Circulation

ORIGINAL RESEARCH ARTICLE

Inhibiting Fibronectin Attenuates Fibrosis and Improves Cardiac Function in a Model of Heart Failure

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BACKGROUND: Fibronectin (FN) polymerization is necessary for collagen matrix deposition and is a key contributor to increased abundance of cardiac myofibroblasts (MFs) after cardiac injury. We hypothesized that interfering with FN polymerization or its genetic ablation in fibroblasts would attenuate MF and fibrosis and improve cardiac function after ischemia/reperfusion (I/R) injury.

METHODS: Mouse and human MFs were used to assess the impact of the FN polymerization inhibitor (pUR4) in attenuating pathological cellular features such as proliferation, migration, extracellular matrix deposition, and associated mechanisms. To evaluate the therapeutic potential of inhibiting FN polymerization in vivo, wild-type mice received daily intraperitoneal injections of either pUR4 or control peptide (III-11C) immediately after cardiac surgery for 7 consecutive days. Mice were analyzed 7 days after I/R to assess MF markers and inflammatory cell infiltration or 4 weeks after I/R to evaluate long-term effects of FN inhibition on cardiac function and fibrosis. Furthermore, inducible, fibroblast-restricted, FN gene–ablated (Tcf21^{MerCreMer};*Fn*^{flox}) mice were used to evaluate cell specificity of FN expression and polymerization in the heart.

RESULTS: pUR4 administration on activated MFs reduced FN and collagen deposition into the extracellular matrix and attenuated cell proliferation, likely mediated through decreased c-myc signaling. pUR4 also ameliorated fibroblast migration accompanied by increased β1 integrin internalization and reduced levels of phosphorylated focal adhesion kinase protein. In vivo, daily administration of pUR4 for 7 days after I/R significantly reduced MF markers and neutrophil infiltration. This treatment regimen also significantly attenuated myocardial dysfunction, pathological cardiac remodeling, and fibrosis up to 4 weeks after I/R. Last, inducible ablation of FN in fibroblasts after I/R resulted in significant functional cardioprotection with reduced hypertrophy and fibrosis. The addition of pUR4 to the FN-ablated mice did not confer further cardioprotection, suggesting that the salutary effects of inhibiting FN polymerization may be mediated largely through effects on FN secreted from the cardiac fibroblast lineage.

CONCLUSIONS: Inhibiting FN polymerization or cardiac fibroblast gene expression attenuates pathological properties of MFs in vitro and ameliorates adverse cardiac remodeling and fibrosis in an in vivo model of heart failure. Interfering with FN polymerization may be a new therapeutic strategy for treating cardiac fibrosis and heart failure.

Key Words: extracellular matrix ■ fibroblasts ■ fibronectins ■ fibrosis ■ heart failure

Sources of Funding, see page 1250

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Clinical Perspective

What Is New?

- The fibronectin (FN) polymerization inhibitor pUR4 attenuates the pathological phenotype exhibited by mouse and human myofibroblasts by decreasing FN polymerization and collagen deposition into the extracellular matrix, as well as myofibroblast proliferation and migration.
- Inhibiting FN matrix deposition by pUR4 treatment or deleting FN gene expression in cardiac fibroblasts (Tcf21 lineage) confers cardioprotection against ischemia/reperfusion–induced injury by attenuating adverse left ventricular remodeling and cardiac fibrosis, thus preserving cardiac function.

What Are the Clinical Implications?

- Targeting FN polymerization may be a new therapeutic strategy for treating cardiac fibrosis and heart failure, and this principle may also extend to other fibrotic diseases.

Heart failure (HF) is a devastating disease that remains a leading cause of death worldwide and a major socioeconomic burden for Western societies. In the United States alone, HF affects ≈6.5 million people ≥20 years of age,[1] and projections show that by 2030 the prevalence of HF will increase to >8 million people ≥18 years of age with an estimated cost of $70 billion.[2] However, despite many advances, there are few options for efficacious treatment of patients with end-stage HF.

Most of the causes of HF are associated with progressive fibrosis that reduces myocardial compliance and function. Cardiac fibrosis is characterized by exacerbated accumulation of extracellular matrix (ECM) components in the myocardium. This dynamic meshwork is composed of structural and nonstructural proteins that provide an architectural scaffold, surrounding and connecting various cardiac cell populations. In addition to its function in tissue support, the myocardial ECM acts as a signal transducer for cell-cell and cell-ECM interactions that modulate cell motility, survival, and proliferation.[3,4]

The cardiac ECM is comprised primarily of collagen types I and III, which form an intricate 3-dimensional network in which cardiac cell types reside and interact to maintain organ function.[5] Cardiac fibroblasts (CFs) are thought to be the major producers of ECM proteins and are responsible for ECM homeostasis. However, after cardiac injury and under stress stimuli, quiescent CFs transdifferentiate into myofibroblasts (MF) that display exacerbated proliferative, migratory, and contractile abilities, as well as a greater capacity to produce ECM proteins.[6] Although MFs are initially thought to be beneficial by promoting tissue healing after injury, their continued activation and propagation result in adverse tissue remodeling and fibrosis, eliciting cardiac systolic and diastolic dysfunction, thus actively contributing to progression.

Although collagen is the most abundant ECM protein in the heart, cellular fibronectin (FN) is likely to play a pivotal role in cardiac fibrosis. This multifunctional glycoprotein is secreted mainly as a soluble protein by different cell types, including CFs, MFs, or endothelial cells,[7,8] and it is polymerized into the ECM by a cell-dependent process.[9] FN levels are increased in humans with ischemic and dilated cardiomyopathy and in animal models of HF.[10–12] Global FN-null animals are embryonic lethal[13]; prior reports suggests that systemic FN genetic ablation may attenuate hypertrophy/dysfunction after pressure-overload HF[14] and may play a role in stem cell recruitment to the injured heart.[15] Furthermore, FN plays an essential role in the development of lung and liver fibrosis.[16–18] Polymerized FN regulates the deposition, maturation, and stabilization of other ECM proteins, including collagen I[19]; thus, it is recognized that polymerized FN guides aspects of ECM biogenesis.[20] Polymerized FN also influences a variety of crucial cellular processes, including adhesion,[21] growth,[21] proliferation,[22,23] migration,[24,25] survival,[26] and differentiation.[27] Therefore, targeting FN deposition/polymerization and ablating its gene expression in CF populations may be a novel approach to attenuate myocardial fibrosis and HF progression.

pUR4 is a recombinant peptide derived from the bacterial F1 adhesin that exclusively mimics the cell surface binding site for the 5 N-terminal type I modules of FN,[28,29] impeding its polymerization by blocking the binding of soluble FN to the cell surface.[30] As previously reported, pUR4 has a specific affinity for FN that is not shared with other ECM components such as collagen I, laminin, fibrinogen, or vitronectin, indicating a relatively specific bond between pUR4 and FN without affecting additional FN properties.[31]

The potential therapeutic effects of pUR4 in reducing FN accumulation and limiting organ fibrosis in models of liver and flow-induced vascular fibrosis have been recently reported,[31,32] raising the possibility that, by interfering with cardiac FN polymerization in a mouse model of HF, pUR4 peptide will elicit a reduction of pathological ECM deposition and preserve cardiac architecture and function. To assess the therapeutic efficacy and specificity of our FN polymerization inhibitors and to determine the cardiac cell–specific roles of FN inhibition/ablation, we have also used inducible, fibroblast-specific FN-knockout (KO) mice. The basic helix-loop-helix transcription factor Tcf21 is expressed in virtually all resident CFs; these fibroblasts also constitute the source of activated MFs in the infarct region of the heart.[33,34] The inducible Tcf21^{MerCreMer} knock-in mouse

was crossed with *Fn*$^{flox/flox}$ mice to examine the functional role of cellular FN in a temporal and cell type–specific manner.

Here, we demonstrate a potential therapeutic role for inhibiting FN polymerization or ablating local cellular expression in a mouse model of HF in attenuating the excess deposition of both FN and collagen that occurs during adverse cardiac remodeling, thereby limiting the pathogenesis of cardiac fibrosis and preserving cardiac function.

METHODS

The data, analytical methods, and study materials that support the findings of this study are available from the corresponding author on reasonable request.

