PDGF to promote AHDF survival [32] and promoted tissue survival in peri-burn tissue, I investigated whether P12 had a similar effect under acute nutrient deprivation. For this purpose, I employed initially a nutrient deprivation system [46] with or without hypoxia (1% oxygen). As an indicator for cell viability I used the XTT assay, which measures mitochondria dehydrogenase activity. Since the presence of hypoxia had no obvious additional effect on cell viability compared to nutrient deprivation alone, in subsequent studies I used only nutrient deprivation.

As shown in **Figure 2.2**, P12 enhanced the ability of PDGF-BB to support AHDF in Hank's balanced salt solution (HBSS) devoid of all growth factors and amino acids. P12 increased cell metabolism at a range of PDGF-BB doses (**Figure 2.2a**) and protected cells from nutrient deprivation-induced apoptosis (**Figure 2.2b** and **c**). AHDFs were rounded and clumped after 3 days under these starvation conditions while P12/PDGF-BB-treated AHDFs were well-spread and maintained healthy morphology (**Figure 2.2b**). Almost 40% of the AHDFs underwent apoptosis after 3 days of starvation. While PDGF-BB reduced apoptosis to 30%, P12/PDGF-BB treatment lowered apoptosis to less than 10% (**Figure 2.2c**). To determine whether cells treated with P12/PDGF-BB survived 72h starvation better than cell treated with PDGF-BB alone, cells were switched to full-serum medium and assayed by the XTT 24h later (**Figure 2.2d**). Indeed metabolism was significantly higher with P12/PDGF-BB than PDGF-BB alone. Similar results were obtained with cells cultured under nutrient deprivation conditions and hypoxia (1% oxygen) (data not shown).

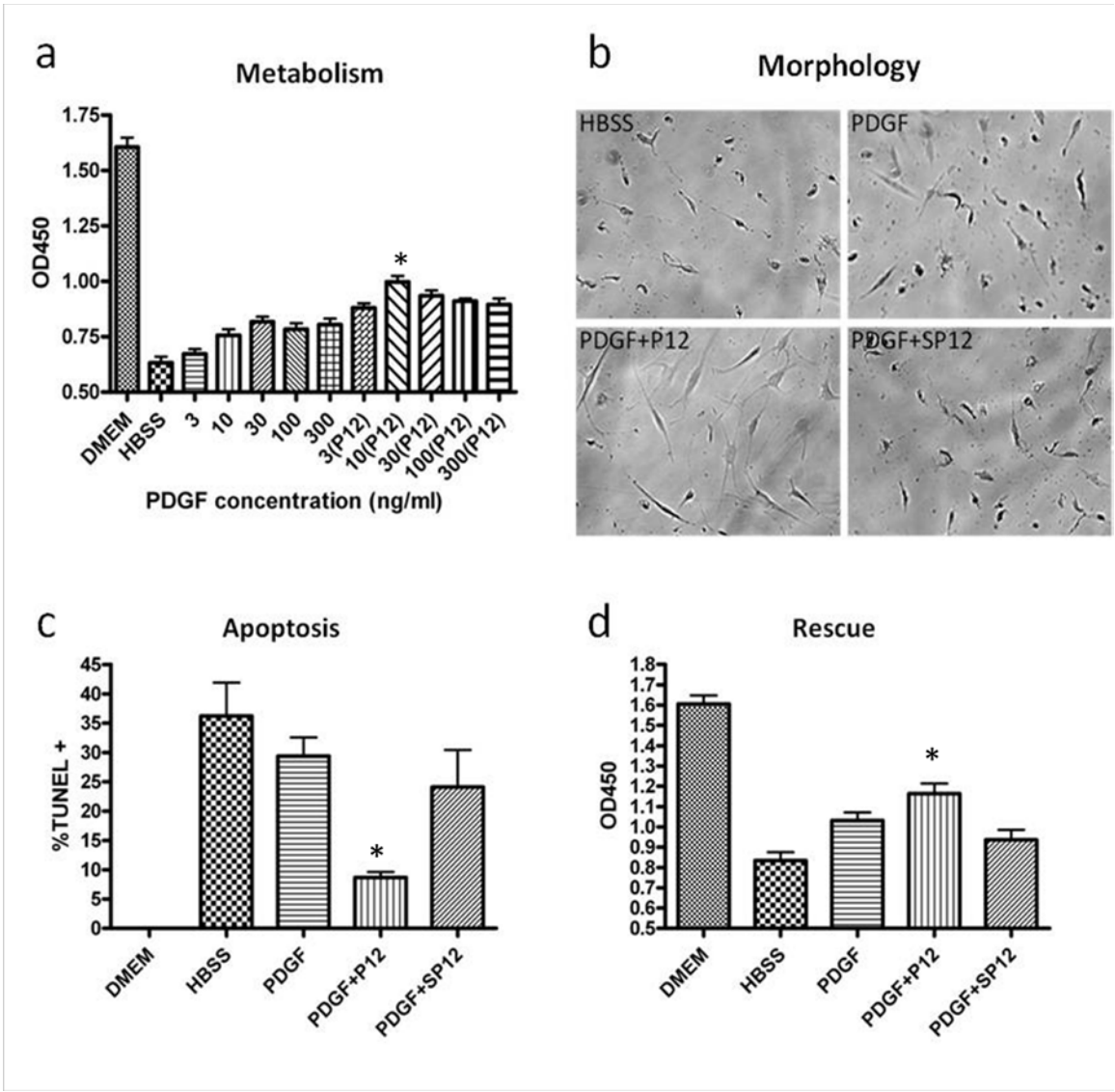


Figure 2.2 P12/PDGF promoted AHDF survival under nutrient deprivation. AHDFs were treated with PDGF+/- P12 in HBSS for 72 h. Unless otherwise indicated, all experiments were performed on AHDF in Hank's Balanced Salt Solution (HBSS) with 1nM PDGF and 10 μM P12. **a)** Cell metabolism was measured by the XTT assay (asterisk, $P<0.05$ compared to PDGF alone, n=5), error bar indicates standard deviation. **b)** Cell morphology as judged by phase contrast microscopy. (SP12=scrambled P12) The control is in HBSS without any PDGF. **c)** Apoptosis was measured by the TUNEL assay. %TUNEL+ = TUNEL positive/ DAPI positive. >800 cells counted (asterisk, $P<0.05$ compared to PDGF alone, n=3). **d)** After 72h of nutrient deprivation, cells were rescued by 10% FBS for 24h and metabolism measured by the XTT assay (asterisk, $P<0.05$ compared to PDGF alone, n=3).

Cell proliferation was not observed in AHDF under nutrient deprivation

Also, cell proliferation was measured by DNA replication with Cyquant kit. The results from Cyquant experiments showed no statistical significant difference in cell proliferation

between the treatment conditions (**Figure 2.3**). This indicates that the difference I observed in cell metabolism (XTT assay) was not a result of differential cell proliferation and instead it reflects a difference in cell survival. This is complimentary to the apoptosis results and the rescue data showing that P12 promoted cell survival instead of proliferation in AHDF under nutrient deprivation.

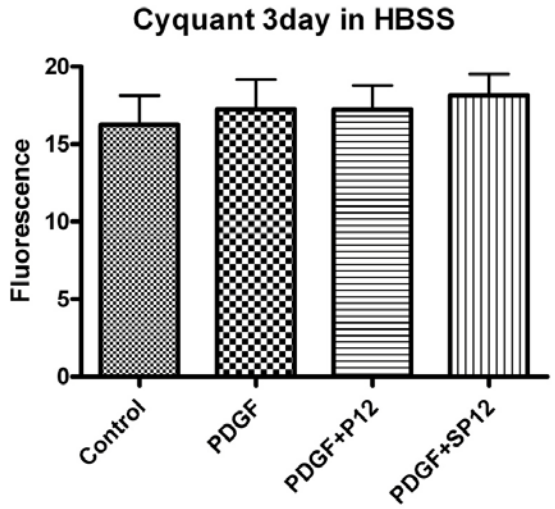


Figure 2.3 Cell proliferation was not observed in AHDF under nutrient deprivation. AHDF cell treated with PDGF+/- P12 (SP12) were cultured on serum coated surfaces in HBSS for 3 days. Cell proliferation was measured by Cyquant cell proliferation assay based on DNA synthesis. Experiment was repeated 3 times. Error bar indicates SD.

P12 does not affect PDGF signaling on a plastic surface.

Since P12 works synergistically with PDGF-BB to promote cell survival, I next studied if P12 has any effect on PDGF-BB signaling pathways. I first examined the effect of P12 on PDGFR-β activation. The first effort to characterize P12 effect on PDGF-BB signaling pathway was not successful (**Figure 2.4**). Here, AHDF cell were plated without serum on tissue culture plates. So the only ECM available is the ECM secreted by the cells themselves. After 24h of serum starvation, I stimulated the cell with PDGF-BB+/- P12 for the indicated time and measured signaling molecules in PDGF-BB signaling pathways. As shown in figure 2.4, PDGF-BB activation of PDGFR-β, p-SHP2 and p-ERK1/2 was not affected by the presence of P12.

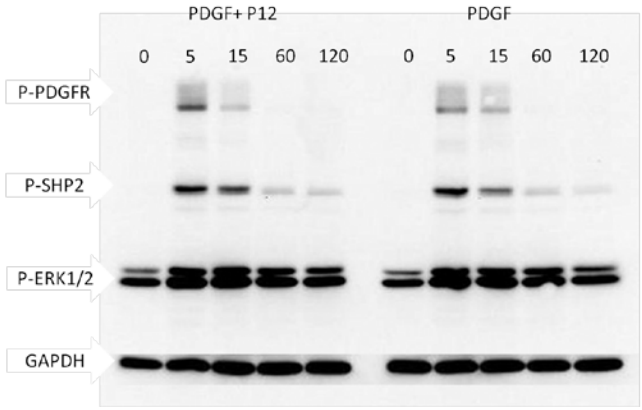


Figure 2.4 P12 does not affect PDGF signaling on a plastic surface. AHDFs were serum-starved overnight and then treated with PDGF-BB +/- P12 (SP12) in DMEM. Western blots were performed with antibodies against p-PDGFR-β, p-SHP2 and p-ERK1/2. GAPDH was used as a loading control.

P12 enhanced PDGFR-β activation on FN coated surface.

Previously, Dr. Mctigue in the lab has demonstrated that in AHDF grown on FN coated surface, P12 in the presence of PDGF-BB changed mRNA levels of important signaling relay proteins such as Akt and survivin after 20h. As a logical extension of that finding, I went on to study if P12 can affect PDGF-BB signaling pathway at earlier time points on FN coated surface. Here, AHDF grown on FN coated surface treated with P12 in the presence of PDGF-BB exhibited enhanced PDGFR-β activation at Y751 compared to cells treated with PDGF-BB alone (**Figure 2.5**). It is worthwhile to mention here that when I first started the project, I examined the effects of P12 treatment alone on cell signaling/metabolism under various conditions. However, P12 alone showed no effect on cell metabolism or signaling. In other word, P12 promotes cell survival only in the presence of PDGF-BB (or in FBS that contains PDGF-BB).

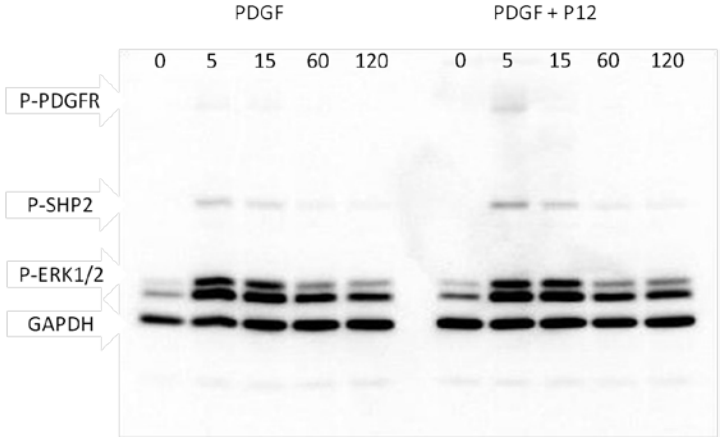


Figure 2.5 P12 enhanced PDGFR-β activation on FN coated surface. AHDFs were serum-starved overnight and then treated with PDGF-BB +/- P12 (SP12) in DMEM. Western blots were performed with antibodies against p-PDGFR-β, p-SHP2 and p-ERK1/2. GAPDH was used as a loading control.

