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Extracellular Ubiquitin Modulates Cardiac Fibroblast Phenotype and Function via Its Interaction with CXCR4

Stephanie L.C. Scofield¹, Christopher R. Daniels¹, Suman Dalal^{1,2}, Jonathan A. Millard¹, Mahipal Singh¹, and Krishna Singh^{1,2,3}

¹Department of Biomedical Sciences, James H Quillen College of Medicine

²Center of Excellence for Inflammation, Infectious Disease and Immunity East Tennessee State University, Johnson City, TN 37614

³James H Quillen Veterans Affairs Medical Center, Mountain Home, TN 37684

Abstract

Aims: β -adrenergic receptor (β -AR) stimulation increases extracellular levels of ubiquitin (UB), and exogenous UB plays an important role in β -AR-stimulated myocardial remodeling with effects on heart function, fibrosis and myocyte apoptosis. Cardiac fibroblasts are vital for maintaining the normal function of the heart, and in the structural remodeling of the heart in response to injury. Here we hypothesized that extracellular UB modulates cardiac fibroblast phenotype and function via its interaction with CXC chemokine receptor type 4 (CXCR4).

Main Methods: Serum starved adult cardiac fibroblasts were used to identify CXCR4 as a receptor for UB. Fluorescent microscopy, co-immunoprecipitation, western blot, proliferation, migration and collagen contraction assays were performed to investigate the role of UB/CXCR4 axis on cell signaling, and modulation of fibroblast phenotype and function.

Key findings: Using fluorescent microscopy and co-immunoprecipitation assay, we provide evidence that extracellular UB interacts with CXCR4. CXCR4 antagonist, AMD3100, inhibited interaction of UB with CXCR4. UB activated ERK1/2, not Akt. It enhanced VEGF-A expression, while decreasing β 3 integrins expression. Two mutated UB proteins (V70A and F4A; unable to interact with CXCR4) failed to affect the expression of VEGF-A and β 3 integrins. UB treatment inhibited migration of cells into the wound and FBS-stimulated cell proliferation. UB enhanced expression of α -smooth muscle actin (marker of myofibroblast differentiation) and contraction of fibroblast-populated collagen gel pads. Most of the effects of UB were negated by AMD3100.

Significance: The data presented here suggest that UB interacts with CXCR4, and UB/CXCR4 interaction affects intracellular signaling, and modulates fibroblast phenotype and function.

Correspondence: Krishna Singh, PhD., FAHA, FAPS, Dept of Biomedical Sciences James H Quillen College of Medicine East Tennessee State University PO Box 70582, Johnson City, TN 37614 Ph: 423-439-2049 Fax: 423-439-2052., singhk@etsu.edu.

Disclosures

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Keywords

Ubiquitin; Fibroblast; CXCR4; Heart

Introduction

Ubiquitin (UB), a highly conserved protein of ~8.5 kDa, is found in all eukaryotic cells. The most important intracellular function of UB is to regulate protein turnover via the ubiquitin-proteasome pathway¹. The ubiquitin-proteasome pathway is suggested to regulate receptor internalization, hypertrophic response, apoptosis, and tolerance to ischemia and reperfusion in cardiac myocytes². UB is a normal constituent of plasma. Elevated levels of UB are described in the serum or plasma of patients with parasitic and allergic diseases³, alcoholic liver disease⁴, type 2 diabetes⁵, β 2-microglobulin amyloidosis⁶ and chronic hemodialysis⁷. Patients with traumatic brain injury are shown to have increased UB levels in the cerebrospinal fluid⁸. Extracellular UB is proposed to have pleiotropic functions including regulation of immune response, anti-inflammatory and neuroprotective activities^{9–12}, as well as regulation of growth and apoptosis in hematopoietic cells¹³. Previously our lab has shown that β -adrenergic receptor (β -AR) stimulation increases extracellular levels of UB, and treatment of adult rat ventricular myocytes with UB inhibits β -AR-stimulated apoptosis¹⁴. *In vivo*, exogenous UB decreased β -AR-stimulated increases in cardiac myocyte apoptosis and myocardial fibrosis¹⁵.