Mouse CFs and Cardiomyocytes Isolation and Culture

Adult CF were collected from 10 to 12-week-old C57BL6/J wild-type (WT) mouse hearts by enzymatic digestion. Briefly, mice were given 100 µL heparin (100 U/mL) via intraperitoneal injection and anesthetized with isoflurane. The heart was quickly excised and retrograde-perfused using a Langendorff apparatus under constant pressure (60 mm Hg; 37°C, 4 minutes) in Ca^{2+}-free perfusion buffer containing 113 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 5.5 mmol/L glucose, 0.6 mmol/L KH_2PO_4, 0.6 mmol/L Na_2HPO_4, 12 mmol/L $NaHCO_3$, 10 mmol/L $KHCO_3$, 10 mmol/L Hepes, 10 mmol/L 2,3-butanedione monoxime, and 30 mmol/L taurine. Digestion was achieved by perfusing for 3 minutes with Ca^{2+}-free perfusion buffer containing collagenase II (units per 1 mL) (600 U/mL of collagenase II in perfusion buffer; Worthington LS004177) followed by 8 minutes of perfusion with digestion buffer containing 12.5 µmol/L $CaCl_2$. Subsequently, it was removed from the apparatus and gently teased into small pieces with fine forceps in the same enzyme solution. Heart tissue was further dissociated mechanically with 2-, 1.5-, and 1-mm-diameter pipettes until all large heart tissue pieces were dispersed. The digestion buffer was neutralized with buffer containing 10% FBS and 12.5 µmol/L $CaCl_2$ and cell suspension was filtered through 200-µm mesh. Cardiomyocytes (CMs) were pelleted by gravity (20 minutes), and the supernatant was collected and stored on ice. CMs were resuspended in perfusion solution containing 5% FBS and 12.5 µmol/L $CaCl_2$ and subsequently allowed to settle for 20 minutes. The 2 supernatants were pooled, and both non–myocyte-containing CF fractions were centrifuged at 500*g* for 7 minutes. CFs were resuspended and plated on the desired culture plates previously coated with 1% gelatin in culture media containing Iscove modified Dulbecco medium, 10% FBS (ESCell FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 103 U ESGRO Supplement (Millipore), 10 ng/mL epidermal growth factor, and 20 ng/mL fibroblast growth factor (37°C, 5% CO_2).

Human Fibroblast Isolation

Failing human CFs were isolated from ventricular tissue excised during left ventricular (LV) assist device implantation surgery under the Institutional Review Board protocol 2013-1386. Tissue was washed in Hanks balanced salt solution and cut into 1-mm cubed pieces with scissors. Digestion mixture, consisting of 100 mg collagenase (Worthington), 1 mg trypsin (Worthington TLR3), and 15 mg BSA in DMEM (HyClone SH30022.01), was applied to dissected tissue, and the mixture was placed in a shaker at 125 rpm for 20 minutes at 37°C. After incubation, a 10-mL pipette was used to gently dissociate cells, and supernatant was collected and combined with neutralization media containing DMEM+10% FBS; this process was then repeated until the tissue pieces were completely digested. The combined supernatants were centrifuged at 1300 rpm for 7 minutes to collect the nonmyocyte pellet, which was then resuspended in growth media (as described above) and plated on 1% gelatin–coated dishes.

Animals

All animal procedures were conducted according to the Department of Laboratory Animal Medicine and the University Committee on Animal Resources at Cincinnati Children's Hospital Medical Center.

C57BL6/J WT 10 to 12-week-old male mice (The Jackson Laboratories) were used for systemic peptide administration. *Fn*$^{flox/flox}$ mice were kindly provided by Dr Reinhard Fässler and crossed with mice expressing tamoxifen-inducible Cre recombinase under the control of the basic helix-loop-helix transcription factor Tcf21 promotor (Tcf21^{MerCreMer})[33] to generate double transgenic (DTG) inducible fibroblast-specific FN-KO mice (Tcf21^{mERCremER}x*Fn*$^{flox/flox}$; CF-FN-KO). DTG without tamoxifen or tamoxifen-treated *Fn*$^{flox/flox}$ littermates served as controls. Animals were fed tamoxifen chow (400 mg/kg; Harlan TD.130860) 2 weeks before cardiac surgery and 4 weeks after injury to induce FN ablation in Tcf21 lineage fibroblasts (quiescent before surgery and activated after injury).[34]

Peptides and Treatment

pUR4 is a peptide derived from a surface protein of *Streptococcus pyogenes* called F1 adhesin[30] and the first described inhibitor of FN polymerization. pUR4 mimics the binding site required for soluble FN to anchor into the cell surface, inhibiting its adhesion without interfering with the critical FN-integrin cell binding domain. pUR4 specifically binds to FN but does not bind to other ECM proteins.[35] In addition, FN polymerization tightly regulates the assembly of different ECM proteins, including collagen type I.[19,20] pUR4 has been shown to reduce the deposition of endogenous collagen I into matrix fibrils both in vitro and in vivo.[32,35] We have used a control peptide called III-11C that is based on sequences in the III-11 module of FN[36] that has proven not to have biological effects in vitro or in vivo.[35]

The pUR4 and III-11C peptides were produced as described[35] and dialyzed in PBS, and endotoxin was removed from peptides with the Acrodisc Unit with Mustang E membrane 0.2 µm (VWR MSTG25E3). Subsequently, endotoxin levels were measured with a Limulus amebocyte lysate assay kit (Lonza 50-647U). After purification, endotoxin levels were <0.1 endotoxin units per 1 mg peptide.

Mice received daily intraperitoneal injections of pUR4 or III-11C (25 $mg \cdot kg^{-1} \cdot d^{-1}$) for 7 consecutive days starting the same day of ischemia/reperfusion (I/R) surgery or for 14 days

starting 4 weeks after I/R injury. Cells received pUR4 or III-11C (500 nmol/L) for 72 hours.

Statistical Analysis

Data are reported as mean±SEM. For single biochemical and physiological observations, the Student *t* test was applied. A paired *t* test was used when different treatments (pUR4 and III-11C) were applied to cells isolated from the same animal. An unpaired *t* test was applied when pUR4 and III-11C were administered to different animals. Multiple responses were analyzed by 1-way or 2-way ANOVA. Post hoc analysis was performed as indicated if statistical significance ($P \leq 0.05$) was achieved. Calculations were performed with GraphPad Prism 6.0.

An expanded Materials and Methods section is available in the online-only Data Supplement.

RESULTS

pUR4 Attenuates FN Deposition in Adult Mouse CFs In Vitro

A key function of CF is to produce structural proteins that make up the myocardial ECM and regulate its homeostasis. Polymerized FN regulates the deposition and maturation of several ECM proteins, including collagen I, and actively plays a role in cellular survival, proliferation, migration, and differentiation. To explore the functional role and efficacy of pUR4 in reducing FN assembly into the ECM, in vitro studies were performed with primary CF cultures. Primary adult mouse CFs from unchallenged hearts were isolated and treated for 72 hours with either the FN polymerization inhibitor pUR4 or the control (III-11C) peptide, which binds FN but does not possess inhibitory activity.[31] pUR4-treated cells displayed a robust reduction of ECM network organization compared with cells treated with the control III-11C peptide (Figure 1A); however, no effects were observed on cellular expression of FN mRNA after pUR4 administration (Figure 1B). Furthermore, to explore whether there were cellular or ECM-related changes in FN protein content, the cellular and ECM fractions were isolated separately from CF cultures. Although pUR4 treatment did not alter FN intracellular protein content compared with III-11C (Figure 1C), pUR4-treated cells exhibited attenuation of FN deposition into the ECM (Figure 1D). However, the normal stoichiometry of total extracellular FN expression was maintained; pUR4 increased FN abundance in cell culture media measured by ELISA (Figure 1E). These data demonstrate that pUR4 efficiently blocks FN polymerization within the ECM of CFs with no effect on cellular transcript or protein level.

pUR4 Attenuates Pathological Mouse Cardiac MF Activation Profile In Vitro

CFs actively participate in the complex and dynamic intercellular cross-talk between the myriad cardiac cells to maintain cardiac structure and function. After cardiac insult, this cross-talk is dysregulated and elicits the transition of CFs to MFs, resulting in exacerbated synthesis and deposition of ECM, as well as enhanced proliferation and migration. To explore the impact of inhibiting FN polymerization on cell behavior, primary activated MFs were isolated from WT hearts 5 days after myocardial I/R injury. First, the activation profile of MF was confirmed by identifying increased fibrotic and inflammatory cytokine gene expression (Figure I in the online-only Data Supplement). Subsequently, experiments investigated whether pUR4 reduces FN polymerization in MF ECM to the same extent as observed in unchallenged CFs. pUR4 exhibited a robust inhibitory effect on FN polymerization in MFs isolated from injured mouse hearts compared with III-11C–treated cells (Figure II in the online-only Data Supplement). FN polymerization also tightly regulates the assembly of collagen I; when MFs were cultured in the presence of pUR4, deposition of collagen into matrix fibrils was reduced (Figure 2A and 2B). Neither intracellular protein content nor collagen I mRNA transcript levels were affected by pUR4 treatment (Figure 2C and 2D).