P12 enhanced PDGFR-β activation and P85 recruitment.

To determine how P12 affects PDGF signaling, I first examined the effect of P12 on PDGFR-β activation. AHDF were treated with PDGF +/- P12 in HBSS followed by isolation of internalized PDGFR-β (see Material & Methods). Enhanced PDGFR-β activation at Tyr751 was observed in cells treated with P12/PDGF-BB compared to those treated with PDGF-BB alone (**Figure 2.6a**). Furthermore, when biotinylated PDGFR-β was pulled-down by streptavidin beads and probed with an antibody to the PI3K p85 subunit, I observed increased recruitment of p85 to PDGFR-β in P12/PDGF-BB treated cells compared to cells treated with PDGF-BB alone (**Figure 2.6b**). These data suggests that P12 can enhance PDGFR-β activation by PDGF-BB and lead to increased PI3K signaling that is required to activate downstream survival pathways [47].

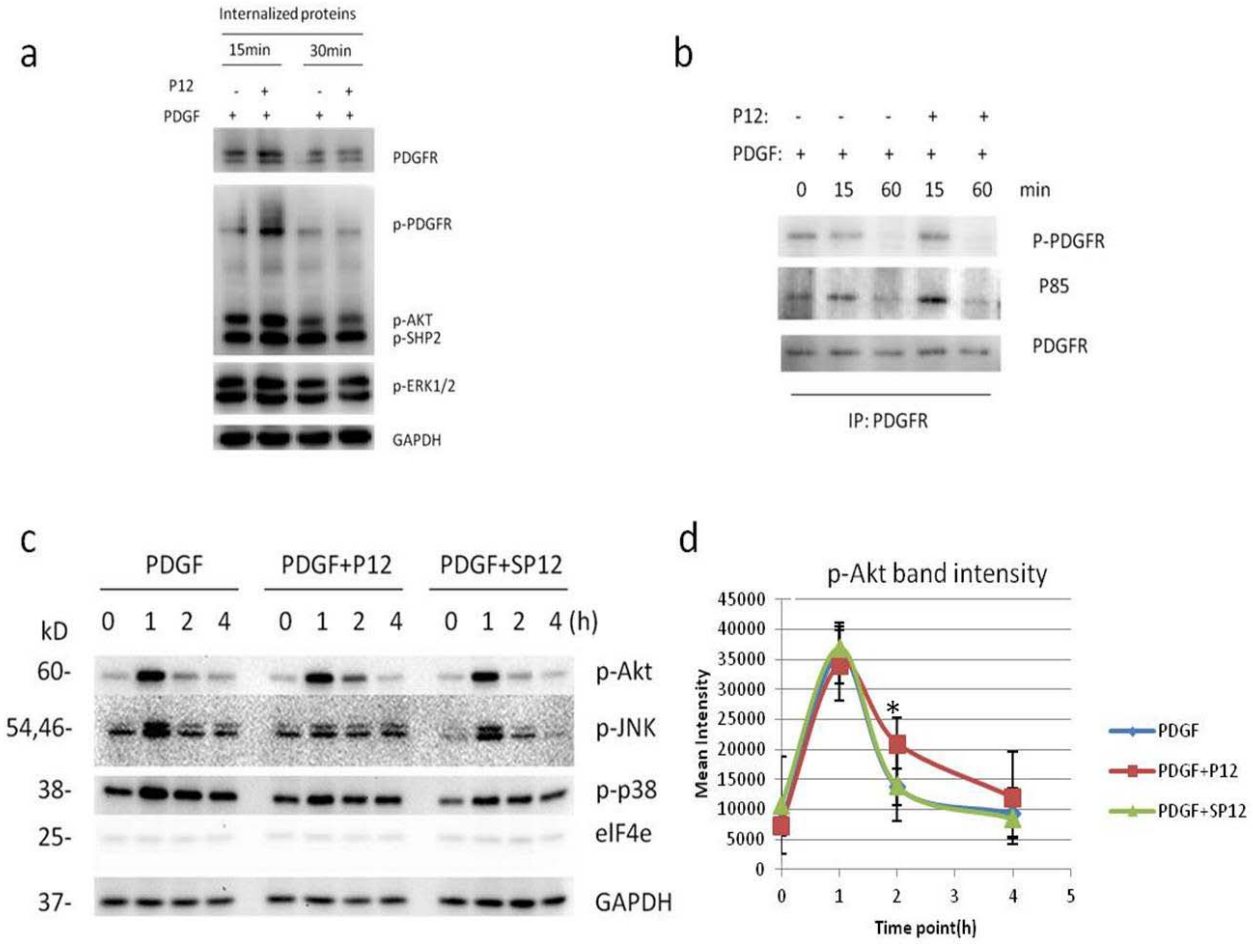


Figure 2.6 P12 enhanced PDGFR-β activation, P85 recruitment to PDGFR-β and prolonged Akt phosphorylation. a) AHDFs were treated with PDGF-BB +/- P12 in HBSS. The PDGFR-β internalized during treatment was separated (See methods) and probed with antibodies against p-PDGFR-β (Tyr751) and p-ERK1/2. GAPDH was used as loading control. **b)** AHDF cells were treated with PDGF+/-P12 in HBSS. PDGFR-β in total cell lysate was immunoprecipitated. p-PDGFR (Y751) and p85 were probed. **c)** AHDFs were treated with

PDGF-BB +/- P12 (SP12) in HBSS. Western blots were performed with antibodies against p-Akt, p-JNK, p-p38 and elF4e. GAPDH was used as a loading control. **d)** Digital quantification of p-Akt band intensity using Kodak IM ver4.0.3. Data from 3 independent experiments (asterisk, $P<0.05$ compared to PDGF alone, n=3). Error bar indicates SD.

P12 prolonged Akt phosphorylation induced by PDGF-BB.

To test whether there is a link between differential PDGF-BB trafficking into the cell and enhanced cell survival, I studied whether P12 also affected PDGF-BB survival signals downstream of PI3K. The Akt/PI3K signaling pathway is one of the major signaling pathways activated by PDGF-BB and is important for cell survival under conditions including oxidative stress, ER stress and nutrient deprivation [48]. Based on increased PDGFR-β activation and P85 recruitment in P12 treated cells, it is possible that P12 can enhance PDGF-BB signaling downstream in the Akt/PI3K pathway under nutrient deprivation. Indeed, on serum-coated surfaces, P12/PDGF-BB, compared to PDGF-BB alone, prolonged Akt phosphorylation (Ser473) and inhibited JNK phosphorylation (T183/Y185) (**Figure 2.6c&d**) while not effecting p38 phosphorylation and elF4e protein levels 1 hour after treatment. Since prolonged Akt and decreased JNK phosphorylation are linked to increased cell survival under starvation [49], these data are consonant with the supposition that P12 may promote cell survival through modulating PDGF signaling. Similar results were obtained with AHDF (AG09605) from another individual (data not shown).

Because collagen is the normal ECM available for AHDF in the dermis, I next tested P12 effect on PDGF-BB signaling pathways in AHDF grown on collagen coated plates. Similar to the results on serum coated surface, which mimics acute cutaneous injury where cells are in contact with serum ECM, I observed a prolonged Akt phosphorylation in the presence of P12 (**Figure 2.7**). Interestingly, there was no p-JNK signal from nutrient deprivation stress in AHDF grown on collagen. This may be due to the ECM regulation of GF signaling. This phenomenon may be expanded into another project but is not followed in this study since I am focused on P12 activity. ERK1/2 phosphorylation was found to be potentiated by P12 in AHDF on collagen possibly due to ECM regulation of GF signaling.

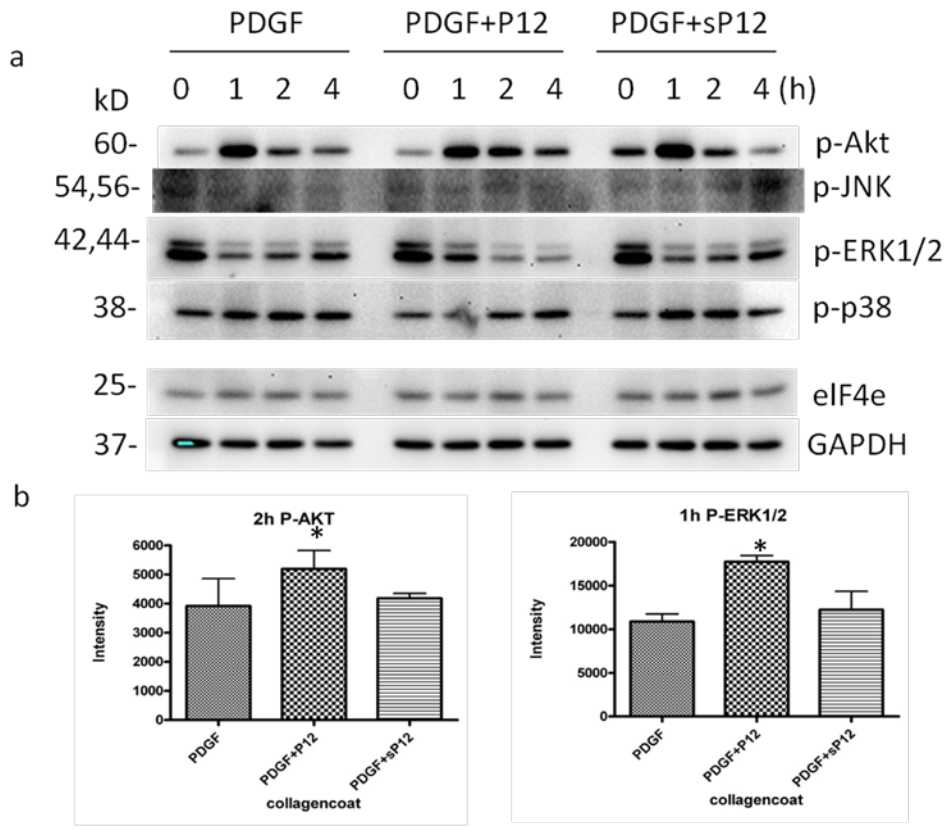


Figure 2.7 P12 enhanced prolonged Akt phosphorylation on collagen coated surface. a) AHDFs were treated with PDGF-BB +/- P12 (SP12) in HBSS. Western blots were performed with antibodies against p-Akt, p-JNK, p-p38 and elF4e. GAPDH was used as a loading control. **b)** Digital quantification of p-Akt band intensity using Kodak IM ver4.0.3. Data from 3 independent experiments (asterisk, $P<0.05$ compared to PDGF+SP12, n=3). Error bar indicates SD.

Taken together, Akt phosphorylation was consistently prolonged by P12 in AHDF grown on both surfaces and sustained Akt phosphorylation was shown to lead to enhanced cell survival under starvation [50].

To put this finding in the context of wound healing, I next tested biologically relevant doses of PDGF-BB (**Figure 2.8**). In acute wound, the concentration of PDGF-BB ranges from 0.1 nM to 1 nM while the PDGF-BB concentration in serum was shown to be about 1 nM [51]. Western blots data showed that P12 increased p-Akt at PDGF-BB levels observed in both acute wound fluid and in serum [51, 52]. However, this boost in p-Akt happens at an earlier time point for the lower PDGF-BB doses probably because the PDGF-BB given to the cells were depleted sooner. An interesting explanation for this P12 effect observed is that after binding to PDGF-BB, P12 somehow slowed the internalization/degradation of PDGF-BB and that led to the enhanced signaling.

Because P12 does not bind P12 (as shown by the Biacore experiment), I used EGF as a control for the P12 effect. As expected, P12 had no effect on EGF-stimulated Akt phosphorylation under nutrient deprivation (**Figure 2.8**). This supported the notion that the P12 effect on PDGF-BB depends on its binding affinity to PDGF-BB.