The G protein-coupled receptor CXC chemokine receptor type 4 (CXCR4) is expressed by a variety of cell types, including cardiac fibroblasts and myocytes¹⁶. CXCR4 plays an important role in a variety of biological processes, and is suggested to be involved in the pathophysiology of various disease processes, such as cancer, HIV, ischemic myocardial injury, and angiogenesis¹⁷. Stromal derived factor-1 α (SDF-1 α) is a cognate ligand for CXCR4¹⁸. SDF-1 α /CXCR4 axis is shown to have cardioprotective effects against ischemia/reperfusion injury by promoting anti-apoptotic program¹⁸. Interestingly, CXCR4 is identified as a receptor for UB in THP1 leukemia cell line¹⁹. UB-CXCR4 interaction follows a two-site binding mechanism in which the hydrophobic surfaces surrounding Phe-4 and Val-70 are important for receptor binding, whereas the flexible C terminus facilitates receptor activation²⁰. We have provided evidence that UB, acting via CXCR4 receptor, increases expression of angiogenic molecules and stimulates angiogenesis in cardiac microvascular endothelial cells²¹.

Fibroblasts play a critical role in maintaining the normal function of the heart, and in the remodeling process of the heart that occurs in response to myocardial infarction, hypertension and heart failure²². The objective of this study was to investigate the role of UB/CXCR4 axis in the modulation of fibroblast phenotype and function. The data presented here suggest that extracellular UB interacts with CXCR4, and affects fibroblast phenotype and function, at least in part, via the involvement of CXCR4.

Materials and Methods

Vertebrate animals:

All experiments and procedures were reviewed and approved by the East Tennessee State University Committee on Animal Care. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Male Sprague-Dawley rats (average weight: 200–225g; Harlan, Indianapolis, IN) were used for isolation of resident cardiac fibroblast cells. Rats were anesthetized using a mixture of isoflurane (2.5%) and oxygen (0.5 l/min), and the heart was excised following a bilateral cut in the diaphragm.

Fibroblast isolation and treatment:

Adult rat cardiac fibroblasts were isolated as described^{15, 23}. The cells were grown to confluence and serum-starved for 48 h before use. Experiments were performed using first and second passage cells. The cells were pretreated with AMD3100 (AMD; 10 μ M; Sigma) or PD98059 (PD; 15 μ M; Calbiochem) for 30 min followed by treatment with UB (10 μ g/ml; Sigma), biotin-UB (bUB; 500 ng/ml; Boston Biochem), or mutated UB proteins (V70A and F4A; 10 μ g/ml; Boston Biochem) for indicated time points. In mutated UB proteins, Val-70 (V70A) and Phe-4 (F4A) are mutated to Ala. To investigate cellular interaction of UB, cells were treated with FITC-UB (500 ng/ml; Boston Biochem) or FITC-labeled secondary antibodies (negative control) for 30 min. The concentration of UB (10 μ g/ml; Sigma) is selected based on our previously published report in adult rat cardiac myocytes¹⁴, where we observed that lower concentrations (0.01, 0.1, and 1 μ g/ml) partially inhibit β -AR-stimulated myocyte apoptosis. However, a concentration of 10 μ g/ml almost completely inhibits β -AR-stimulated apoptosis. Normal human serum is described to have UB concentrations ranging from 30 ng/mL to 120 ng/mL. Pathological conditions increase serum UB concentrations. Burn injury in humans associates with increased serum UB levels of ~800 ng/mL¹²

Co-immunoprecipitation assay:

Serum-starved cells were treated with bUB for 30 min. Total cell lysates (300 μ g proteins) prepared in TNT buffer (20 mM TrisHCl, pH 7.5, 137 mM NaCl, 20 mM NaF, 5 mM EDTA, 1 mM PMSF, 10 mM sodium pyrophosphate, 0.2 M sodium orthovanadate, 8 μ g/ml aprotinin and 2 μ g/ml leupeptin, digitonin 0.05%, and 1% Triton X-100) were incubated overnight at 4°C with 3 μ g of anti-CXCR4 antibody (Abcam). Protein A/G beads (60 μ g, Thermo Scientific, Rockford, IL) were then added to the mixture and incubated for an additional hour. The beads were washed six times with TNT buffer and resuspended in sample buffer. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were then incubated overnight in extra-avidin peroxidase (Sigma; 1:2000 dilution in TBST) and chemiluminescence reagents to detect bUB. The membranes were also probed with anti-CXCR4 antibodies.