To examine whether pUR4 attenuates MF activation, MFs were treated with pUR4 or III-11C peptides, and different cellular functions were analyzed. In vitro MF proliferation was evaluated by counting the cell number and by the expression of the mitosis marker phospho-histone H3 72 hours after peptide administration. Cell proliferation and phospho-histone H3–positive cells were significantly reduced after pUR4 treatment in activated cardiac MFs (Figure 2E and 2F). Reduced FN polymerization resulted in decreased transcript expression of proliferation-related genes such as c-myc and auroraB kinase (Figure 2G). In line with these results, a cell cycle analysis was performed in activated MFs at different time points after peptide administration (12, 24, 36, 48, 56, and 72 hour); a significant decrease was observed in the percentage of cells in the S phase at 24, 36, 48, and 56 hours (Figure IIIA and IIIB in the online-only Data Supplement). However, this decrease was not associated with changes in signaling via the extracellular signal-regulated kinase or AKT pathways (Figure IIIC in the online-only Data Supplement). It is surprising that inhibiting FN polymerization did not affect cell proliferation in unchallenged CFs isolated from healthy animals (Figure IVA in the online-only Data Supplement).

Another central property of MFs is their enhanced migratory ability after cardiac injury. The effect of pUR4 in MF migratory capacity was evaluated by performing a scratch-wound healing assay. pUR4-treated cells displayed a significant reduction in MF migration (Figure 2H). This finding was also observed in unchallenged CFs (Figure IVB in the online-only Data Supplement). In this context, integrins contribute to the recruitment and activation of crucial signaling proteins

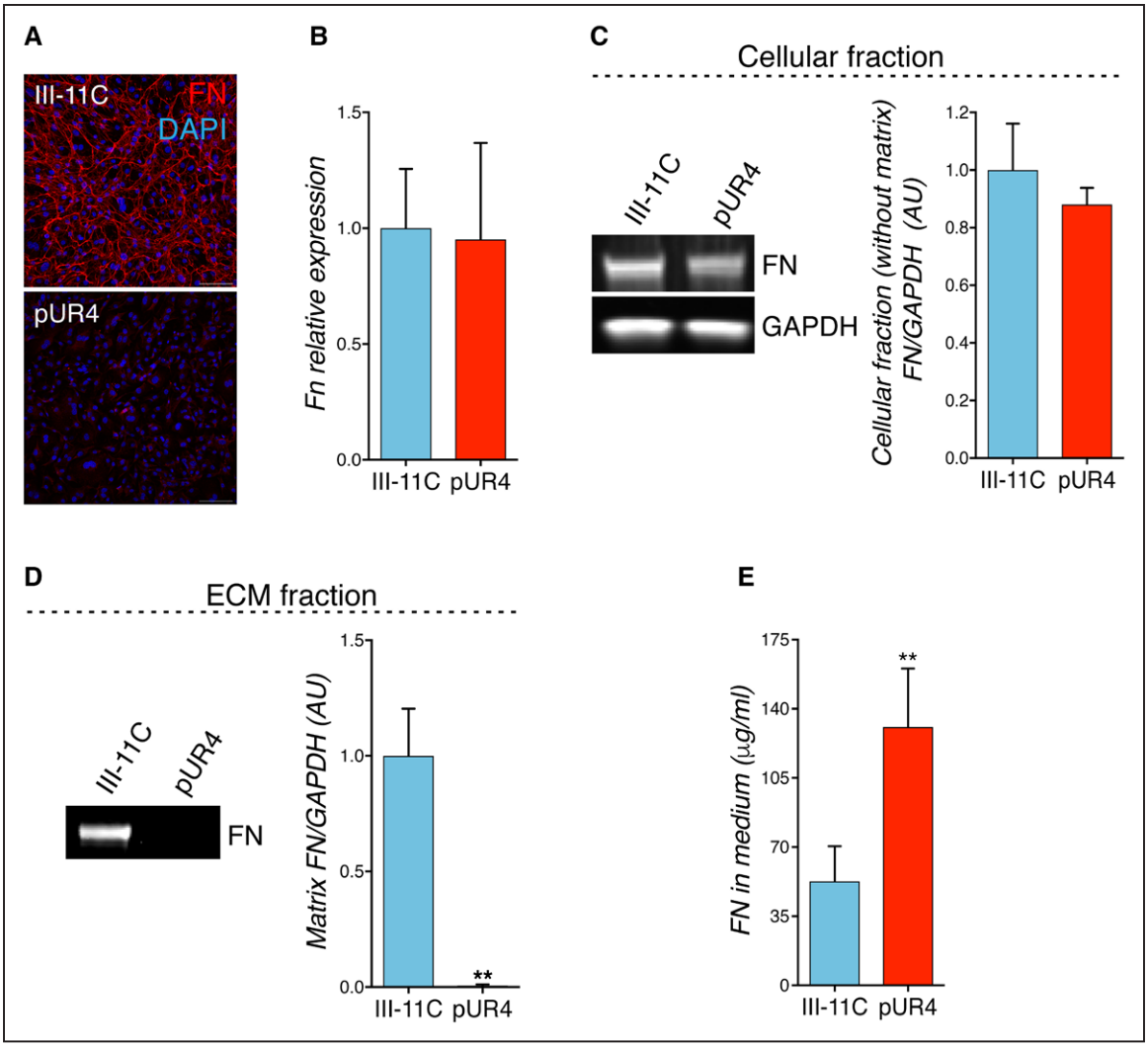

Figure 1. pUR4 attenuates fibronectin (FN) polymerization in unchallenged primary mouse cardiac fibroblasts (CFs) 72 hours after treatment. **A**, FN immunofluorescence staining in healthy CFs. Scale bars, 100 μm. **B**, FN transcript level expression. n=5. **C**, Cellular FN protein level expression (without matrix) (representative immunoblots, **left**; densitometry, **right**). n=5. **D**, Matrix FN deposition in the extracellular space of CF is attenuated with pUR4 (representative immunoblots, **left**; densitometry, **right**). n=5. **E**, FN is accumulated in the cultured media measured by ELISA. n=5. Data are presented as mean±SEM. AU indicates arbitrary units; and ECM, extracellular matrix. Statistical significance was determined with paired *t* test. **P*<0.05. ***P*<0.01.

involved in cell migration, including focal adhesion kinases (FAKs). A significant increase of integrin internalization was observed after pUR4 treatment (Figure 2I). Because FAKs are among the most prominent proteins involved in ECM-integrin signaling pathways crucial for cell migration, the abundance of phospho (p)-FAK was assessed after peptide treatment. p-FAK protein content was also reduced in pUR4-treated cells compared with control III-11C–treated cells (Figure 2J). Furthermore, pUR4 decreased the expression of α-smooth muscle actin (α-SMA) and reduced the complexity of fiber formation (Figure 2K) without affecting vimentin expression (Figure 2L). Together, these data suggest that pUR4 exhibits a principal role in diminishing MF proliferation via the c-myc signaling axis and reduces cell migration by promoting integrin internalization, decreasing FAK activation, and ameliorating the activated MF phenotype.

pUR4 Does Not Alter Mitochondria Content, Oxidative Stress, or Cell Metabolism in Mouse Cardiac MFs

Our data suggest that it is possible to attenuate the pathological cardiac MF phenotype by interfering with FN polymerization. Our next aim was to determine whether pUR4 treatment affected cell survival or metabolism, which may explain the changes overserved in cell behavior. To this end, the impact of inhibiting FN polymerization on cell apoptosis, reactive oxygen species production, and metabolic state was determined. pUR4 administration did not result in a significant difference in apoptosis measured by flow cytometry (Figure 3A). In addition, the accumulation of total and mitochondrial reactive oxygen species, known to be increased after cardiac injury, did not differ between control- and pUR4-treated cells (Figure 3B and