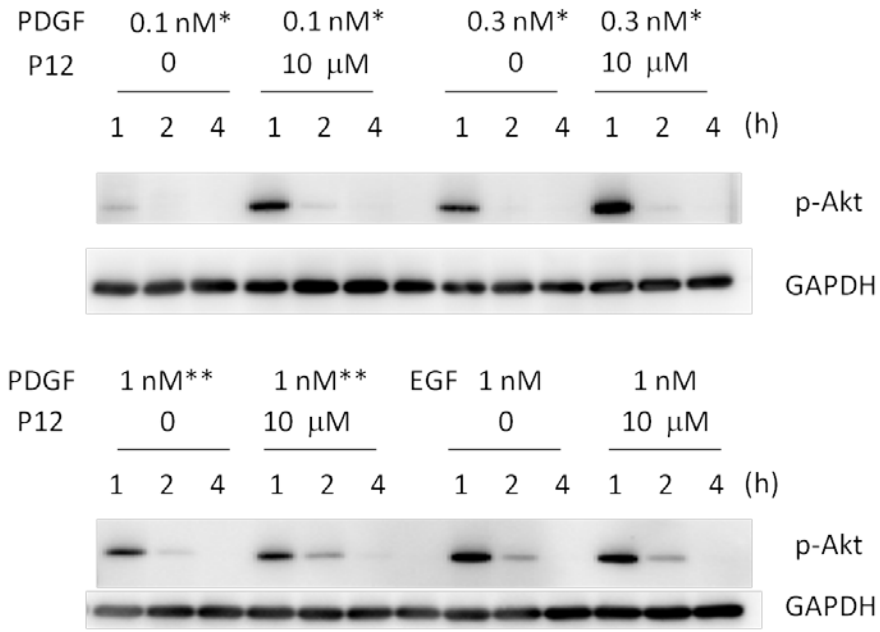


Figure 2.8 P12 increased p-Akt stimulated by various doses of PDGF-BB but not EGF. AHDFs were treated with PDGF-BB +/- P12 or EGF +/- P12 in HBSS at the doses indicated in the figure. Western blots were performed with an antibody against p-Akt and GAPDH was used as loading control.

2.4 Discussion

Although the mechanism of P12 on supporting cell survival under various stress conditions is not clear, it is reasonable to posit that P12 acts as a co-factor of PDGF-BB or other growth factors to support cell survival synergistically. Indeed, it has been reported that survival signal from FN and growth factors converge at the level of the focal contact (15) and are co-stimulatory (16) for progression through the cell cycle (17). For example, fibroblast attachment to surface-bound FN is requisite for activation of ERK1/2 in the MAP kinase signaling pathway for cell cycle progression by PDGF (5).

The finding of P12 and its bioactivities reveals a novel mechanism of tissue cell survival regulated by extracellular matrix proteins under various stress conditions including nutrition deprivation, oxidative stress, and ER stresses. These stresses are associated with the developments of numerous diseases including neurodegenerative diseases, diabetes, and tissue injury progression such as stroke, heart attack, and burn injury progression. P12 has been found not only associate with PDGF-BB but also interacts with other growth factors including vascular endothelial growth factor, basic fibroblast growth factor, transforming growth factor- β, insulin and insulin-like growth factor (**Table 2**). It is reasonable to posit that clustering of ECM receptors and GF receptors on the cell surface and ensembles of ECM ligands and GFs in the ECM might allow for an ordered robust amplification of signals that activate cell migration, proliferation, protein synthesis.

The blood vessel occlusion observed in the periburn tissue raises concern about the bio-availability of PDGF-BB in the zone of ischemia imediately around burn site. However, previous data in the lab showed macrophage penetration into the deep dermis before 24h post burn. So PDGF-BB can be secreted by macrophage in the zone of ischemia. Also, immediately surrounding the zone of ischemia is a zone of hyperemia [53]. The increased blood flow is likely to expose cells in that region to PDGF-BB and fibronectin from serum.

Chapter 3

P12 Redirected the Endocytosis of PDGF-BB/PDGFR-β to a Macropinocytosis-like Pathway

3.1 Introduction

3.1.1 Endocytic signaling of GFR

Signaling by cell surface receptors appears to be relatively straight-forward: ligand binds to the extracellular domain of the receptor and biochemical changes are communicated into the cell. However, this process is more complex than it first seems due to the various endocytic process that regulate signaling [54]. Various GFR have been shown to generate signals after they are internalized into endosomes or other vesicles and the endocytic route and subsequent sorting/trafficking determines the duration, specificity and the nature of the signals.

3.1.2 Internalization of GFR

Internalization of cell surface receptors can be divided into clathrin-independent and clathrin-mediated endocytosis, depending on the route of entry into the cell. Studies from the past decades have demonstrated that clathrin-mediated endocytosis (CME) is the major pathway of internalization for ligand-occupied GFR [55]. Because many conceptual findings have been made using the EGFR system and later reproduced in other GFR, EGFR has become the prototypic model system to study GFR endocytosis. Thus we will rely on EGFR model for the demonstration of the basic mechansims here.

CME is the fastest internalization pathway (rate constant Ke~ up to 0.6 min-1). Kinetics analysis of EGFR endocytosis suggested that CME is saturated when a large number of surface EGFRs are activated by EGF, and the contribution of a slower clathrin-independent endocytosis (CIE) increases with the increase of EGF concentration and EGFR expression levels, leading to overall reduction in apparent internalization rate [56].

The basic events associated with ligand-stimulated, clathrin-mediated endocytosis have been fairly well delineated for some time [57], although new molecular details continue to emerge. Briefly, the ligand-bound EGFR monomers dimerize and translocate along the plasma membrane until it associates with a membrane domain that is enriched with clathrin on the intracellular face. This domain invaginates to form a clathrin-coated pit, which pinches off forming a clathrin-coated vesicle. The clathrin is shed from this vesicle to produce an intermediate vesicle that fuses with and delivers the EGF: EGFR complex to the early endosome. In the early endosome, the ligand: receptor complex is readied for its ultimate cellular fate—either (1) the early endosome matures into a late endosome and delivers the cargo to the lysosome [58]; (2) a recycling endosome pinches off the early endosome and the ligand and receptor recycle back to the plasma membrane [59]; or (3) an endosome forms to deliver the receptor to some other intracellular organelle (i.e., mitochondria [60], trans-Golgi network [61],

and endoplasmic reticulum [62]). It should also be noted that endocytic trafficking is distinct from the movement of the EGFR and EGFR fragments to the nucleus [63, 64]. It is not entirely clear which cellular components determine the trafficking itinerary, although there is strong evidence that the stimulating ligand, receptor density, and cell type each have a contributing role[54]. EGFR ubiquitination following tyrosine phosphorylation during signaling plays a key role in targeting the receptor for lysosomes and degradation.

3.1.3 Endocytic signaling of GFR

Each endocytic route has a very different consequence on EGFR signaling. Trafficking to lysosomes will result in attenuated signaling due to receptor degradation. Receptors that recycle back to the plasma membrane have the opportunity to be re-stimulated by extracellular ligands that are present. When the ligand: receptor complex traffics to other subcellular organelles, receptor: effector interactions may occur due to enhanced localized effector concentrations that impact a defined cell biology. When a cell's normal trafficking itinerary is perturbed, the duration, magnitude, and specificity of receptor signaling will be altered and can affect cell biology.

Based on many of the early studies, EGFR endocytosis was thought to be strictly a mechanism to control how long a ligand-bound receptor stayed active. However, for almost 20 years, it has been appreciated that the endocytic pathway provides spatial regulation of signaling. Work by Vieira et al. that examines endocytosis-deficient EGFRs indicated that for some effectors, maximal activity requires that the receptor be internalized [65]. Thus, the endocytic pathway can be a positive regulator of signaling as well. In the last decade there was increasing evidence that PDGF/EGF signals from endosomes. It was shown that after blocking membrane signaling, endosome signaling of PDGF alone is enough to support cell survival [66].

Other examples on how the route of internalization influences the final signaling output have been recently provided in the case of IGF-1R [67, 68] and PDGFR-β [69]. In both cases, it has been proposed that they can enter through both clathrin-dependent and -independent pathways depending on the amount of ligand used to stimulate cells, similarly to what has been shown for the EGFR system. This again impacts on the final biological response [70]. For instance, in the case of PDGFR-β, cells switch from a migrating to a proliferating phenotype in response to an increasing PDGF gradient. It was proposed that the decision to proliferate or migrate relies on the distinct endocytic route followed by the receptor in response to ligand concentration. A recent study demonstrated that in H-Ras transformed cells, PDGFR-β internalization was shifted from CME into macropinocytosis. And PDGFR-β can generate enhanced survival signals in macropinosomes [71]. Although these studies remain at the phenomenological level with no mechanistic insights, they confirm the idea that integration of different internalization pathways is crucial to decode signal information and to specify the signaling response.

3.1.4 Cell penetrating peptide

The ability of HIV transcriptional activator (TAT) protein to enter mammalian cells is conferred by a localized region in the protein known as the protein transduction domain. Furthermore, the isolated peptide sequence, sometimes referred to as a cell-penetrating peptide (CPP), retains the transduction properties of the native protein. After the TAT peptide, a number of CPPs were discovered and used in the delivery of macromolecules into mammalian cells.

Some of them were naturally occurring protein transduction domains e.g. TAT peptide and penetratin, some were artificially constructed based on the typical structures of TAT e.g. oligoargine peptides, pep1.

These cell-penetrating peptides comprise a class of short (<20 amino acid) cationic peptides that have the ability to traverse the cell membranes of many different types of mammalian cells. A wide variety of macromolecules has been attached to these peptides and subsequently internalized (Figure 1.3). Moreover, after uptake the cargo maintains its activity. The ability of cell-penetrating peptides to translocate biologically active molecules into cells makes these peptides promising candidates for drug delivery applications. But current use is limited by a lack of cell specificity in CPP-mediated cargo delivery and insufficient understanding of the modes of their uptake.

Many studies have been designed to specify the endocytic pathways of these CPPs. However, the results varied depending on the type of CPP, the type of cells and the size of the cargo [72]. A number of research groups have proposed macropinocytosis as the mechanism of uptake for cell-penetrating peptides [73-77]. Macropinocytosis involves the formation of large vesicles of irregular size and shape, generated by actin-driven invagination of the plasma membrane. Macropinosomes have no coat and do not concentrate receptors. They vary in size, sometimes being as large as 5 μm in diameter [78]. Studies have shown dose-dependent inhibition of TAT peptide uptake when cells are pretreated with amiloride, an inhibitor of the Na+/H+ exchange required in macropinocytosis [74]. Additionally, cytochalasin D, an inhibitor of actin polymerization, and the macropinocytosis inhibitor ethylisopropylamiloride have been shown to significantly suppress uptake of the arginine-rich peptides into HeLa cells [77].

In Chapter 2 my results gave some ideas to explain the mechanism of action of the P12 effect in the pig studies. Namely, P12 can bind to and act as a co-factor of PDGF-BB, augmenting its survival signaling to promote AHDF cell survival under nutrient deprivation. But it remains a mystery how P12 was able to achieve this on a molecular level after binding to PDGF-BB.

A clue to this is that P12 has the structural characteristics of a cell-penetrating peptide (**Table 1**). CPPs are generally cationic peptides of 10 to 20 amino acids in length that can bring other cargoes (proteins, peptides, nucleic acids, etc.) into mammalian cells in an energy- and temperature-dependent manner [79]. It has been proposed that CPP function is dependent on the electrostatic interaction with negatively charged molecules, especially glycosaminoglycans (GAGs), on the cell surface [80]. A lot of efforts have been put into characterizing the entry pathway of CPP but the results varied depending on cargo, cell type and experimental conditions [74, 79, 81]. More likely than not, the endocytic route of CPP will be different from the one PDGF-BB/PDGFR-β usually takes (clathrin-mediated endocytosis). Since P12 fits the structural definition of a typical CPP (4 cationic residues out of a total of 14), it may work as a CPP to change endocytic entry of PDGF-BB/PDGFR-β.

Since the binding affinity between PDGF-BB and PDGFR-β (Kd~0.4 nM) is stronger than that between PDGF-BB and P12 (Kd~200 nM), it is likely that P12 will bind better to the PDGF-BB/PDGFR-β complex on the cell surface than to PDGF-BB alone.

P12: **PSHISKYILRWRPK**

Penetratin: **RQIKIWFQNRRMKWKK**

Pep1: **KETWWETWWTEWSQPKKKRKV**

TAT: **GRKKRRQRRRPPQ**

Table 1. Sequence comparison between P12 and typical cell penetrating peptides. Positively charge amino acids are shown in red letters. Penetratin: Antennapedia peptide, derived from the homeodomain of Antennapedia. Pep1, a synthetic CPP that was proposed to enter through a physically driven mechanism. TAT peptide, derived from the transactivator of transcription (TAT) of HIV, is used by the virus to deliver TAT protein into mammalian host cells.