Western blot analysis:

Cell lysates (20 µg), prepared using lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 1% Triton X-100, and 1 mM PMSF], were resolved by 10% SDS-PAGE. The proteins were transferred onto PVDF membrane. The membranes were probed with primary antibodies directed against p-Akt (1:1000; Cell Signaling Tech.), p-ERK1/2 (1:2000; Cell Signaling Tech.), β3-integrin (1:1000; Santa Cruz), VEGF-A (1:200; Santa Cruz), or α-SMA (1:20,000; Sigma). The membranes were then stripped and probed with Akt, ERK1/2, actin (Chemicon) or GAPDH (Santa Cruz) antibodies to normalize protein loading. Band intensities were quantified using Kodak photo documentation system (Eastman Kodak Co.).

Proliferation assay:

An equal number of fibroblasts (3×10^4) were plated on p60 culture dishes containing glass coverslips and incubated in DMEM supplemented with 10% fetal bovine serum (FBS) for 24 h at 37°C. The cells were then serum starved for 24 h (0 h control) followed by treatment with UB (10 µg/ml) in the presence or absence of FBS (1%) for 24 h. The cells were fixed using methanol (−20°C) for 10 min followed by washing with PBS. The cells were then incubated in blocking solution (5% BSA in PBS) for 1 h followed by incubation with anti-Ki-67 antibodies (Abcam) at 1:100 dilution overnight at 4°C. After washing, the cells were incubated with FITC- labeled secondary antibodies for 1 h at room temperature in the dark. The cells were then washed and stained with Hoechst 33342 (10 µM; nuclear stain). After washing and mounting, the cells were visualized using a fluorescent microscope (EVOS). Images were acquired using Life Technologies EVOS FL Auto microscope. A cell was determined to be actively proliferating if FITC-Ki-67 positive staining co-localized with Hoechst 33342 staining. The percentage of Ki-67-positive cells relative to the total number of nuclei (Hoechst positive) was determined by counting five randomly chosen fields per coverslip. The percentages of proliferating cells were normalized to 0 h control and are expressed as fold-change vs the 0 h control.

Migration assay:

Movement of cells through a wound introduced in a cell monolayer was measured as described²¹. Briefly, fibroblasts were grown as a confluent monolayer. After serum starvation for 48 h, a wound was created in the center of the cell monolayer by gentle removal of attached cells using a sterile plastic pipette tip. Cell debris was removed by a PBS wash and images of the wound were acquired using a Nikon TE-2000 microscope equipped with a Retiga-1300 color cooled camera (0 h). The cells were then incubated in serum-free DMEM containing UB with or without AMD for 24 and 48 h. Images were again acquired 24 and 48 h after treatment. The wound area was measured using Bioquant Image Analysis software (Bioquant Image Analysis Corp., Nashville, TN). The ability of cells to migrate into the wound area was assessed by comparing micrographs at time 0, 24 and 48 h along the wounded area. The percentage of recovered wound area was calculated by dividing the wound area after 24 h or 48 h by the initial wound area at time 0 multiplied by 100.

Collagen gel pad contraction assay:

Collagen gel pad contraction assay was performed as described²⁴ Briefly, serum starved fibroblasts were mixed with collagen solution (1 mg/ml) to achieve the final cell count of 3×10^5 cells/ml. 500 μ l of this suspension was aliquoted into a 24 well culture plate and allowed to polymerize at 37°C for 30 min. The gels were released from wells, transferred to p60 culture dishes, floated in DMEM and incubated at 37°C for 24 h. Fibroblast containing gels were treated with UB (10 μ g/ml) or AMD (10 μ M) for 24 h. For AMD+UB, AMD was added 30 min prior to UB treatment. TGF- β 1 (10 nM) treatment was used as a positive control. Images were acquired at 0 h and 24 h to assess the degree of gel contraction. The area of each collagen gel pad was measured using NIS elements software (Nikon).