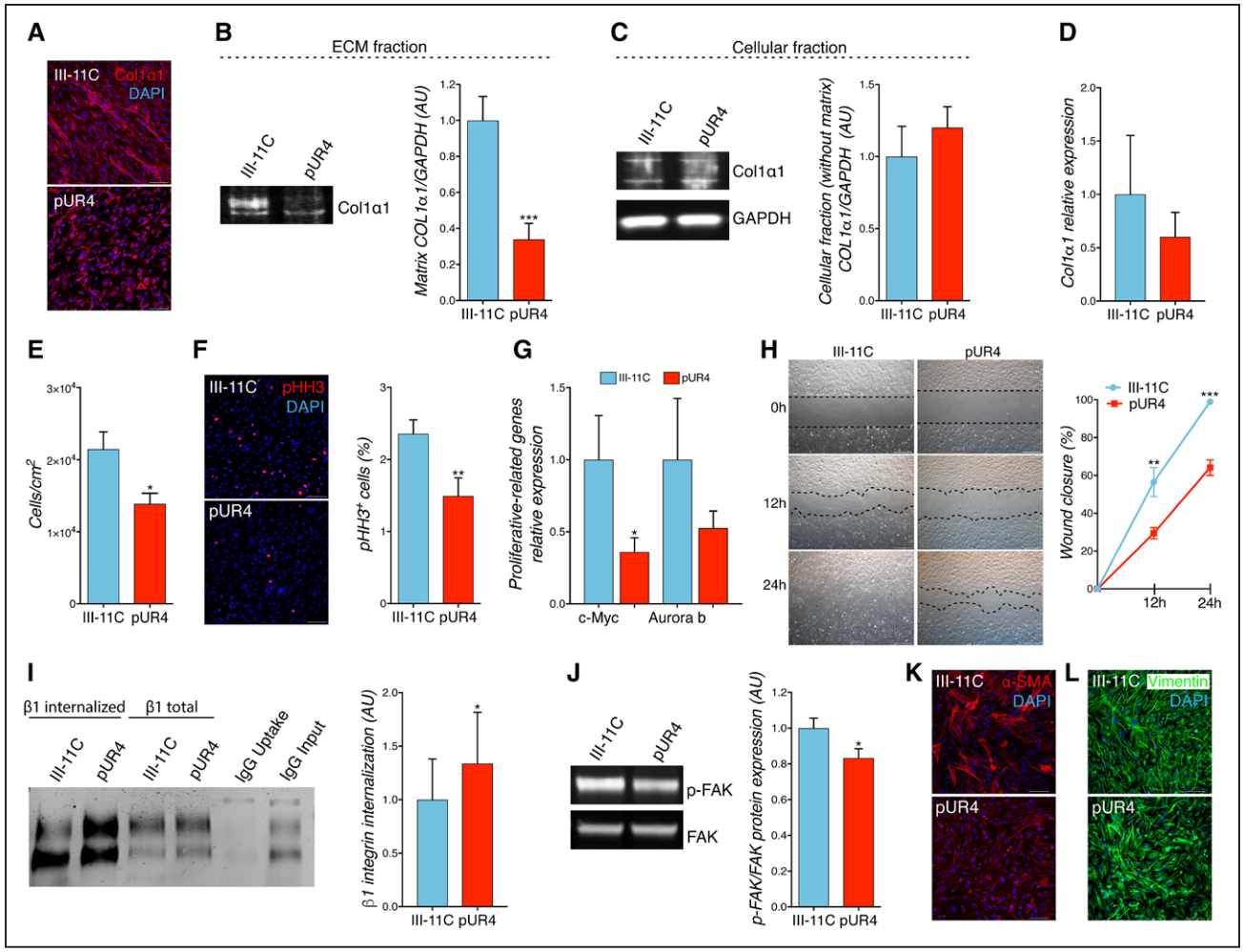

Figure 2. pUR4 attenuates mouse pathological myofibroblast (MF) phenotype.
A, pUR4 decreases collagen I staining in cardiac fibroblasts (CFs) isolated after cardiac ischemia/reperfusion injury by immunofluorescence. Scale bars, 100 µm. **B**, Matrix collagen (COL) I deposition in activated MFs is attenuated on pUR4 treatment (representative immunoblots, **left**; densitometry, **right**). n=5. **C**, Cellular collagen I protein level expression (without matrix) (representative immunoblots, **left**; densitometry, **right**). n=5. **D**, Collagen I transcript level expression. n=6. **E**, pUR4 decreases cell proliferation monitored by counting cell number with a hemocytometer. n=6. **F**, Phospho-histone H3 (pHH3) staining in mouse MFs shows a decrease in pHH3⁺ cells in pUR4-treated cells (representative pictures, **left**; quantification, **right**). Scale bars, 100 µm. n=3. **G**, Reverse transcription–quantitative polymerase chain reaction of proliferative-related genes in pUR4- and III-11C–treated mouse MFs. n=6. **H**, Scratch wound-healing assay in mouse CF cell migration; pictures were taken at 0, 12, and 24 hours after scratch. Black dotted lines denote the wound borders (representative photographs, **left**; mobility quantification, **right**). Scale bars, 1000 µm. n=6. **I**, Increased β1 integrin internalization was found in MFs treated with pUR4 compared with III-11C peptide (representative immunoblots, **left**; densitometry, **right**). n=5. **J**, Expression of phosphorylated (p-) focal adhesion kinase (FAK) was evaluated by Western blotting and quantified relative to total FAK expression (representative immunoblots, **left**; densitometry, **right**). n=4. **K**, α-Smooth muscle actin (α-SMA) staining in III-11C– and pUR4-treated MFs. Scale bars, 100 µm. **L**, Vimentin staining in peptide-treated cells. Scale bars, 100 µm. Data are presented as mean±SEM. ECM indicates extracellular matrix; and IgG, immunoglobulin G. Statistical significance was determined with paired *t* test. **P*<0.05. ***P*<0.01. ****P*<0.001.

3C). Furthermore, cellular mitochondrial content was examined to evaluate whether the limited proliferative and migratory abilities exhibited by pUR4-treated MFs may be explained by an overall reduction of energy production. Flow cytometry analysis with Mitrotracker combined with TOM20 cell staining revealed no differences in total mitochondrial content after inhibiting FN polymerization (Figure 3D and 3E). Last, to evaluate whether pUR4 treatment affects the metabolic state of cardiac MFs, cellular ATP levels and oxygen consumption were measured in MF-treated cells. No differences were detected in cells lacking an FN network (Figure 3F and 3G). In addition, cellular glycolysis and fatty acid oxidation were measured with no effect on the aforementioned pathways after pUR4 administration (Figure 3H and 3I).

These findings demonstrate that inhibiting FN polymerization does not alter cell survival or cellular metabolic state, highlighting the direct ECM-mediated effect of pUR4 in attenuating MF migration and proliferation.

pUR4 Reduces the Pathological Activation of Human Failing Cardiac MFs In Vitro

Human CFs regulate normal cardiac function and organ homeostasis but also are the main mediators of pathological myocardial remodeling after cardiac injury that contributes to the progression of organ fibrosis and