Endocytic entry of growth factor receptors following ligand binding plays an important role in the regulation of growth factor signaling [82]. After internalization, epidermal growth factor (EGF) can still generate survival signals from endosomes [83, 84]. Moreover, ERK1/2 activation is dependent on endocytosis of the EGF receptor (EGFR) [85]. Furthermore, the specific route of entry, what downstream adaptor/signal relay proteins are present, and how long EGF resides in those vesicles before it is sent for degradation or recycled back to the plasma membrane dictate the amplitude, specificity and duration of signal [82]. As another example, specific endocytic pathways used to internalize the PDGFR-β generate different signals, leading either to proliferation, or migration [69]. Most importantly, endosomal signaling from ligand-bound PDGFR-β has been reported to be sufficient for cell survival [66].

Several studies have suggested that CPP can bring protein cargo into cells through macropinocytosis [74, 86]. So it is possible that after P12 binding to PDGF-BB/PDGFR-β, P12 can bring the PDGF-BB/PDGFR-β complex into macropinosomes. Macropinosomes were shown to be a unique signaling platform for PDGF-BB to generate robust survival signals [87]. Most recently, the same group demonstrated that H-Ras-transformed tumor cells can employ macropinocytosis to generate enhanced survival signals from PDGF-BB [71]. The increased localization of PDGFR-β in macropinosomes led to stronger receptor activation, prolonged Akt phosphorylation and enhanced cell survival.

Although PDGF-BB induces macropinocytosis, activated PDGFR-β is mainly internalized through clathrin-mediated endocytosis [71, 88] or, at high PDGF-BB doses, through caveolin-mediated endocytosis [69]. During both clathrin- and caveolin-mediated endocytosis, the GTPase dynamin is required for vesicle fission, whereas macropinocytosis occurs independently of dynamin activation [89, 90]. In this chapter, I investigated the effect of P12 on PDGF-BB endocytosis and PDGF signaling.

Our results suggest that by shifting the balance between clathrin-/dynamin-dependent and independent endocytic pathways, P12 slowed internalization and degradation of PDGF-BB, augmented survival signal, and promoted cell survival under nutrient-removal stress.

3.2 Materials and methods

Materials: P12, scrambled P12 and C-terminal Cys-tagged P12 were purchased from American Peptide (Sunnyvale, CA). Antibody against p-PDGFR-β (Tyr751), p-Akt (Ser473), p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Tyr204), p-P38 (Thr180/Tyr182) and GAPDH are purchased from Cell signaling Technology (Denver, MA). AHDF (CF31) primary cells are isolated from dermis of a 31 year old female. Hank's Balanced Salt Buffer from Sigma (St. Louis, MO). Cell Proliferation kit II (XTT) and In Situ Cell Death Detection kit (TUNEL reaction mixture) was purchased from Roche Diagnostics (Indianapolis, IN). SiRNA for CHC KD (sc-35067) was purchased from Santa Cruz Biotechnology (Dallas, TX). Allstar negative control siRNA and Hiperfect transfection reagents were purchased from Qiagen (Valencia, CA). PDGFR extracellular domain was purchased from R&D Systems.

Cell culture and Transfections: AHDFs were maintained in DMEM (Gibco) with 10% FBS at 37°C and 5% CO2 in a humidified atmosphere. Cells from passage 8-12 were used. SiRNA and mock was used at 10nM for transfection. Cells were transfected twice with Hiperfect Transfection Reagent according to manufacturer's recommendations at 36-h intervals.

Peptide Labeling: 100uM Cys-P12 was incubated with 150uM Alexa Fluor 488 C5 maleimide in 500ul of PBS for 2h at room temperature. Then the labeled P12-488 was purified by cation exchange chromatography (CM-C50). The column was washed, and labeled peptides (colored band) were eluted with pH 11.5 Hepes buffer. The pH was adjusted to 7.5 with HCl and concentration of P12-488 is determined by OD 280nm.

Confocal Microscopy: AHDF cells grown in Lab-Tek Chambers were incubated with 1μM of P12-Alexa488 for 1 h at 37 °C, and washed. Plasma membranes were stained with 5μg/ml Cellmask Deep Red plasma membrane stain for 2min at 37 °C, fixed with 3.7% formaldehyde, and analyzed by confocal microscopy (Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System) with excitation light at 488nm and 633nm.

Flow cytometry: 2 x 10^5 cells were incubated with 1μM of P12-488 dissolved in DMEM medium. Subsequently, cells were incubated for 2 min with 0.25% trypsin to detach the cells and remove surface-bound peptide. Cell suspension fixed with 3.7% formaldehyde, and washed in PBS. Alternatively, cells were treated with 1nM PDGF + /- 10μM P12 for 1 h then fixed with 3.7% formaldehyde and permeabilized with 0.4% Triton-X100; blocked with 1% BSA and stained with primary and secondary antibodies each for 1 h. Cells were washed with PBS and analyzed by flow cytometer.

Immunocytochemistry: Cultured AHDF cells were seeded to 4 well lab-tak chambers before processing. Cells were treated with 1nM PDGF + /- 10μM P12 for the indicated time before fixing in fixation buffer (Santa Cruz) for 15 min. Cells were permeabilized with 0.4% Triton X-100 in PBS for 5 min, and blocked in 2% BSA for 30 min. Samples were then incubated with a rabbit polyclonal antibody to EEA1 or a monocolonal antibody to PDGFR-β at 1:200 dilution in PBS for 1 h, followed by three washes of 5 min each in PBS. Samples were then incubated with Alexa 488-conjugated goat anti- rabbit secondary antibody (Invitrogen) at 1:200 dilutions in PBS for 1 h, again followed by three washes of 5 min each in PBS. Samples were washed with PBS and analyzed with confocal microscope.

Biacore experiments: The PDGFR-β extracellular domain was fixed to a CM5 chip through amine coupling with NHS and EDC. Real-time interaction of PDGF-BB with PDGFR-β was determined by plasmon surface resonance (BiaCore 2000) with or without 10μM P12 at different doses of PDGF-BB (50nM, 25nM, 12.5nM, 6.25nM, 3.125nM). The chip cell without PDGFR-β was used as a reference. Sensorgrams are representative of three different experiments. Dissociation constants (K_d) were derived from the ratio of kinetic dissociation constants (kd) divided by kinetic association constants (ka).

3.3 Results

P12 was internalized into AHDF cells like a CPP.

The structure of P12 resembles a CPP. Therefore, I hypothesized that P12 can penetrate the mammalian cell plasma membrane like a CPP. To test this hypothesis Cys-tagged P12 was labeled with Alexa Fluor 488 (P12-Alexa488), incubated with AHDF, and cells were examined by one micron step-sections with immunofluorescence confocal microscopy (**Figure 3.1a**). Importantly, N- or C-terminal blocking with amidation, acetylation or Cys-tagging did not change P12 bioactivity (data not shown). P12-Alexa488 was detectable in endocytic structures within 1 h at 37°C. FACS analysis demonstrated that internalization required P12, as Alexa488 itself did not enter AHDF (**Figure 3.1b**). To differentiate between association and uptake, cells were trypsinized to remove surface P12 before the FACS analysis.

To further characterize the P12 internalization pathway, P12-Alexa488 was incubated with cells at low temperature, with ATP depletion or chondroitin sulfate removal since CPP internalization requires active metabolism and interaction with cell surface GAGs [91]. Labeled P12 uptake was almost completely inhibited at 4°C or when cells were pre-incubated with sodium azide and deoxy-glucose to deplete cellular ATP, and greatly inhibited after digestion with chondrointinase ABC (**Figure 3.1c** and **d**). Together these data support the contention that P12 behaved like a cell penetrating peptide.

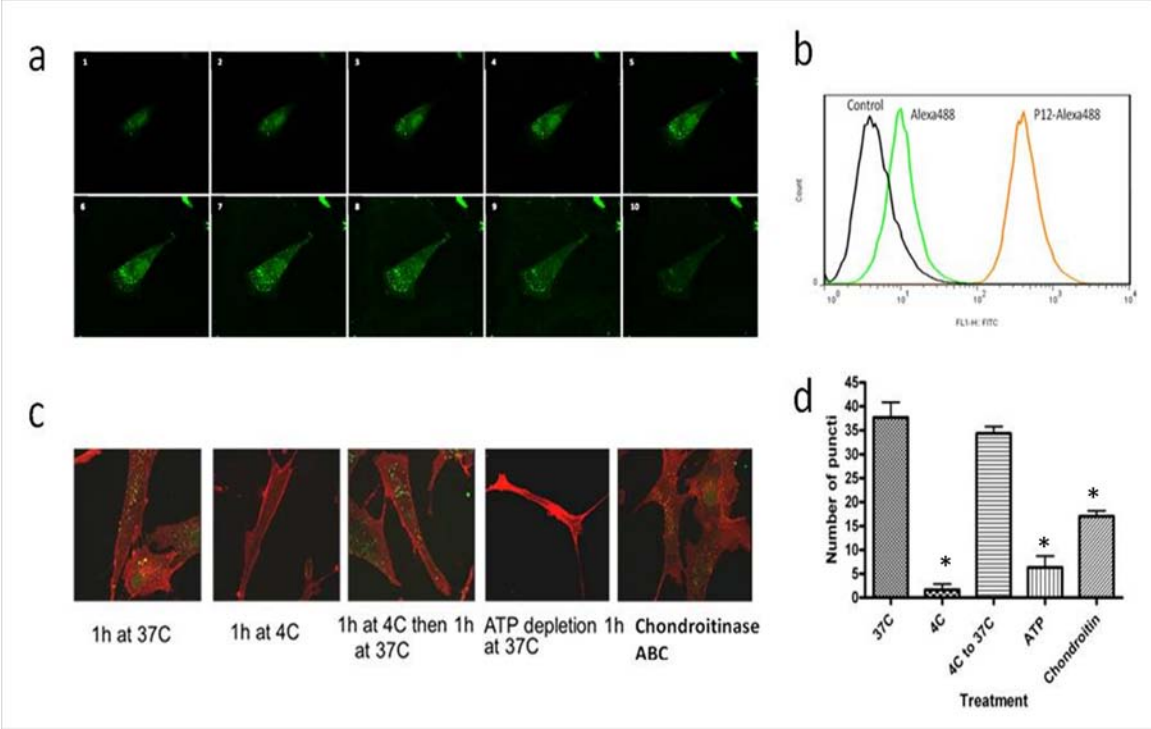


Figure 3.1 P12 acted like a cell penetrating peptide. a) AHDF were treated with 1 μM of Alexa488-P12 for 1 h at 37°C then cross-setion images were taken by confocal microscope. **b)** AHDF were treated with 1μM of Alexa488-P12 or 1 μM Alexa488 alone for 1 h at 37°C then analyzed by flow cytometry for fluorescence intensity. **c)** AHDF were treated with 1μM of Alexa488-P12 for 1 h at 37°C, 1 h at 4C, 1 h at 4°C then 1 h at 37°C, after ATP depletion with 10mM of sodium azide and 2-deoxyglucose for 1 h at 37°C, or after 0.1U/ml chondroitinase ABC digestion for 1 h at 37°C. Red signal is Cellmask^{TM} membrane stain. **d)** Quantification of puncta number in individual cells. Over 50 cells were counted for each condition (asterisk, $P<0.05$ compared to 37°C, n=3).

P12 slowed PDGF-BB/PDGFR-β internalization/degradation.

Although PDGF-BB can induce macropinocytosis, activated PDGFR-β is mainly internalized through clathrin- or caveolin-mediated endocytosis [83, 133]. By contrast, CPPs are internalized through a slower clathrin/dynamin-independent pathway similar to macropinocytosis [73, 92]. Because P12 acts as a CPP and binds strongly to PDGF-BB, it is possible that P12 can change the internalization pathway of PDGF-BB/PDGFR-β and thereby affect the internalization/degradation rate. To test this possibility, I analyzed Alexa Fluor-350 labeled-PDGF-BB uptake in HBSS. Importantly, Alexa Fluor-350 did not affect PDGF-BB bioactivity (**Figure 3.2a**). After incubation with AHDF for 1 hour at 37°C, cellular uptake (indirectly measured by residual PDGF in the medium) was slower in the presence of P12 for both labeled and unlabeled PDGF-BB (**Figure 3.2b** and **c**).