Statistical analysis:

Data are expressed as the mean $\pm$ SEM. Data was analyzed using Student's *t* test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Probability (P) values of <0.05 were considered to be significant.

Results

UB interacts with CXCR4:

To investigate if extracellular UB interacts with fibroblast, non-permeabilized cells were treated with FITC-UB for 30 min. As a negative control, cells were incubated with FITC-labeled secondary antibodies (1:500) for 30 min. Visualization of cells using fluorescent microscope showed clear staining of cells with FITC-UB. No staining was observed in control cells or cells incubated with FITC-labeled secondary antibodies (Fig 1A).

CXCR4 is identified as a receptor for UB in THP-1 cells¹⁹ To show interaction of UB with CXCR4, cells were pretreated with AMD for 30 min followed by incubation with bUB for 15 min, 3 h and 24 h. Western blot analysis of cell lysates using extra-avidin peroxidase showed clear presence of bUB in the cell lysates at all time-points. Pretreatment with AMD interfered with the presence of bUB at all different time-points (Fig 1B). Co-immunoprecipitation is considered as a standard method to determine if two proteins of interest form a complex. To confirm interaction of UB with CXCR4 receptor, fibroblasts were treated with bUB for 30 min. Cell lysates were immunoprecipitated with anti-CXCR4 antibody. The resultant immunoprecipitates were analyzed by western blots using extra-avidin peroxidase to detect bUB. This analysis showed the presence of bUB in both samples immunoprecipitated with anti-CXCR4 antibodies (Fig 1C).

UB/CXCR4 interaction affects activation of ERK1/2, and expression of β 3 integrin and VEGF-A:

A variety of growth factors activate ERK1/2²⁵. UB treatment is also shown to activate ERK1/2 in THP-1 cells²⁶. To analyze the activation ERK1/2 in cardiac fibroblasts and the involvement of CXCR4 receptors in UB signaling, cells were pretreated with AMD or PD (a selective inhibitor of ERK1/2 pathway) for 30 min followed by treatment with UB for 15 min. Western blot analysis of cell lysates using phospho-specific anti-ERK1/2 antibodies showed increased phosphorylation (activation) of ERK1/2 in response to UB. PD completely

inhibited UB- mediated ERK1/2 activation, while AMD partially inhibited UB-stimulated increase in ERK1/2 activation (CTL, 1 ± 0.01 ; UB, $4.73 \pm 0.8^*$; AMD+UB, $2.4 \pm 0.8^{*\$}$; PD+UB 0.05 ± 0.02 ; AMD, 0.93 ± 0.38 ; * $P < 0.05$ vs CTL; $^{\$}P < 0.05$ vs UB; $n = 5$; Fig 2A).

Previously, we have provided evidence that inhibition of PI3-kinase inhibits the protective effects of UB in β -AR-stimulated apoptosis in adult rat ventricular myocytes¹⁴ PI3-kinase acts upstream in the activation of Akt, and activation of Akt is generally considered as an anti-apoptotic signal²⁷ Western blot analysis of cell lysates using phospho-specific anti-Akt antibodies showed no significant change in Akt phosphorylation in response to UB in the presence or absence of AMD (Fig 2B).

VEGF-A is suggested to play a role in tissue repair²⁸. Extracellular UB increases VEGF-A expression in microvascular endothelial cells²¹. Western blot analysis of cell lysates using anti- VEGF-A antibodies showed a significant increase in the expression of VEGF-A in UB-treated (24 h) samples compared to CTL (Fig 3A). AMD alone had no effect on VEGF-A expression. However, it almost completely inhibited UB-mediated increase in VEGF-A expression (CTL, 1.00 ± 0.01 ; UB, 3.22 ± 0.6 ; AMD+UB, 1.49 ± 0.3 , AMD, 1.15 ± 0.1 ; * $P < 0.05$ vs CTL; $n = 5$; Fig 3A). The UB/CXCR4 interaction is suggested to involve the hydrophobic surfaces surrounding UB residues Phe-4 and Val-70²⁰. To investigate the role these amino acid residues in UB signaling, cells were treated with two different mutated UBs proteins; V70A and F4A. Western blot analyses of cell lysates showed no effect of mutated UBs on VEGF-A protein levels.