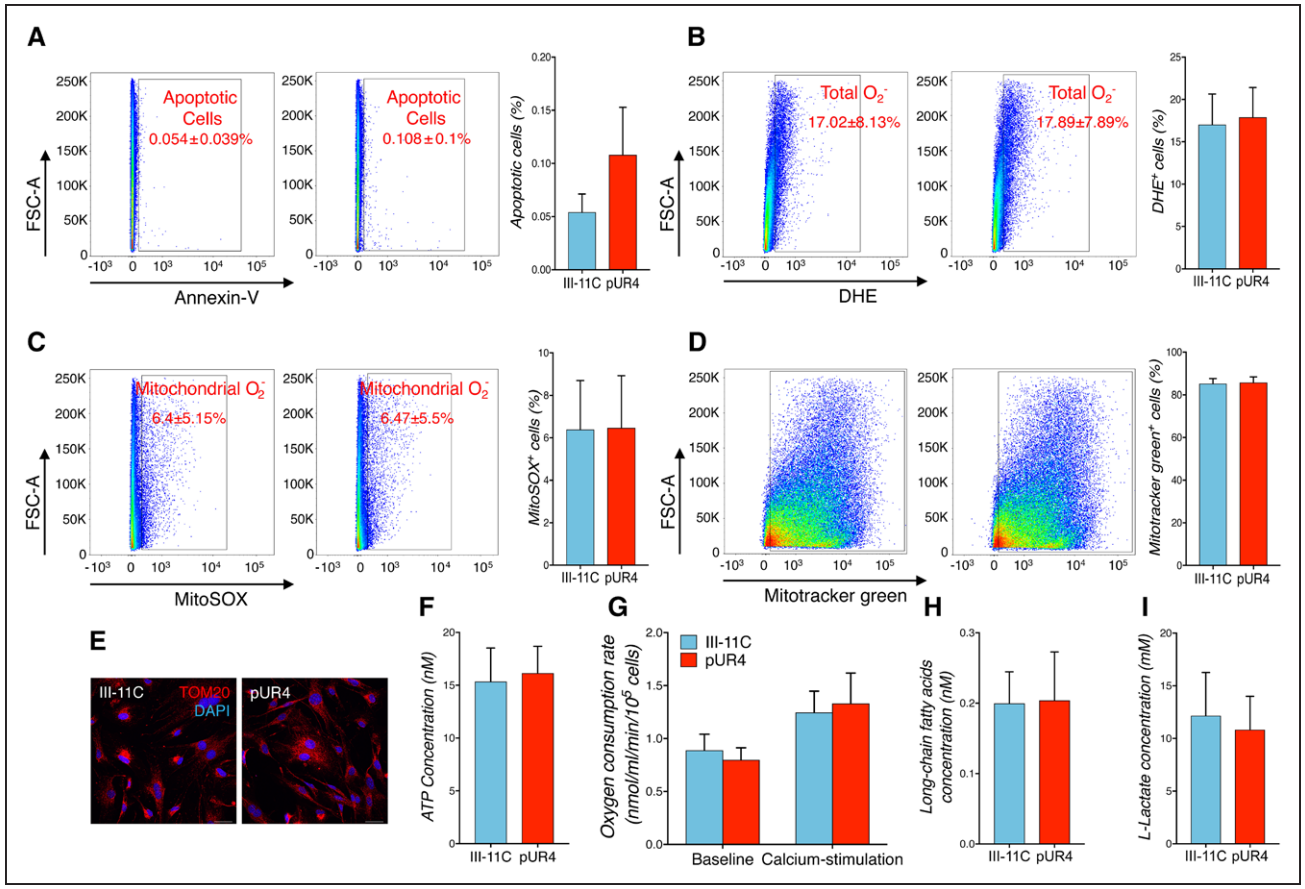

Figure 3. pUR4 does not alter myofibroblast (MF) cell survival, reactive oxygen species (ROS) production, mitochondrial content, and metabolism after 72 hours of peptide treatment.

A, Flow cytometry analysis of annexin-V staining of apoptotic MFs after pUR4 or III-11C peptide treatment. n=5. **B**, MFs treated either with pUR4 or III-11C were stained with superoxide anion–specific dye dihydroethidium (DHE). Oxidized version of DHE was detected by flow cytometry. n=5. **C**, Levels of mitochondria-associated ROS in peptide-treated MFs were analyzed by MitoSOX labeling and analyzed with flow cytometry. n=5. **D**, Mitochondria were stained with MitoTracker Green and analyzed by flow cytometry. n=5. **E**, Mitochondria immunostaining with TOM20 in pUR4- or III-11C–treated cells. Scale bars, 20 μm. **F**, ATP content was measured in peptide-treated MFs after 72 hours. n=5. **G**, Oxygen consumption was determined without or with calcium stimulation in MFs treated with III-11C or pUR4. n=7. **H**, Mitochondrial fatty acid oxidation was measured by long-chain fatty acid concentration existent in the cellular content. n=5. **I**, Glycolysis activity was measured by L-lactate concentration in the cultured media. n=5. Absence of statistical significance was determined with paired *t* test. Data are presented as mean±SEM. FSC-A indicates forward scatter area.

HF. To determine the effects of FN polymerization inhibition on human activated cardiac MFs, primary failing human MFs were isolated from the hearts of patients with end-stage HF undergoing LV assist device implantation, characterized (Figure V in the online-only Data Supplement) and treated with pUR4. As with murine MFs, pUR4 treatment impaired the deposition of FN fibrils into the ECM of human MFs (Figure 4A). pUR4 significantly reduced in vitro cell growth, as monitored by total cell counts and phospho-histone H3–positive cells (Figure 4B and 4C). In addition, pUR4 reduced the expression levels of proliferative and inflammation-related transcripts in human failing cardiac MFs (Figure 4D and 4E) and significantly blunted their migratory ability in a scratch-wound healing assay (Figure 4F). Furthermore, pUR4-treated cells exhibited a reduced p-FAK expression (Figure 4G and 4H) and a reduction of F-actin patterning expression (Figure 4I). In addition, human cardiac MFs exhibited reduced expression of α-SMA and complexity of fiber formation (Figure 4J) without affecting vimentin expression (Figure 4K) on inhibition of FN polymerization. Collectively, these data show the potential therapeutic effect of inhibiting FN fibril formation in reducing the pathological phenotype of human failing cardiac MFs.

Neither pUR4 Nor III-11C Affects Baseline Cardiac Function in Unchallenged Hearts In Vivo or Isolated CMs In Vitro

To explore the effects of pUR4 peptide administration on cardiac function at baseline in vivo, WT mice were injected intraperitoneally with III-11C or pUR4 once daily for 7 consecutive days. A fluorescent dye was covalently bound to the peptides to verify peptide localization in vivo 24 hours after the last administration. As shown in Figure VI in the online-only Data Supplement, the peptide was detected throughout the animal body in addition to the heart.