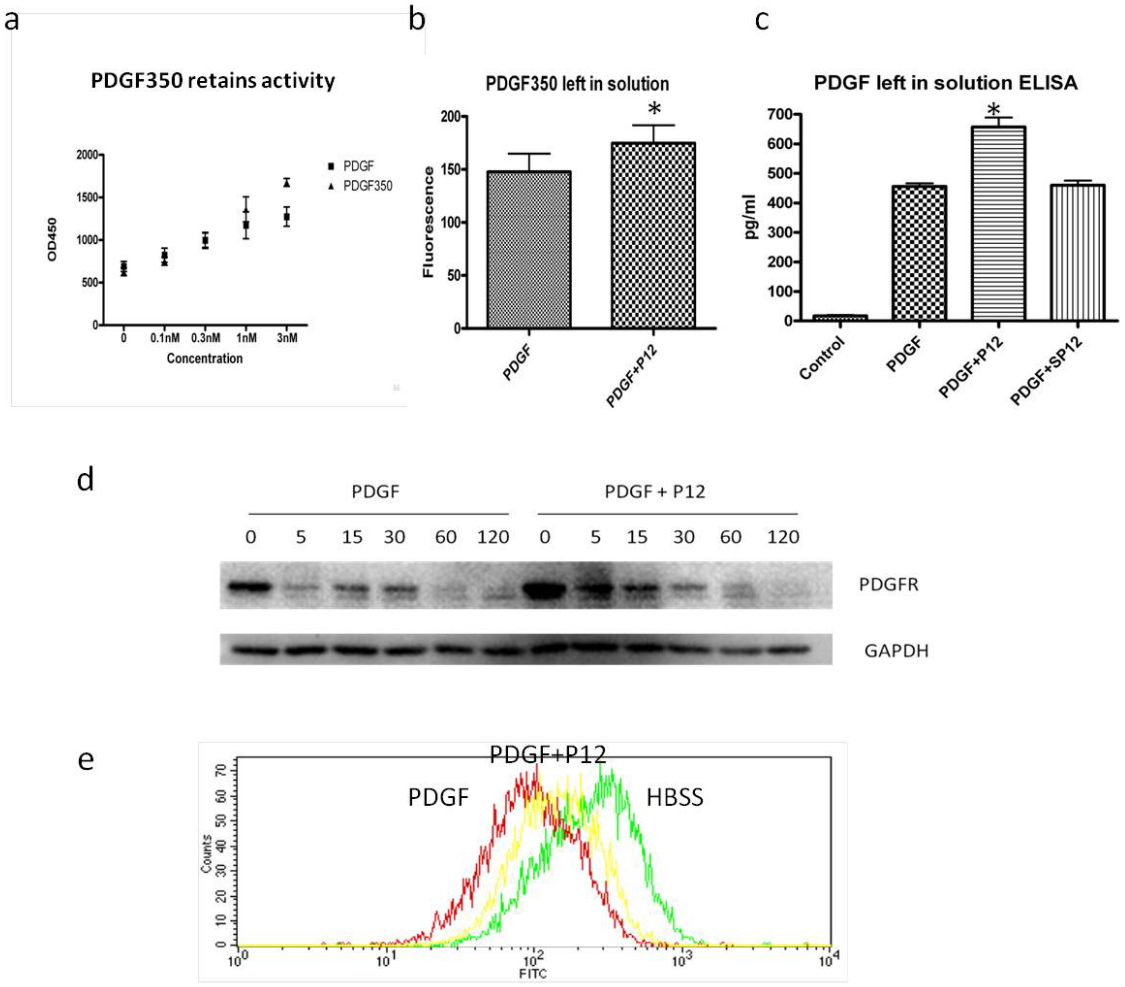


Figure 3.2 P12 slowed PDGF-BB/PDGFR-β internalization/degradation under nutrient deprivation. a) AHDFs were treated with PDGF-BB or PDGF-BB-Alexa350 for 5 days in DMEM. Cell metabolism was measured by the XTT assay. **b)** AHDFs were treated with PDGF-Alexa350 or PDGF ± P12 for 1 h at 37°C. PDGF left in the medium was measured by fluorescence. Control = no additions (asterisk, P>0.05 compared to control, n=3). Error bar indicates SD. **c)** AHDFs were treated with PDGF-Alexa350 or PDGF ± P12 for 1 h at 37°C. PDGF left in the medium was measured by PDGF ELISA. Control = no additions (asterisk, P<0.05 compared to control, n=3). Error bar indicates SD. **d)** AHDFs were treated with PDGF-BB ± P12 in HBSS for the indicated times. Western blots were performed with an antibody against PDGFR-β. GAPDH was used as a loading control. **e)** AHDFs were treated with PDGF-Alexa350 ± P12 for 1 h at 37°C. PDGFR-β was immunostained and analyzed by flow cytometry. Red: PDGF-BB, Yellow: PDGF+P12 and Green: HBSS.

The internalization through clathrin-mediated endocytosis [93] and subsequent receptor degradation is a negative regulation that prevents sustained signaling from PDGFR-β on the cell surface [38]. In our case however, by acting as a CPP, P12 may have altered the internalization of PDGF-BB pathway, slowed the rate of internalization and thus leaving more PDGF in the

medium. In order to see if PDGFR-β degradation is also affected by P12, I next used Western blot and immunofluorescence to analyze PDGFR-β levels in cells. After PDGF-BB +/- P12 treatment in HBSS, I found that P12 slowed PDGFR-β degradation significantly (**Figure 3.2d** and **e**).

Taken together, the results showed that P12 slowed the internalization/degradation of both PDGF-BB and PDGFR-β. One possible explanation of these findings is that P12-binding to PDGF-BB interfered with receptor recognition. However, Biacore experiments showed that the interaction between PDGF-BB and PDGFR-β extracellular domain was not affected by the presence of P12 (**Figure 3.3**). An alternate possibility is that P12 slowed PDGF-BB/PDGFR-β internalization and degradation by shifting endocytosis from a clathrin-mediated to slower macropinocytosis route.

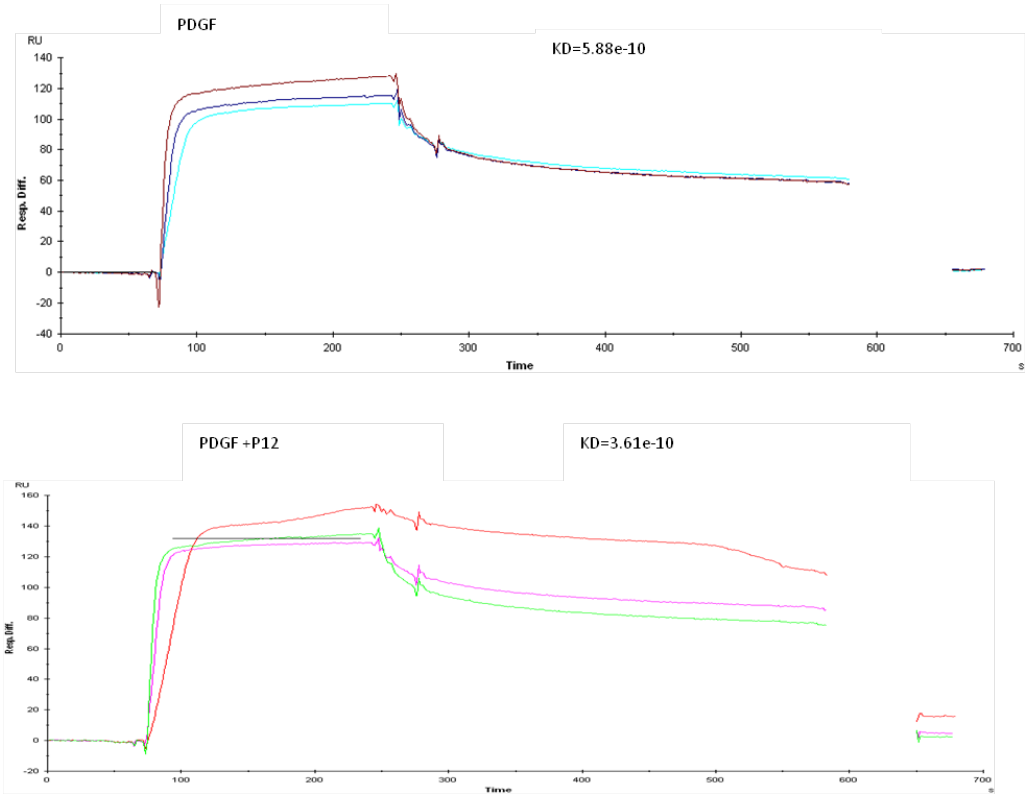


Figure 3.3 P12 does not affect binding of PDGF-BB to PDGFR-β. The PDGFR-β extracellular domain was fixed to a CM5 chip through amine coupling. Real-time interaction of PDGF-BB with PDGFR-β was determined by plasmon surface resonance (BiaCore 2000) with or without 10μM P12. The chip cell without PDGFR-β was used as a reference. Sensorgrams are representative of three different experiments. Dissociation constants (K_d) were derived from the ratio of kinetic dissociation constants (kd) divided by kinetic association constants (ka).

P12 shifted the endocytic pathway of PDGF-BB/PDGFR-β

To study how P12 altered PDGF-BB/PDGFR-β entry into the cell, we first attempted to use Alexa Fluor 488-label PDGF-BB. However, the sensitivity was too low at physiological concentrations of PDGF-BB. I next tried Q-dot labeled PDGF-BB [94, 95]; however, the PDGF-

Q-dot complex was internalized by macropinocytosis probably secondary to the large size of Qdots (data not shown). Therefore, a MCF-7 cell line expressing GFP-PDGFR-β [96] was used to track PDGF-BB/PDGFR-β complexes. Alexa Fluor-594 labeled transferrin (TFN) was used as a marker for vesicles that enter through CME. In control cells (without PDGF-BB) most of the GFP signal was localized on the cell surface with a uniform distribution (**Figure 3.4**). When PDGF-BB was added for 15min, GFP signal was observed in vesicles that stained for TFN. However, when P12 was present, the co-localization of GFP-PDGFR-β and TFN-594 was lower than PDGF treatment alone as shown in the quantification. This indicates that P12 directed the entry away from CME which is the canonical pathway of stimulated PDGFR-β entry. This change is very small and not statistically significant at 95% confidence interval (**Figure 3.4c**). This may be due to the possibility that P12 only redirected a small part of the PDGFR-β out of CME. However, this small portion of PDGFR-β may generate significant survival signaling as shown in chapter 2, where I saw that the internalized PDGFR-β were hyper-activated.

When the cells were immunostained for EEA1, which is an early endosome marker, at the 30 min time point, I did not observe any significant change in co-localization when P12 was present. One possible explanation is that P12 acted as a CPP and brought PDGF-BB/PDGFR-β into macropinosomes that fused with EEA1 positive vesicles. Alternatively, maybe CME was still the major route of entry when P12 is present and CIE vesicles only accounts for a small part of the signal.