Integrins, transmembrane receptors, are crucial for providing linkages between extracellular matrix (ECM) proteins and the actin cytoskeleton, thereby providing adhesion of cells to the substratum and playing a crucial role in cell migration^{29, 30}. Cardiac fibroblasts predominantly express $\beta 3$ integrins³¹. Western blot analysis of cell lysates using anti- $\beta 3$ -integrin antibodies showed that UB treatment significantly decreases $\beta 3$ integrin protein levels (Fig 3B). AMD alone had no effect, however, pretreatment with AMD almost completely reversed UB-mediated decrease in $\beta 3$ integrin protein levels (CTL, 1.00 ± 0.01 ; UB, $0.45 \pm 0.09^*$; AMD+UB, 0.99 ± 0.22 ; AMD, 0.97 ± 0.18 ; * $P < 0.05$ vs CTL; $n = 5$; Fig 3B). Treatment with mutated UB (V70A and F4A) had no effect of $\beta 3$ integrin protein levels (Fig 3B).

UB treatment affects cell migration and proliferation:

Fibroblast migration is an essential early event in wound healing and deposition of fibrosis³². To examine the effect of UB on cell migration, we used an *in vitro* migration (scratch) assay. Migratory potential of fibroblasts into the wound was clearly present in the untreated cells as observed by the decrease in the percent wound area recovered 24 and 48 h after incubation. Interestingly, UB treatment significantly inhibited the percent wound area recovered at both time points (Fig 4). AMD pretreatment negated the effects of UB on cell migration at both time points (% wound area recovered; 24 h; CTL, 45 ± 3 ; UB, $14 \pm 4^*$; AMD +UB, $34 \pm 6^{\$}$; AMD, 47 ± 3 ; 48 h; CTL, 64 ± 2 ; UB, $35 \pm 6^*$; AMD+UB, $59 \pm 5^{\$}$; AMD, 63 ± 5 ; * $P < 0.05$ vs CTL; $^{\$}P < 0.05$ vs UB; $n = 5$; Fig 4B & C). AMD alone had no effect on the migration of cells into the wound.

Ki-67 is found in the nucleus of proliferating cells and is commonly used as an index of proliferation³³. It is undetectable during G0 phase, but peaks during the G2/mitosis phase³⁴. Treatment of cells with UB alone for 24 h had no effect on the percentage of Ki-67-positive cells versus control. FBS significantly increased the number of Ki-67-positive cells versus control. UB treatment significantly reduced FBS-stimulated increase in the number of Ki-67-positive cells (fold change vs 0 h control; CTL, 1.63 ± 0.13 ; FBS, $3.76 \pm 0.85^*$; FBS + UB, $1.56 \pm 0.16^{\$}$; UB, 1.31 ± 0.25 ; * $p < 0.05$ vs CTL; $^{\$}p < 0.05$ vs FBS; $n = 3-4$; Fig 5).

UB treatment increases α -SMA expression and contraction of fibroblast-populated collagen gel pads:

Expression of α -SMA serves as a marker of myofibroblasts³⁵. To investigate if extracellular UB affects α -SMA expression, serum-starved fibroblasts were treated with UB for 48 h. Western blot analysis of cell lysates showed a significant increase in α -SMA expression in UB-treated cells vs CTL (Fig 6A). Collagen gel contraction assay is frequently used to evaluate the ability of fibroblasts to reorganize and contract the collagen matrix *in vitro*³⁶. To investigate the role of UB in this process, collagen gel contraction assay was performed using a protocol received from Dr Frangogiannis (Albert Einstein College of Medicine, Bronx, NY)²⁴. This analysis showed that UB significantly increases the contraction of fibroblast-populated collagen pads, an observation similar to that of TGF- β 1 (used as positive control). AMD alone had no effect on collagen gel contraction. However, it significantly reduced UB-mediated increase in collagen gel pad contraction (Fig 6B).