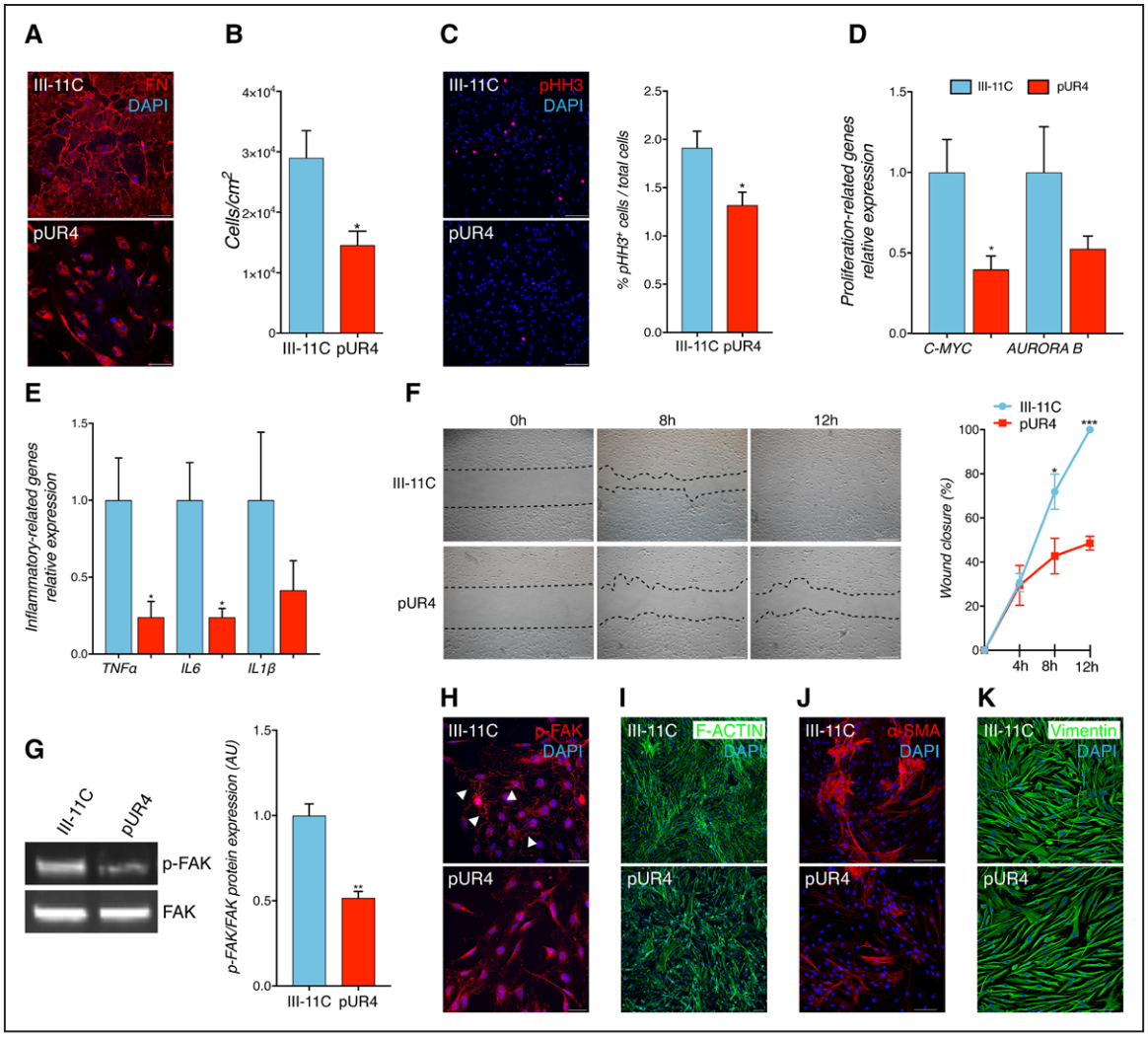

Figure 4. pUR4 decreases proliferation and migration of human failing cardiac fibroblasts (CFs).
A, pUR4 reduces fibronectin FN staining. Scale bars, 100 μm. **B**, pUR4 decreases cell proliferation. **C**, pUR4 decreases phospho-histone H3 (pHH3)–positive human heart failure CFs (representative pictures, **left**; quantification, **right**). Scale bars, 100 μm. **D**, pUR4 downregulates proliferation-related genes. **E**, Expression levels of proinflammatory cytokine transcripts are significantly reduced after pUR4 treatment in failing human CFs measured by reverse transcription–quantitative polymerase chain reaction. IL1β indicates interleukin 1β; Il6, interleukin 6; and TNFα, tumor necrosis factor-α. **F**, Cardiac fibroblasts were scratch wounded and recorded at 0, 4, 8, and 12 hours after scratch. Black dotted lines denote the wound borders (representative photographs, **left**; mobility quantification, **right**). Scale bars, 500 μm. **G**, Phosphorylated (p-) focal adhesion kinase (FAK) protein level expression (representative immunoblots, **left**; densitometry, **right**). **H**, Immunofluorescence analysis of p-FAK in pUR4- and III-11C–treated human myofibroblasts (MFs) (arrowheads). Scale bars, 10 μm. **I**, F-actin staining in pUR4-treated cells indicated a compromised defect in cellular organization compared with the control group. Scale bars, 100 μm. **J**, α-Smooth muscle actin (α-SMA) staining in III-11C– and pUR4-treated human MFs. Scale bars, 100 μm. **K**, Vimentin staining in peptide-treated cells. Scale bars, 100 μm. Data are presented as mean±SEM. n=5.Statistical significance was determined with paired *t* test. **P*<0.05. ***P*<0.01. ****P*<0.001.

No effects on cardiac function or morphometry parameters were detected after 7 days of peptide exposure (Figure VIIA and VIIB and Table I in the online-only Data Supplement). Furthermore, pUR4 administration did not result in CM hypertrophy in healthy hearts (Figure VIIC in the online-only Data Supplement). To determine whether there was any effect on the cardiac inflammatory milieu after exposure to the peptides, flow cytometry analysis was performed, and no significant changes in the inflammatory cell population of the heart were observed (Figure VIID in the online-only Data Supplement). In addition, FN inhibition in healthy animals did not result in a significant decrease of collagen deposition (Figure VIIE in the online-only Data Supplement). To further explore possible effects on cardiac muscle cell contractility, primary CMs were isolated from unchallenged hearts and assessed for contractile function. No basal differences in sarcomeric shortening were observed with either peptide (Figure VIIF in the online-only Data Supplement). CMs isolated 5 days after I/R and treated immediately with III-11C and pUR4 for 1 hour did not show significant alterations in contraction (Figure VIIG in the online-only Data Supplement). Overall, these data suggest that peptide administration has no significant effect on baseline cardiovascular function.

Inhibition of FN Polymerization Attenuates MF Markers and Inflammatory Cell Infiltration 7 Days After Injury In Vivo

Our in vitro data suggest that blocking FN deposition into the ECM attenuates the pathological phenotype exhibited by activated MFs isolated from failing mouse and human hearts. Therefore, we sought to examine whether inhibition of FN polymerization by pUR4 treatment may be salutary after myocardial injury. To test this hypothesis, WT mice were subjected to myocardial ischemia followed by 7 days of reperfusion (Figure 5A). First, FN expression was markedly upregulated 7 days after I/R (Figure VIII in the online-only Data Supplement) as previously described.[37,38] Seven days of pUR4 treatment after cardiac injury trended toward a reduction of the expression of FN by 30% (Figure 5B), as well as fibrosis-related transcripts such as α-SMA and collagen 3 (Figure 5C). In addition, pUR4 significantly reduced proliferative fibroblast numbers in both the infarcted and remote areas of the heart 7 days after injury as observed by EdU administration (Figure 5D).

ECM homeostasis relies on a tight balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which collectively regulate ECM components in the process of cardiac remodeling.[39] However, exacerbated MMP activity is detrimental for cardiac output.[39] To characterize MMP activity, we studied the expression of MMPs and TIMPs, as well as their enzymatic activity. Analysis in pUR4-treated hearts revealed a trend toward a reduction of MMP-9 activity (Figure IXA in the online-only Data Supplement) and its transcript level (Figure IXB in the online-only Data Supplement). TIMP-1 transcript level was significantly reduced by pUR4 treatment (Figure IXB in the online-only Data Supplement).

The role of FN in the recruitment of inflammatory cells into different tissues has been extensively described.[40,41] The robust inflammatory response triggered after cardiac insult is initially crucial for cardiac repair, but it is also involved in pathological tissue remodeling and HF at later stages.[42] CM necrosis triggers an acute inflammatory response characterized by immune cell recruitment and a rapid increase in cytokine and chemokine levels.[43] Toll-like receptors (TLRs) play a pivotal role in recognizing endogenous "danger signals" released during cell death.[43] Because FN is a possible ligand for TLR-2 and TLR-4, we sought to investigate the role of FN inhibition in TLR expression and inflammatory progression after I/R injury. TLR-2, but not TLR-4, expression exhibited a significant reduction in the LV of pUR4-treated hearts (Figure 5E). Furthermore, the different immune subsets residing in the heart were evaluated by flow cytometry 7 days after I/R injury in pUR4- and III-11C–treated animals (Figure 5F). The recruitment of neutrophils into the heart was significantly attenuated after pUR4 treatment (Figure 5G), whereas the total number of $CD45^+$ cells recruited was not significantly altered (Figure 5H), nor was the number of macrophages, dendritic cells, and other compartments affected by pUR4 treatment (Figure XA through XF in the online-only Data Supplement). In addition, we did not detect a significant decrease in the mRNA expression of relevant cytokines such as interleukin-1β and -6 in the LV of pUR4-treated animals 7 days after organ reperfusion (Figure XG in the online-only Data Supplement).

Given the observed decrease in neutrophil infiltration in the heart after I/R in mice treated with pUR4, we hypothesized that pUR4 would impair neutrophil adhesion to heart endothelial cells as a mechanism for the observed in vivo defect in recruitment. To test whether pUR4 had any effect on neutrophil adhesion, mouse heart endothelial cells (MHECs) were evaluated under shear flow conditions in vitro. Treatment of neutrophils or MHECs with pUR4 did not alter the expression of the neutrophil markers Ly6G and CD11b, the expression of neutrophil adhesion molecules such as P-selectin glycoprotein ligand-1, or the MHEC monolayer integrity, and it efficiently inhibited FN polymerization in MHECs (Figure XI in the online-only Data Supplement). It is striking that pUR4 treatment of MHEC resulted in significantly reduced total accumulation and firm adhesion of neutrophils to MHECs compared with III-11C control treatment (Figure 5I and 5J) without affecting neutrophil rolling on MHECs (Figure 5K). This decreased adhesion observed in pUR4-treated cells was not related to an alteration in the expression of integrins involved in neutrophil adhesion such as very late antigen-4 and lymphocyte function-associated antigen 1[44] (Figure XIIA and XIIB in the online-only Data Supplement). Subsequent experiments tested whether FN polymerization regulated the expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), which are the endothelial ligands for neutrophil very late antigen-4 and lymphocyte function-associated antigen 1,[45] on tumor necrosis factor-α stimulation. Although flow cytometry data showed that pUR4 does not significantly decrease VCAM-1 and ICAM-1 surface expression in MHECs treated for 72 hours (Figure XIIC and XIID in the online-only Data Supplement), cellular MHEC VCAM-1 and ICAM-1 staining demonstrated overall reduction of protein expression (Figure XIIE and XIIF in the online-only Data Supplement). Taken together, these results suggest that pUR4 reduces neutrophil adhesion and arrest on MHECs under inflammatory conditions by a putative decrease of total ICAM-1 and VCAM-1 protein expression.