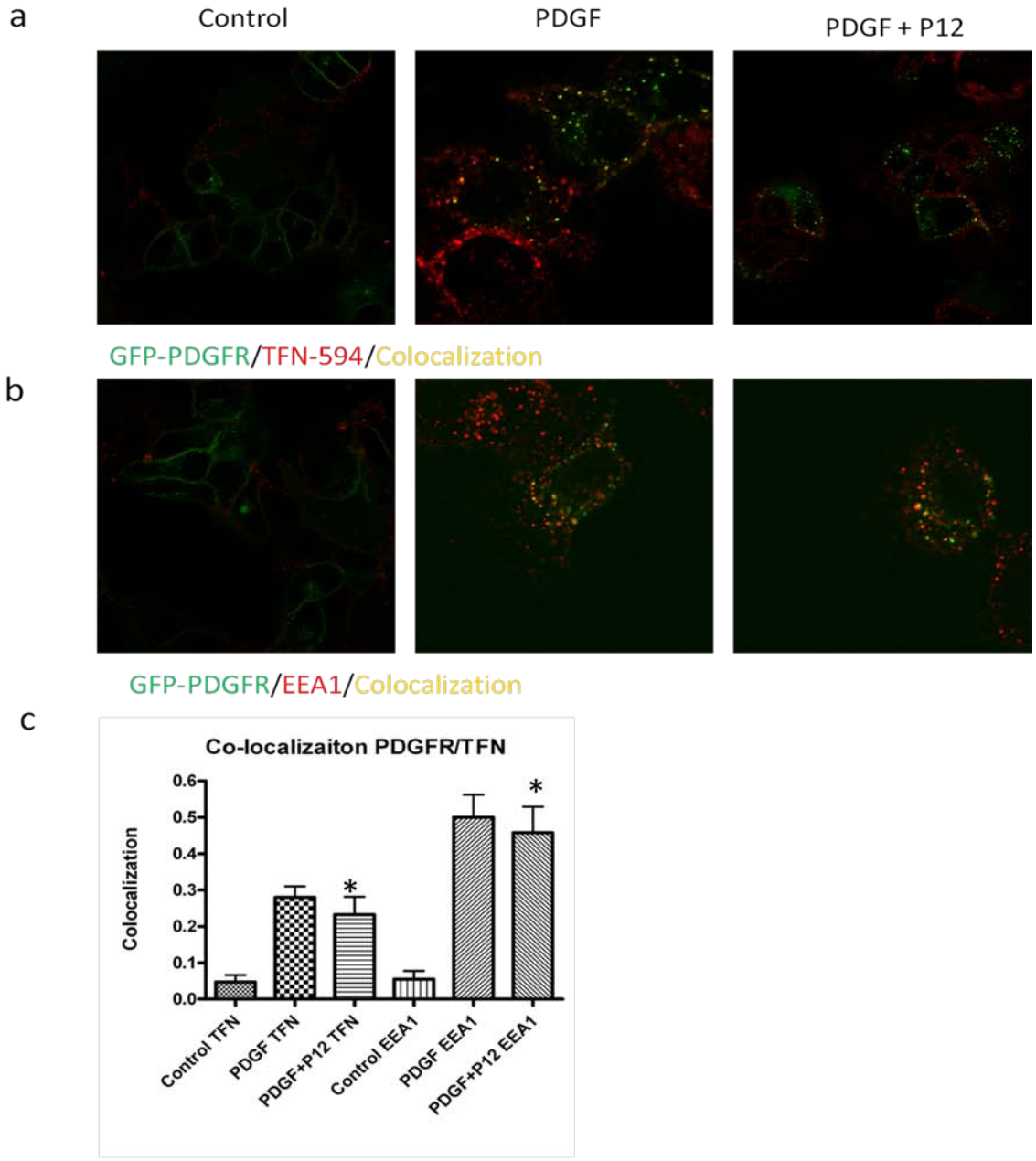
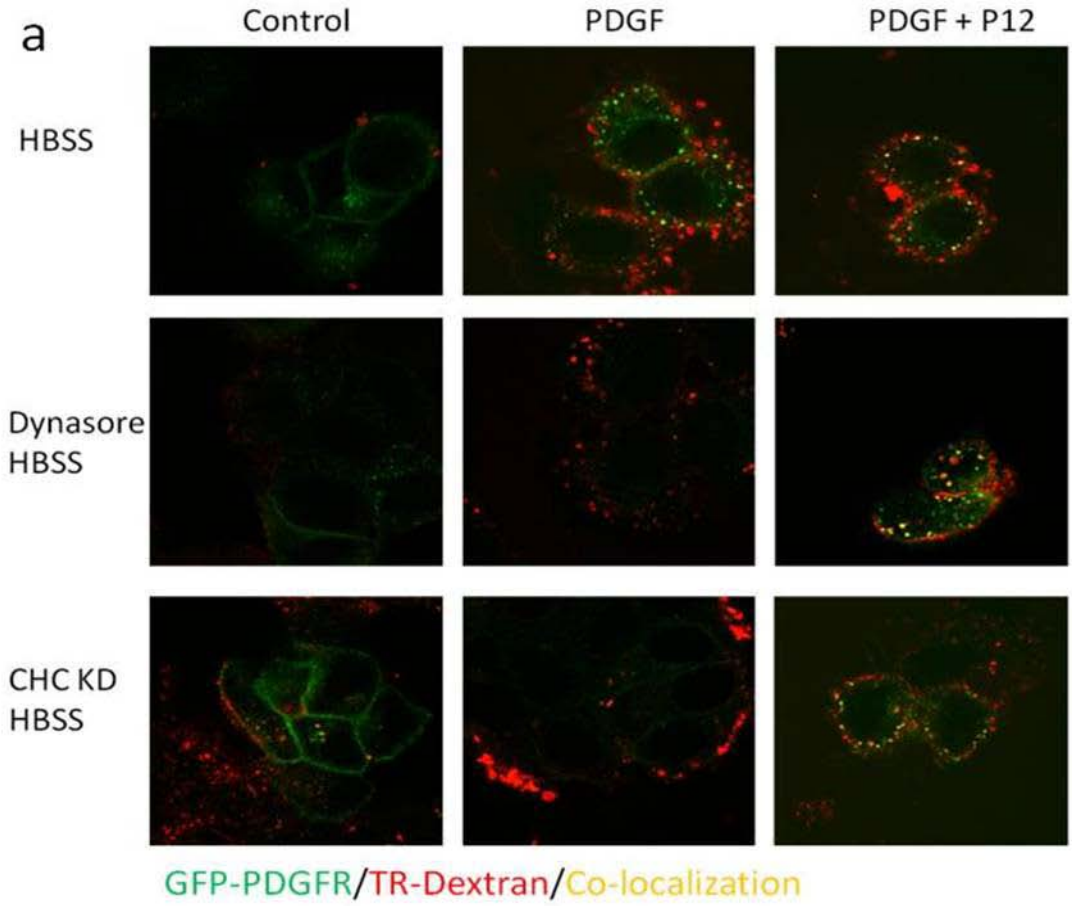


Figure 3.4 P12 redirected the endocytic pathway of PDGFR-β away from CME upon PDGF-BB stimulation. **a**) MCF-7 Cells expressing GFP-PDGFR-β were treated with Alexa Fluor-594 transferrin (TFN-594) and PDGF-BB+/-P12 for 15min in HBSS, and then analyzed by confocal microscope. **b)** MCF-7 Cells expressing GFP-PDGFR-β were treated with PDGF-BB+/-P12 for 30min in HBSS, and then immunostained for EEA-1. **c**) Percentage of co-localization of red and green signal was used to indicate the degree of co-localization between PDGFR-β and TR-dextran (Threshold for both channels were set to 70). More than 50 cells per condition were counted. The bar graph represents the co-localization index, calculated as the number of yellow pixels divided by the number of yellow + green pixels (asterisk, P>0.05 compared to PDGF alone, n=3). Error bar indicates SD.

In order to further study the endocytic pathway that P12 is involved in, I used 70kD dextran-Texas red (TR-dextran) as a macropinosome marker [97]. Macropinosomes were earlier described as vesicles larger than 0.5 μm in diameter. Here, I refer to macropinosome as vesicles positively stained with 70kD dextran-Texas red. In control cells (without PDGF-BB) most of the GFP signal was localized on the cell surface with a uniform distribution (**Figure 3.5**). When PDGF-BB was added, GFP signal was observed in vesicles that did not stain for Texas red. However, P12/PDGF promoted co-localization of PDGFR-β and TR-dextran, consistent with the hypothesis that P12 shifted PDGF entry to a macropinocytosis-like pathway.

To further verify that P12 shifted the endocytic pathway of PDGFR-β, I used dynasore, a small chemical inhibitor of dynamin that blocks clathrin- and caveolae-dependent endocytosis [98]. In the presence of dynasore, PDGF-BB could only stimulate minimal internalization of GFP-PDGFR-β. However, P12 in the presence of PDGF-BB stimulate GFP-PDGFR-β uptake into vesicles that co-localized with TR-dextran (**Figure 3.5**).

Complementary to this, PDGFR-β degradation was also slowed significantly by dynasore, and that P12 partially overcame the dynasore block of receptor degradation (**Figure 3.6**). Also, P12 in the presence of PDGF-BB slowed PDGFR-β degradation compared with PDGF-BB alone. This is compliant with the idea that P12 shifted PDGF-BB/PDGFR-β entry from CME to a slower, macropinocytosis-like pathway.



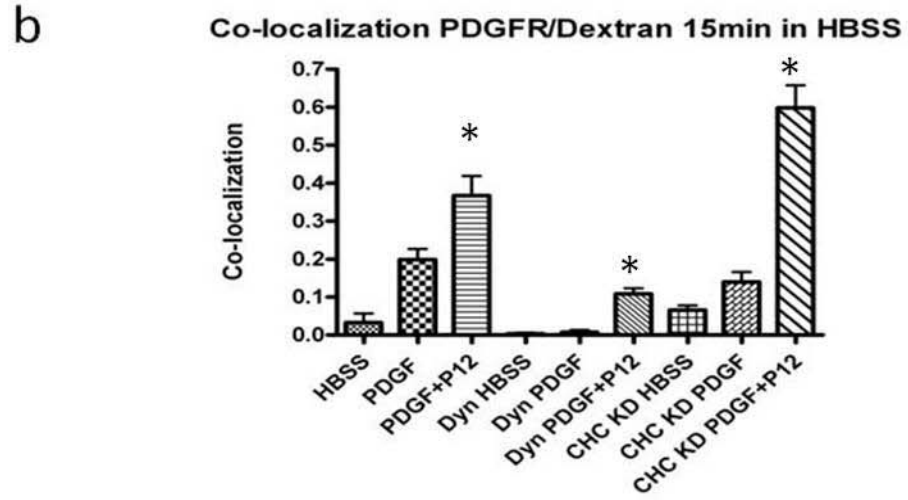


Figure 3.5 P12 increased PDGF-BB/PDGFR-β internalization into macropinosomes. a) MCF-7 cells expressing GFP-PDGFR-β were treated with TR-dextran and PDGF-BB+/-P12 for 15 min in HBSS, and then analyzed by confocal microscope. For dynamin inhibition, cells were pre-incubated with 80 μM dynasore at 37°C for 1 h in serum-free-DMEM. See **Figure S4** for CHC KD methods. **b)** The percentage of co-localization of red and green signal was used to indicate the degree of co-localization between PDGFR-β and TR-dextran (Threshold for both

channels were set to 70). More than 100 cells per condition were counted. The bar graph represents the co-localization index, calculated as the number of yellow pixels divided by the number of yellow + green pixels. Experiments were repeated 3 times (asterisk, $P<0.05$ compared to PDGF alone, n=3). Error bar indicates SD.

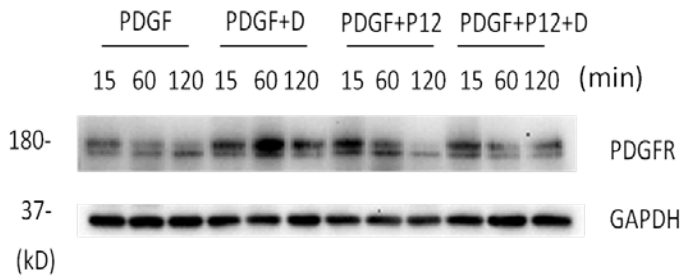


Figure 3.6 P12 slowed PDGFR-β degradation and bypassed dynamin blockage of PDGFR-β internalization/degradation. AHDFs were pre-incubated for 1 hour in DMEM with 80 uM dynasore and then treated with PDGF-BB +/- P12 for the indicated time in HBSS. Western blots on total cell lysate were performed with an antibody against PDGFR-β. GAPDH was used as a loading control. Data is representative of 3 independent experiments.

Besides dynasore, clathrin heavy chain knock-down (CHC KD) blocks clathrin-mediated endocytosis [99]. Therefore, to further define P12/PDGF-BB trafficking into cells I used siRNA to knock down CHC expression in MCF-7 cells (**Figure 3.7**). Indeed, PDGF-BB-stimulated PDGFR-β entry into the cell was significantly suppressed by CHC knockdown, but addition of P12 overcame this block (**Figure 3.5).** Together these results support the contention that P12 shifted PDGF-BB/PDGFR-β entry into cells from clathrin-mediated endocytosis to a slower, macropinocytosis-like pathway.

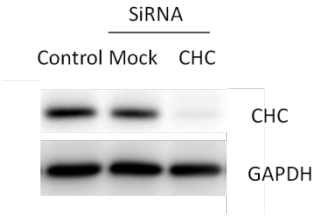


Figure 3.7 Clathrin heavy chain knockdown in MCF-7 cells. CHC siRNA or control siRNA was used to knockdown CHC expression in MCF-7 cells. The protein level dropped significantly after 2 consecutive transfections (36 hours apart) with Hiperfect transfection reagents according to kit instruction.