Discussion

Cardiac fibroblasts play a critical role in maintaining normal function of the heart, and in cardiac remodeling during pathological conditions²². A major finding of this study is that UB modulates intracellular signaling, phenotype and function of fibroblasts via the involvement of CXCR4. The data presented here demonstrate that UB interacts with CXCR4. UB/CXCR4 interaction activates intracellular signaling, and affects expression of β 3 integrins and VEGF-A. It inhibits the migration of cells into the wound and FBS-stimulated cell proliferation, while enhancing α -SMA expression and contraction of fibroblast populated collagen gel pads.

CXCR4 (a cognate receptor for stromal derived factor-1 α ; SDF-1 α) is identified as a receptor for extracellular UB in THP1 leukemia cells¹⁹. Although both UB and SDF-1 α can signal via CXCR4, important differences have been identified in their receptor binding and signaling. For example, SDF-1 α not UB, binds to CXCR7²⁶. Binding affinity of SDF-1 α with CXCR7 is 10-fold higher than with CXCR4³⁷. Interaction of UB with CXCR4 occurs independent of the N-terminus of CXCR4 and does not follow the typical two-site binding mechanism of chemokine-receptor²⁶. UB/CXCR4 interaction differentially affects receptor desensitization³⁸ and exhibits weaker chemotactic activity (vs SDF-1 α /CXCR4) in cell migration assay and does not reduce HIV-1 infectivity^{17, 19}. CXCR4 amino acid residues (Phe-29, Phe-189, Lys-271) play an important role in UB/CXCR4 interaction. However, these residues do not contribute to the binding of SDF-1 α with CXCR4²⁰. Thus, UB contains separate receptor docking and activation sites, and signals through CXCR4 via a

unique binding mechanism. Myocytes and non-myocyte cell populations of the heart express CXCR4³⁹. Previously, we provided evidence for CXCR4 as a receptor for extracellular UB in microvascular endothelial cells²¹. Here, we provide evidence for CXCR4 as a potential receptor for UB in cardiac fibroblasts. CXCR4 antagonist, AMD3100, decreased levels of bUB in the cell lysates. bUB also co-immunoprecipitated with CXCR4. Other signaling, phenotypic and functional parameters were also negated by AMD treatment. Use of mutated UB proteins (F4A and V70A) further supports the involvement of CXCR4 in UB signaling. It is interesting to note that CXCR4 antagonist, AMD3100, almost completely inhibits UB-mediated changes in the expression of VEGF-A and $\beta 3$ integrin. However, inhibition of ERK1/2 was partial. In THP-1 cells, UB is shown to induce intracellular signaling, including phosphorylation of ERK1/2 and Akt²⁶. In adult rat ventricular myocytes, UB alone had no effect on the activation of JNKs and glycogen synthase kinase-3 β (GSK-3 β)¹⁴. The reasons for this differential response may reflect cell-type specific differences and/or differences in binding affinities of AMD and UB. Furthermore, CXCR4 can act as a homodimer or heterodimer with CXCR7⁴⁰, $\beta 2$ -adrenergic receptor ($\beta 2$ -AR)⁴¹ or $\alpha 1A$ -AR^{42, 43}. Future investigations are needed to clarify the role of these other receptors in UB signaling.

Fibroblasts are capable of modulating their microenvironment through autocrine and paracrine signaling, thereby playing a role in tissue remodeling following tissue injury. Fibroblasts are also shown to play a role in the angiogenic process^{44, 45}. Fibroblasts secrete growth factors, including VEGF-A⁴⁶. VEGF-A is considered as a potent angiogenic inducer. In this study, we found that UB treatment increases VEGF-A expression. Increased expression of VEGF-A is consistent with our previous finding in microvascular endothelial cells where UB treatment increased VEGF-A expression²¹. Increased expression of VEGF-A may act as a paracrine factor for cardiac microvascular endothelial cells to induce angiogenesis.