Systemic Treatment With pUR4 Reduces Pathological Cardiac Fibrosis and Preserves Cardiac Function Up to 4 Weeks After I/R

To examine whether pUR4 treatment limits long-term cardiac injury and fibrosis in vivo, mice were subjected to

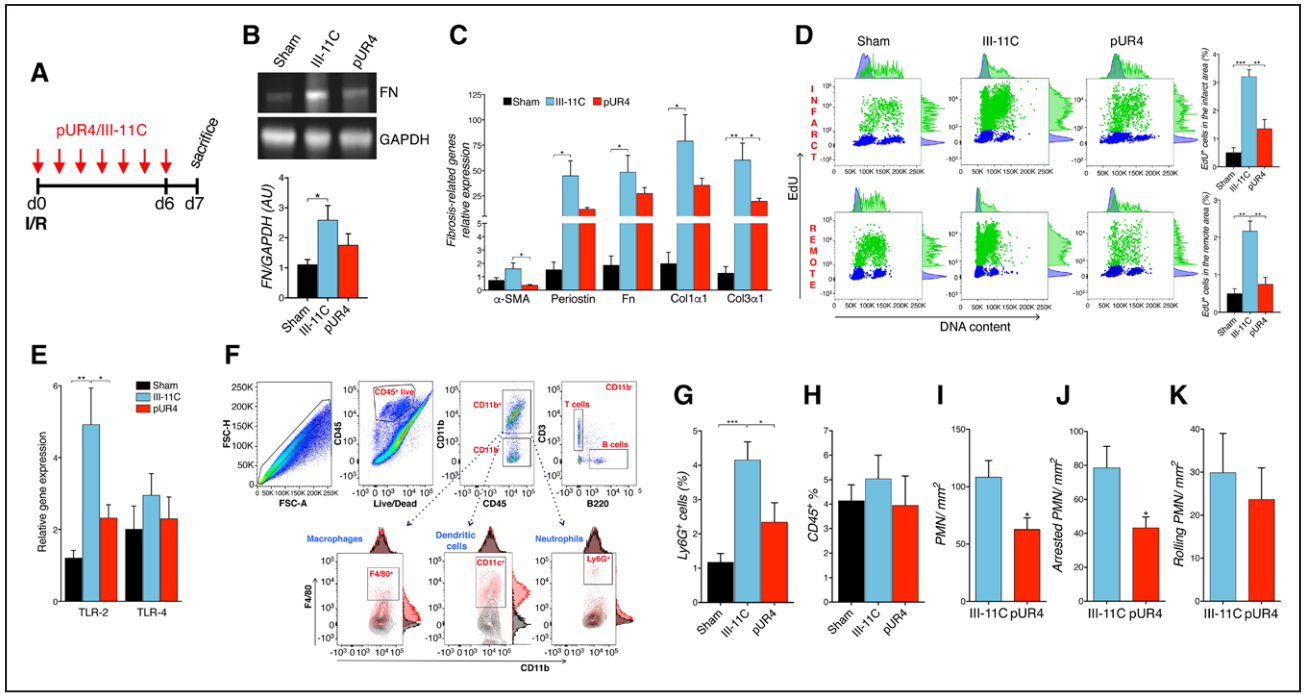

Figure 5. Inhibition of fibronectin (FN) polymerization attenuates fibrotic mediators and inflammatory cell infiltration 7 days after ischemia/reperfusion (I/R) in vivo.

A, Schematic of the experimental timeline showing initiation of pUR4 or III-11C treatment after the I/R injury. **B**, FN protein expression in the heart (representative immunoblots, **top**; densitometry, **bottom**). n=5. **C**, Fibrosis-related gene expression in left ventricular tissue of peptide-treated hearts. α-SMA indicates α-smooth muscle actin; and Col, collagen. n=5 to 6. **D**, Cardiac proliferative cells, S phase (EdU+ staining, green), detected in the infarct and remote areas by flow cytometry (representative plots, **left**; quantification, **right**). n=3 to 4. **E**, Toll-like receptor (TLR)-2 and TLR-4 expression 7 days after infarction in the specified treatment groups. n=5 to 6. **F**, Flow cytometry strategy workflow to analyze the different immune cell compartments. **G** and **H**, Quantification with flow cytometry of neutrophils **(G)** and CD45+ cells **(H)** in the hearts of sham and pUR4- or III-11C–treated animals. n=5 to 8. **I**, Quantification of total accumulation of neutrophils to mouse heart endothelial cells (MHECs). **J**, Quantification of firm adhesion of neutrophils to MHEC. **K**, Quantification of neutrophil rolling on tumor necrosis factor-α–activated MHEC pretreated with the indicated peptides for 72 hours. Neutrophils were perfused at 1 dyne/cm^2, and interactions with MHECs were quantified in 10-minute videos and additional fields of view. Data are presented as mean±SEM. Statistical significance determined by 1-way ANOVA with Tukey post hoc analysis. *P<0.05. **P<0.01. ***P<0.001. FSC-H indicates forward scatter height.

I/R injury and treated daily with pUR4 or III-11C for the first 7 days, followed by evaluation of cardiac function and histology 4 weeks after I/R (Figure 6A). Seven days of pUR4 treatment significantly preserved myocardial function up to 4 weeks after I/R as measured by ejection fraction and end-systolic volume (Figure 6B and 6C and Table II in the online-only Data Supplement), suggesting an injury-reducing effect after just short-term (7 days) pUR4 administration. Attenuation of systolic dysfunction observed in pUR4-treated animals was possibly the result of a reduction in the infarct size, either through direct effects of the peptide on CM survival or through attenuation of inflammatory CM injury. To further evaluate these possibilities, the infarct size was assessed 24 hours after injury with concomitant peptide treatment followed by quantification of percent of infarcted area compared with the area at risk (Figure XIIIA in the online-only Data Supplement). In addition, terminal deoxynucleotidyl transferase dUTR nick-end labeling was performed to measure the number of apoptotic cells in the infarct area 24 hours after treatment and I/R (Figure XIIIB in the online-only Data Supplement). No significant differences were observed either in the area of infarct or in the number of apoptotic cells between treatments.

pUR4-treated animals also exhibited an attenuation in pathological hypertrophy detected in cardiac morphometry (Figure 6D). The observed attenuation of cardiac hypertrophy was confirmed by a reduction of CM size after pUR4 administration (Figure 6E), although pUR4 appeared not to have a direct effect on CM size in response to the prohypertrophic signal angiotensin II using primary neonatal rat CMs in vitro (Figure XIV in the online-only Data Supplement).

A reduction of the pathological fibrotic response was detected in pUR4-treated animals 4 weeks after I/R. FN content in the LV as analyzed by quantitative polymerase chain reaction, immunofluorescence, and Western blotting was reduced after peptide administration (Figure 6F through 6H). At 4 weeks after injury, pUR4 administration resulted in decreased fibrosis and collagen I accumulation, as evidenced by Picrosirius Red (Figure 6I), reduction of transcript level expression (Figure 6J), and immunostaining (Figure 6K). Furthermore, second harmonic generation represents a powerful tool to image collagen at high resolution, with diffraction-limited resolution (<300 nm), that allows the study of collagen architecture, orientation, and crystallinity at a microscopic level better than is possi-

ble by other microscopy or histological techniques.[46,47] Second harmonic generation imaging was performed to further study collagen organization in peptide-treated groups. A notable reduction of collagen deposition was observed in pUR4-treated animals comparted with those treated with III-11C peptide (Figure XV in the online-only Data Supplement). In addition, the total number of CD45^{+} cells localized in the infarcted area was reduced in the LV of pUR4-treated animals (Figure 6L). Collectively, these data demonstrate the salutary effects of pUR4 treatment in preserving cardiac function and attenuating myocardial hypertrophy and fibrosis after organ injury.