Previous reports demonstrated that dynasore can block sustained p-Akt and p-ERK1/2 signaling from CSF-1 [100]. In our system (nutrient deprivation) I observed a similar inhibition of PDGF-BB stimulated p-Akt signal in the presence of dynasore. However, addition of P12 bypassed the dynamin blockade and restored PDGF-BB signaling to control levels (**Figure 3.8**).

These results supported the contention that P12 can mediate PDGF signaling that is independent of dynamin function and that a dynamin-independent pathway is likely involved.

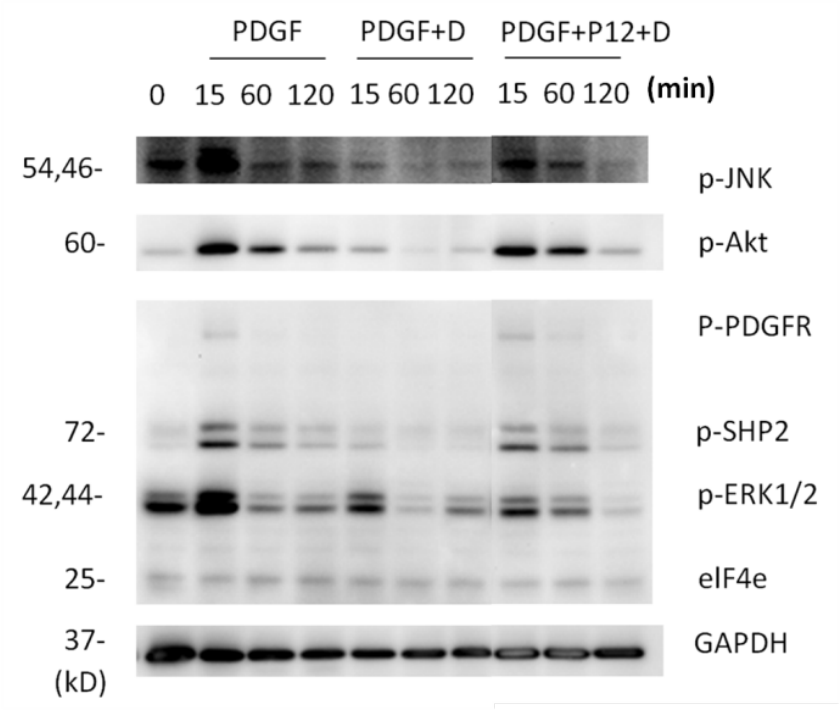


Figure 3.8 P12 bypassed dynamin blockade to activate the Akt/PI3K pathway. AHDFs were put pre-incubated for 1 hour in HBSS with 80uM dynasore and then treated with PDGF-BB +/- P12 in HBSS. Western blots were performed with antibodies against phospho-Akt, phospho-JNK, phospho-ERK1/2, phospho-SHP2 and elF4e. GAPDH was used as loading control. Data is representative of 3 independent experiments.

P12 bypassed clathrin heavy chain knockdown to stimulate cell metabolism.

Clathrin heavy chain (CHC) knockdown has been shown to block clathrin-mediated endocytosis of growth factor receptors [99]. Here I used siRNA to knockdown the CHC in AHDF cells and measured cell metabolism after 3 days of nutrient deprivation (**Figure 3.9a**). Mock transfection had little or no effect on either PDGF- or PDGF/P12-sustained metabolism (**Figure 3.9b,** compared to **Figure 2.2a**). In contrast, PDGF-stimulated cell metabolism dropped in the KD cells almost to control level, while P12 abrogated this effect (**Figure 3.9c**). This is consonant with P12 shifting PDGF-BB trafficking from clathrin-mediated endocytosis to a macropinocytosis-like pathway, thus generating stronger survival signals.

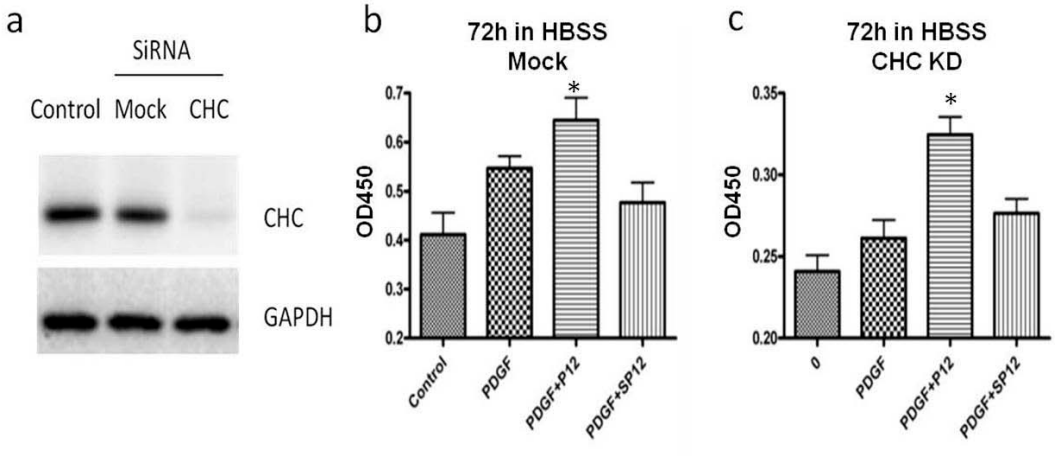


Figure 3.9 P12 rescued PDGF survival activity in CHC knock down cells. a) CHC SiRNA was employed to knockdown CHC expression in AHDF cells. The protein level dropped significantly after 2 consecutive transfections (36 hours apart) with Hiperfect transfection reagents. **b)** Mock cells were treated with PDGF+/-P12 (SP12) in HBSS for 3 days and cell metabolism was measured with XTT assay. Experiment was repeated 3 times (asterisk, $P<0.05$ compared to PDGF alone, n=3). Error bar indicates SD. **c)** CHC knockdown cells were treated with PDGF+/-P12 (SP12) in HBSS for 3 days and cell metabolism was measured with XTT assay. Experiment was repeated 3 times (asterisk, $P<0.05$ compared to PDGF alone, n=3). Error bar indicates SD.

3.5 Discussion

Numerous clinical trials with recombinant growth factors have been largely disappointing. Most growth factors alone have minimal to no activity toward improving wound repair. Interestingly, the trials that demonstrated growth factor benefit all contained a material carrier, suggesting that spatiotemporal control over growth factor bioactivity is crucial to achieve tangible therapeutic effect [17]. We believe that the absence of fibronectin (FN) due to protease degradation is one of the reasons that chronic wounds and burns fail to heal [3, 101, 102]. It is possible that the deficiency in growth factor activity in chronic wounds and burns is partially secondary to the absence of FN growth factor- binding sites. If this is so, administered P12 might increase growth factor bioactivity to a level that would promote wound healing.

Multiple layered interactions between ECM and growth factors have created a rapidly developing and complex field of study. For example, ECM proteins in the cell microenvironment can generate signals through integrins that act in synergy with growth factor signaling [19]. ECM can also bind soluble growth factors and regulate their distribution, activation and presentation to cells [11]. Specifically, growth factor binding-domains on FN have been proposed to function as reservoir for growth factors that provide spatial and temporal regulation for growth factor signaling [12]. Also, proteolytic processing of the ECM proteins generate matrikines that directly activate growth factor receptors [103]. Our study demonstrated for the first time that a growth factor-binding peptide from ECM possibly released by proteolytic processing can regulate growth factor signaling by affecting growth factor endocytosis and

thereby enhance survival signals. This finding adds another layer to the complexity between ECM and growth factor signaling.

During the last decade, there have been many reports on functional protein cargo delivered with CPP [104-106]. One example is the delivery of neuroglobin delivered with CPP protected brain tissue against ischemic stress [107]. However, to date, no CPP has been reported to function on its own through non-covalent interactions with a naturally occurring growth factor. It has been demonstrated that CPP can bring protein cargo into mammalian cells through macropinocytosis [73, 74, 86]. Thus, it is possible that P12, by acting as a cell penetrating peptide, can bind to PDGF-BB/PDGFR-β and shift its entry into macropinocytosis-like endocytosis. By entering the cell through different endocytic pathways, growth factors can generate different signals due to the exposure to unique adaptor proteins and signal relay proteins in different cell compartments [69, 108-110]. Here I show that P12 promotes cell survival by shifting PDGF-BB/PDGFR-β internalization to a dynamin-independent, macropinocytosis-like pathway and thus transiently generates enhanced survival signals to promote AHDF cell survival under stress.

Molecular modeling data has showed a potential binding pocket of P12 is at the interface between PDGF-BB and PDGFR-β (unpublished observations). Thus, as an alternative to the CPP hypothesis, P12 at the interface between PDGF-BB and PDGFR-β could induce conformational change in PDGFR-β and thus affect downstream signaling. To test this possibility, we have initiated a crystallography study to determine whether P12 binding induces conformational change in PDGF-BB/PDGFR-β. Another possibility is that P12 somehow slowed PDGFR-β degradation from dynamin-dependent endocytosis and the slower degradation of PDGFR-β is responsible for the sustained signaling from PDGFR-β. Because the ubiquitination state of PDGFR-β determines the degradation and signal duration from PDGFR-β [111], one of the future studies will be to investigate if the ubiquitination on PDGFR-β is affected by P12 treatment. Regardless of the exact molecular mechanisms at play, our findings here demonstrate a new role for ECM proteins in modulating growth factor signaling and cell survival. In addition, these observations have important implications for tissue engineering and for growth factor treatments of wounds.

Clathrin-mediated endocytosis has been proposed as a negative feedback mechanism to stop constant signaling from activated PDGFR on the cell surface [71]. Upon ligand binding, PDGFR send a signal through plasma membrane to initiate CME, leading to its internalization and subsequent degradation in the lysosome. However, activated PDGFR can also generate signal inside endocytic vesicles [66]. So the pathway and rate of internalization determines the duration of the signal. Macropinocytosis is slower than CME [55]. As a consequence, PDGFR internalized through this pathway can generate prolonged signal in macropinosomes compared to PDGFR internalized through CME [71]. P12, by acting as a cell penetrating peptide shifting the entry pathway of PDGFR (**Figure 3.5**) is likely to cause a change in the downstream signaling of PDGFR.

Chapter 4
Conclusion and Open Questions

4.1 Summary of results

Previously our lab has discovered P12 based on its binding affinity to PDGF-BB (**Figure 2.1**). In AHDF cells, it was found to work together with PDGF-BB to promote cell metabolism and proliferation. Thus, our lab tested its effect in promoting cell survival in vivo and found that it inhibited burn injury progression in a porcine burn injury model. The general hypothesis is that by promoting cell survival under nutrient deprivation and hypoxia, P12 saved AHDF cells under ischemic stress due to peri-burn blood vessel plugging.

To test this hypothesis, I used HBSS as an acute nutrient deprivation system to stress AHDF cells in vitro with PDGF-BB +/- P12 (SP12). I found that in the presence of PDGF-BB, P12 promoted cell metabolism significantly under nutrient deprivation stress. Also, AHDF underwent apoptosis after 3 days of nutrient deprivation stress (**Figure 2.2**). In the presence of PDGF-BB, P12 was able to reduce the amount of apoptosis and bring back normal cell morphology. When those cells were rescued with 10% FBS, I observed a comeback in cell metabolism in P12 treated cells.

Since P12 works together with PDGF-BB to promote cell survival, I next explored the possibility that P12 affected PDGF signaling by analyzing important signal relay molecules in PDGF signaling pathways. I found that in the presence of P12, Akt phosphorylation was prolonged on both collagen and serum coated surfaces. JNK and ERK1/2 phosphorylation was suppressed on serum-coated surface. By immunoprecipitation of PDGFR-β, I found that PDGFR-β phosphorylation at Y751 (docking site for PI3K) was enhanced in the presence of P12 and more P85 subunit of PI3K was recruited to PDGFR-β. This suggested P12 enhanced PDGFR-β signaling and led to enhanced survival signal from PDGF-BB.