Integrins are cell surface receptors that play a role in cell adhesion and migration, as well as in growth and survival^{30, 47}. Cardiac fibroblasts express $\beta 3$ integrin⁴⁸. $\beta 3$ integrin is shown to play a critical role in cardiac fibroblasts adhesion, migration, proliferation and extracellular matrix production since $\beta 3$ integrin null cardiac fibroblast exhibit a significant reduction in these processes³¹. Here, UB treatment decreased $\beta 3$ integrin protein levels and inhibited fibroblast migration into the wound area. Therefore, it is possible that the UB-mediated decrease in $\beta 3$ integrin may play a role in decreased cell migration in response to UB.

Activated fibroblasts play a critical role in both the reparative and fibrotic processes. Compromising the ECM composition after large injuries such as myocardial infarction (MI) destabilizes the heart and predisposes it to cardiac rupture^{49, 50}. Since fibroblasts play an integral role in composition of the ECM and collagen-based scar formation, it is likely that inhibition of fibroblast proliferation would predispose the heart to cardiac rupture post-MI. Conversely, rapid proliferation of fibroblasts and subsequent deposition of extracellular matrix post-MI also associates with adverse effects on cardiac structure and function⁵¹. Therefore, a well-controlled balance between fibroblast proliferation and ECM deposition is integral in the maintenance of ECM homeostasis. Here, we observed that UB significantly inhibits FBS-mediated increase in proliferation of cardiac fibroblasts. UB alone had no

effect on fibroblast proliferation. In KT-3 and HL-60 cells, UB is shown to inhibit cell growth via selective degradation of the STAT3 transcription factor¹³. Previously, our lab has provided evidence that UB inhibits β -AR stimulated increase in cardiac fibrosis¹⁵. It also enhanced expression of matrix metalloproteinases (MMP-2 and MMP-9) in the heart and cardiac fibroblasts¹⁵. Together, these data suggest that UB has the potential to decrease myocardial fibrosis by modulating expression of MMPs and/or fibroblast proliferation.

In normal tissues, resident fibroblasts are quiescent, producing limited amounts of ECM proteins and exhibiting few actin-associated cell-matrix and cell-cell contacts⁵². Tissue injury induces alterations in the microenvironment, resulting in differentiation of fibroblasts into myofibroblasts. Myofibroblasts are characterized by the presence of microfilamentous contractile apparatus enriched in α -SMA fibers⁵³. Myofibroblasts have the ability to contract to close wounds effectively because of their α -SMA reinforced actin structure⁵⁴. Once the myofibroblasts adhere to multiple points in the wounded or stressed area, the α -SMA reinforced fibers pull the adherence points closer together generating wound contracture. This effect is mirrored in the collagen gel contraction assays *in vitro*⁵⁵. In hepatic stellate cells, stimulation of SDF-1 α /CXCR4 axis is shown to promote the contraction of collagen gels, which was inhibited by pretreatment with AMD⁵⁶. Here UB enhanced expression of α -SMA, a hallmark of differentiated fibroblasts into myofibroblasts. It also increased the contraction of fibroblast-populated collagen pads. AMD negated the effect of UB on collagen pad contraction. These data suggest a role for the UB/CXCR4 axis in modulation of fibroblast function.

Reduced cell migration and proliferation in response to UB treatment suggest an anti-fibrotic role for UB, while the apparent increase in α -SMA expression is indicative of a pro-fibrotic role. Deposition of collagen-rich ECM associates with α -SMA-positive myofibroblasts⁵⁷. However, caution should be exercised while interpreting the results obtained using cardiac fibroblasts *in vitro*. Although *in vitro* cell culture provides a useful tool to understand cell biology, behavior of fibroblasts may vary depending on their microenvironment and culture conditions. Of note, overexpression of α -SMA is shown to attenuate serum-stimulated fibroblast proliferation²⁴.

Conclusion

Cardiac fibroblasts are important in maintaining the normal function of the heart as well as in wound repair following injury to the heart. UB is found in normal plasma. A variety of disease states increase levels of UB in the plasma. Here we provide evidence that UB modulates cardiac fibroblast function and phenotype via the involvement of CXCR4. Further investigation of mechanisms leading to UB-mediated regulation of cardiac fibroblast function and phenotype may help uncover novel strategies to improve cardiac remodeling and function following pathological stress.