Because P12 has significant structural similarity with typical cell penetrating peptides (P12 has 14 amino acids long with 4 positively charged ones). In order to understand the molecular mechanism of P12 activity, I next studied if P12 can act as a CPP. Alexa Fluor-488 was used to label Cys-tagged P12 through a thiol-maleimide reaction. The purified P12-488 was then incubated with AHDF. From cross-section imaging under confocal microscope, I observed that P12-488 penetrated AHDF plasma membrane and localized in endocytic vesicles inside of the cell. Flow cytometry was used to quantify the entry after surface P12-488 was removed with protease digestion. The entry was blocked with low temperature, ATP depletion and partially blocked with surface charge removal. Taken together, the data indicated that P12 indeed acts as a CPP.

Clathrin-mediated endocytosis (CME) is the major route of entry for ligand stimulated GFR [55]. However, CPPs have been shown to facilitate internalization of their binding cargoes through macropinocytosis, a process slower than CME. So in order to see if P12 acted as CPP for PDGF-BB/PDGFR-β complex, I next studied PDGF-BB/PDGFR-β entry. First I labeled PDGF-BB with Alexa Fluor-350 at N-terminal amine with succinimide-ester reaction at pH 6.5. After

the labeling, PDGF-350 activity was compared with native PDGF-BB in stimulating of AHDF metabolism and was found to be intact. The uptake of PDGF-350 was then indirectly measured by fluorescence left in the medium. Compared to PDGF-350 alone, the uptake was slowed in the presence of P12. Similarly, uptake of native PDGF-BB was indirectly measured by ELISA for PDGF-BB left in the medium. Again, the uptake was found to be slowed by P12. Next, PDGFR-β internalization/degradation was analyzed by western blots and immunostain. As expected, PDGFR-β degradation was also slowed by the presence of P12. If P12 inhibited PDGF-BB-PDGFR-β binding, it would also slow down entry. So PDGF-BB interaction with PDGFR-β extracellular domain was analyzed with Biacore experiments and P12 showed no significant effect on PDGF-BB binding to PDGFR-β.

Results from a GFP-PDGFR-β expressing MCF-7 cell line demonstrated that P12 indeed shifted the entry of PDGFR-β from CME into a macropinocytosis-like pathway which is independent on dynamin and clathrin heavy chain. CME of GFP-PDGFR-β was blocked with either dynasore or CHC KD, however, P12 was able to overcome the block and bring GFP-PDGFR-β into vesicles that co-localize with Texas Red-dextran, a macropinosome marker. The endocytic route of PDGF-BB/PDGFR-β effects downstream signaling [69]. Furthermore, PDGF-BB can generate a more robust survival signal through macropinocytosis compared to clathrin-mediated endocytosis [87, 112]. So this shift in entry pathways explained the enhanced survival signal from PDGFR-β.

Importantly, P12 was able to bring back PDGF-BB stimulated Akt phosphorylation that is blocked by a dynamin inhibitor. Also, P12 rescued PDGF-BB stimulated cell metabolism that is suppressed by clathrin heavy chain knockdown after 3 days in nutrient deprivation. Together this data suggested that P12 facilitates PDGF-BB/PDGFR-β entry in a clathrin-independent, macropinocytosis-like pathway (**Figure 4.1**). Thus enhances PDGFR-β signaling and promotes AHDF cell survival under nutrient deprivation.

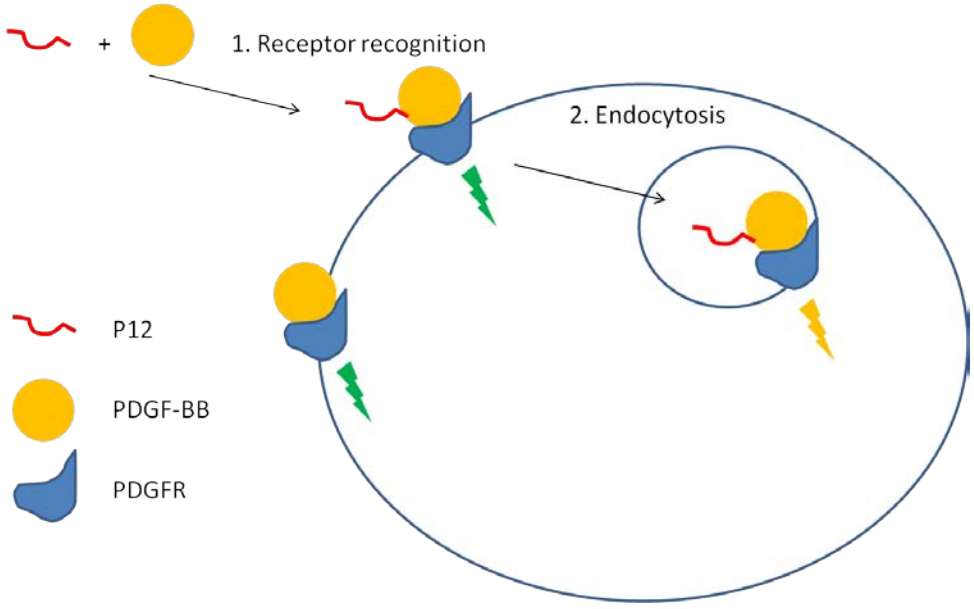


Figure 4.1 P12 acts as a CPP to facilitate PDGF-BB/PDGFR-β entry through a macropinocytosis-like pathway. First, P12 binds to PDGF-BB outside of the cell. Then P12-

PDGF-BB forms a complex with PDGFR-β on the cell surface. Acting as a CPP, P12 initiates macropinocytosis that takes the PDGF-BB/PDGFR-β complex into macropinosomes and generate robust survival signals.

4.2 Open questions

Fibronectin is extremely prone to protease degradation which is prevalent under conditions such as burns and cutaneous wounds. It has been reported that fibronectin generates bioactive FN fragments after proteolytic processing with metalloproteases or plasmin. Theoretical analysis of protease digestion sites indicates that P12 sequence could also be released from FN after exposure to proteases in the wound environment. This may be a naturally existing mechanism for ECM proteins to regulate growth factor activity in the face of adversity. If we can exploit this mechanism and use it to a greater degree, it may provide therapeutic effects that are not achievable with the addition of growth factor alone.

The ubiquitination state of PDGFR-β determines its trafficking and degradation after internalization. One of the questions is that if P12 somehow affected the unbiquitination state of PDGFR-β and then affected its degradation. Another interesting question is P12 effect on PDGF-BB/PDGFR-β signaling complex formation. Because different compartments of the cell contain different sets of signal relay proteins or adaptor proteins, by shifting the endocytic route of PDGFR-β, P12 may change PDGFR-β signaling complex formation. To study this, we will most likely need to employ quantitative proteomics approaches to analyze proteins immunoprecipitated with PDGFR-β with or without P12 treatment.

Biacore experiments showed that P12 binds to different growth factor across growth factor families (**Table 2**). Based on sequence similarity (conserved pattern as judged by PRATT 2.1) among these growth factors, Dr. Fubao Lin has identified a putative P12 binding sequence on PDGF-BB. Peptide synthesized with this sequence can competitively inhibit P12 binding to PDGF-BB. In order to prove that binding to PDGF-BB is important to P12 effect on PDGF-BB signaling, alanine substitution can be performed on this sequence in PDGF-BB to interfere P12 binding to PDGF-BB. If the mutant PDGF-BB retains normal activity and P12 effect is dependent on binding PDGF-BB, then P12 effect should be eliminated in this mutant PDGF-BB.

Growth factors that P12 binds to:

PDGF-BB	(not PDGF-AA)
VEGF-A	
TGF-β1 and TGF-β2	(not BMP 2 or 7)
FGF-2 and FGF-7	(not FGF-1)
IGF-1	(not IGF-2)
NGF	(not EGF or TGF-α)

Table 2. P12 binds growth factors across different families. P12 was fixed to a CM5 chip through amine coupling. Real-time interaction with growth factors was determined by plasmon surface resonance (BiaCore 2000). The chip cell without P12 coupling was used as a reference.

Besides the mechanism proposed here that involves acting as a CPP, P12 could also alter the internalization/activation of PDGFR-β through allosteric structural changes. Molecular modeling data from our collaborator Dr. Wallqvist (Fort Detrick) has demonstrated that the putative docking site of P12 is located at the interface between PDGF-BB and PDGFR-β (**Figure 4.2**). So it is possible that the presence of P12 results in structural changes in its intracellular domain that lead to different endocytic route or enhanced signaling. Our lab has initiated an X-ray crystallography study on PDGFR-β structure changes upon PDGF+/-P12 treatment.

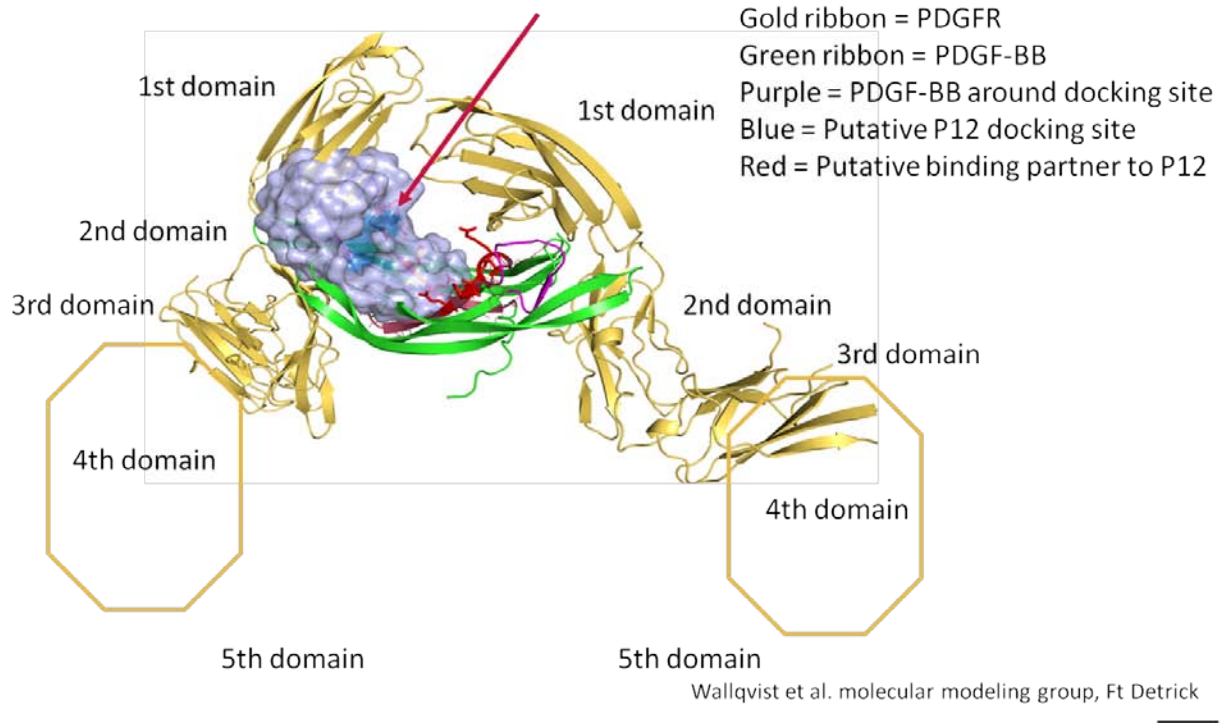


Figure 4.2 Putative docking site of P12 is at the interface between PDGF-BB and PDGFR-β. Molecular modeling was performed with cyclic-P12 to identify its putative docking site on PDGF-BB/PDGFR-β complex. Shown in gold ribbon is PDGFR-β. Green ribbon is PDGF-BB. The purple space filling model indicates the PDGF-BB sequence around P12 docking site. And shown in blue is the P12 docking site. Red ribbon indicates the putative PDGF-BB sequence that is important for P12-PDGF binding.

4.3 Conclusion

Although the commercial market is saturated with cutaneous wound healing products, therapy to limit burn injury progression is an unmet need. In vivo studies using a pig burn injury model showed exciting data supporting P12 efficacy in preventing burn injury progression and P12 has received orphan drug designation from FDA. My study for the first time demonstrated a possible mechanism of action for P12.

All multicellular animals express receptors for growth factors and ECM molecules [15]. Intensive research efforts have revealed that ECM can regulate GF signaling at many levels. The results from my research described a new mechanism how ECM can regulate GF signaling. Namely, an ECM peptide can bind to a GF and change its endocytic trafficking to alter downstream signals. In acute wound environment, increased proteolysis releases many bioactive ECM peptides[23]. My study suggests that these peptides may play an important role in the regulation of GF signaling and the process of wound healing.

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