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Figure 1. Extracellular UB interacts with CXCR4:

A. Cardiac fibroblasts were incubated with FITC-UB (500 ng/ml) or FITC-labeled secondary antibodies (CTL-FITC) for 30 min. The cells were visualized by fluorescent microscope and photographed. CTL, control. **B.** Cells were pretreated with AMD3100 (AMD; 10 μ M) for 30 min followed by treatment with b-UB for 15 min, 3 h or 24 h. Cell lysates were analyzed by western blot using extra-avidin peroxidase. **C.** Fibroblasts were treated with biotin-labeled UB (b-UB; 10 μ g/ml) for 30 min. Cell lysates were immunoprecipitated with anti-CXCR4 antibodies. Immunoprecipitates were analyzed by

western blot using extra-avidin peroxidase to detect bUB and anti-CXCR4 antibodies to detect CXCR4.

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Figure 2. UB treatment activates ERK1/2, not Akt:

Serum starved confluent cultures of fibroblasts were pretreated with AMD3100 (AMD), or PD98059 (PD) for 30 min followed by treatment with UB for 15 min. Cell lysates were analyzed by western blot using phospho-specific ERK1/2 (A) or Akt (serine-473; B) antibodies. The lower panels exhibit mean data normalized to total ERK1/2 or Akt; * $P < 0.05$ versus CTL, $^{\$}P < 0.05$ versus UB, $n = 4-6$.

Figure 3. UB treatment affects protein levels of VEGF-A and $\beta 3$ integrin:

Serum starved confluent cultures of fibroblasts were pretreated with AMD for 30 min followed by treatment with UB (10 μ g/ml) for 24 h. The cells were also treated with mutated UBs proteins (V70A and F4A) for 24 h. Cell lysates were analyzed by western blot using anti-VEGF-A (A) or anti $\beta 3$ -integrin (B) antibodies. The lower panels exhibit the mean data normalized to actin. * $P < 0.05$ versus CTL; \$ $P < 0.05$ versus UB; $n = 6-7$.

Figure 4. UB treatment inhibits migration of fibroblast into the wound:

Confluent fibroblast monolayers were wounded using a sterile pipette tip and pretreated with AMD for 30 min followed by treatment with UB for 24 or 48 h. The ability of cells to migrate into the wound was assessed following 24 or 48 h after UB treatment. Panel A demonstrates the wound area at 0 and 48 h after treatment. Wound healing was calculated as percentage of recovered wound area. Panel B and C represent the mean data of percent wound area recovered 24 and 48 h after UB treatment; * $P < 0.05$ versus CTL; \$ $P < 0.05$ versus UB; $n = 5$.

Figure 5. UB treatment inhibits FBS-stimulated cell proliferation:

Fibroblasts were cultured on glass coverslips for 24 h. Serum-starved cells were then treated with FBS (1%) in the presence or absence of UB for 24 h. The cells were immunostained using anti-Ki-67 antibodies. Nuclei were stained using Hoechst 33258. The cells were visualized using fluorescent microscope. The percentage of Ki-67 positive cells (green fluorescence) versus the total number of nuclei (blue; upper panels) was counted in five randomly chosen fields. The lower panel depicts cell proliferation as fold change versus the 0 h control. *P<0.05 versus CTL; \$P<0.05 versus FBS; n = 3–4.

Figure 6.

A. UB treatment increases α -SMA expression: Serum starved confluent cultures of fibroblasts were treated with UB for 48 h. Cell lysates were analyzed by western blot using anti- α -SMA antibodies. The lower panel exhibits the mean data normalized with GAPDH. * $P < 0.05$ vs control (CTL); $n = 5$. **B. UB treatment enhances contraction of fibroblast-populated collagen gel pads:** Collagen gel pads were populated with fibroblasts and pretreated with AMD3100 (AMD) for 30 min followed by treatment with UB for 24 h. Treatment with TGF- β 1 served as a positive control. Upper panel depicts collagen gel pads

24 h after treatments. Lower panel depicts quantitative analysis of fibroblast-populated collagen gel pad contraction. *P<0.05 versus CTL; \$P<0.05 versus UB; n = 4–6.

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