To investigate whether pUR4 may revert the pre-established scar existing in a later stage of HF, animals underwent I/R cardiac surgery, and pUR4 or III-11C treatment was started 4 weeks after I/R for 2 weeks, followed by 2 more weeks of survival; hearts were harvested 2 weeks after peptide administration (Figure XVIA in the online-only Data Supplement). Delayed pUR4–treated hearts exhibited a trend toward reduction of hypertrophy and fibrosis (Figure XVIB and XVIC in the online-only Data Supplement) with no apparent effect on cardiac function (Figure XVID and Table III in the online-only Data Supplement). Although we did not observe significant changes in cardiac remodeling or dysfunction, these data suggest that pUR4 may hold potential to attenuate the pre-established scar 4 weeks after I/R with no protection in cardiac function at this time point.

Inducible Fibroblast-Specific FN Ablation Is Protective After Cardiac Injury

On the basis of the salutary effects of systemic pUR4 treatment after I/R, the therapeutic efficacy of attenuating local FN polymerization was further evaluated with fibroblast-restricted FN-KO mice. These mice were further treated with III-11C or pUR4 to characterize cell specificity of FN in the heart. *Fn*$^{flox/flox}$ (FN$^{+/+}$) animals were crossed with mice expressing inducible Cre recombinase under the control of Tcf21 promoter (Tcf-21^{mERCremER}).[33] These mice allow conditional ablation of FN gene expression on tamoxifen administration in essentially all cardiac resident fibroblasts and in their activated MF progeny.[34] Tamoxifen chow diet was initiated

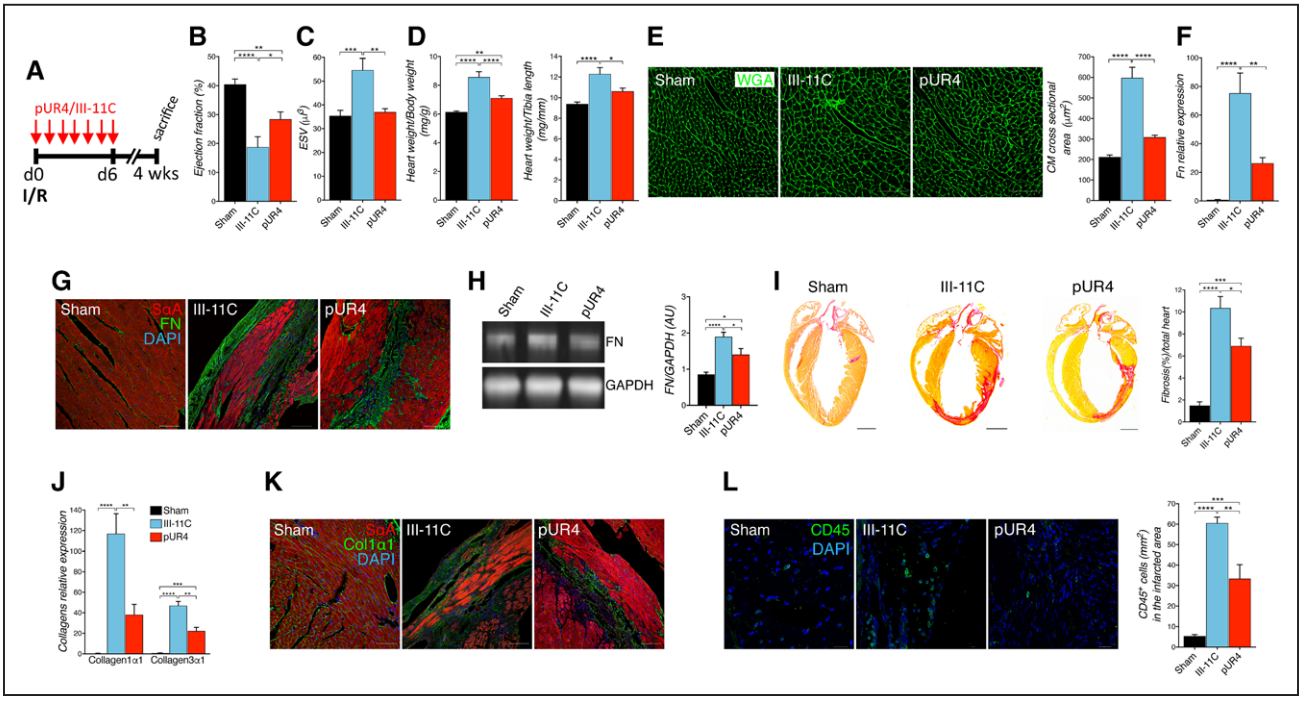

Figure 6. Seven days of pUR4 preserves cardiac function and reduces remodeling up to 4 weeks after ischemia/reperfusion (I/R).
A, Animals received daily intraperitoneal peptide injection for 7 days immediately after cardiac surgery and were followed up for 4 weeks total. **B**, Cardiac function evaluated by echocardiography shown by percent ejection fraction at 4 weeks after I/R. n=8 to 10 per group. **C**, Cardiac dilation assessed by echocardiography shown by end-systolic volume (ESV) 4 weeks after I/R. n=8 to 10 per group. **D**, Cardiac morphometry is shown as heart weight to body weight ratio (**left**) and heart weight to tibia length ratio (**right**). n=11 to 14 per group. **E**, Representative cardiomyocyte cross-sectional images of wheat germ agglutinin (WGA) staining (green) in the infarct border zone of the indicated treatment (**left**) and quantification (**right**). Scale bars, 100 μm. n=5. CF indicates cardiac fibroblasts; and CM, cardiomyocytes. **F**, Fibronectin **(**FN) transcript expression in the left ventricle of the different treatment groups. n=5 to 6. **G**, Representative images of FN staining obtained in the infarcted area of I/R groups or in the left ventricles of sham animals. Scale bars, 100 μm. SαA indicates sarcomeric α-actinin. **H**, FN expression in the left ventricle of the indicated treatments was determined by Western blotting. n=6. **I**, Fibrotic scar formation was evaluated by Picrosirius Red staining (representative pictures, **left**; quantification, **right**). n=5. **J**, Collagen transcript expression in the left ventricle of the different treatment groups. n=5 to 6. **K**, Representative images of collagen staining obtained from infarcted area of I/R groups or in the left ventricles of sham animals. Scale bars, 100 μm. **L**, Inflammatory cells recruited in the infarcted area were detected with CD45 marker (green) (representative pictures, **left**; quantification, right). Scale bars, 10 μm. n=5 to 6. Data are presented as mean±SEM. Statistical significance was determined with 1-way ANOVA with Tukey post hoc analysis. *P<0.05. **P<0.01. ***P<0.001. ****P<0.001.

2 weeks before surgery and continued for 4 weeks to conditionally ablate FN gene expression in both resident and activated fibroblasts (Figure 7A). To determine gene knockdown, quantitative polymerase chain reaction and immunostaining analysis for FN were performed in freshly isolated CFs from DTG mice with or without tamoxifen (Figure XVII in the online-only Data Supplement). It is remarkable that mice in which FN was ablated in Tcf21^{+} fibroblast lineage offered a significant preservation of cardiac function after I/R as measured by improved percent ejection fraction (Figure 7B) and reduced cardiac dilation (Figure 7C) compared with FN$^{+/+}$ control mice (Table IV in the online-only Data Supplement). Administration of the pUR4 FN inhibitor after I/R injury to DTG mice did not further improve cardiac function (Figure 7B and 7C). In addition, restricted CF-FN-KO mice exhibited ameliorated pathological changes in cardiac morphometry treated with III-11C or pUR4 (Figure 7D). The observed attenuation in cardiac hypertrophy was confirmed by a reduction in CM size in CF-FN-KO animals (Figure 7E); however, pUR4 treatment did not further attenuate CM hypertrophy (Figure 7E).

To examine the effects of FN genetic ablation in cardiac fibrosis, hearts of DTG mice with tamoxifen treated with III-11C or pUR4 were histologically assessed. Collagen content was significantly reduced in CF-FN-KO mice with no further changes with pUR4 treatment (Figure 7F). In addition, immunofluorescence staining for FN and periostin revealed a decreased FN expression in DTG mice compared with FN$^{+/+}$ controls after injury and tamoxifen administration (Figure 7G and 7H).

Together, these data demonstrate that animals in which FN was ablated in CF populations exhibited preserved cardiac function and reduced hypertrophy and fibrosis in vivo. These findings may be the result of a reduced post-I/R fibrotic scar as a consequence of the attenuation of pathological FN and collagen deposition derived from CFs.

DISCUSSION

Myocardial ischemia after coronary artery obstruction triggers the recruitment and activation of resident and quiescent CFs. These CFs are stimulated by the release of cytokines, chemokines, and growth factors, as well as by mechanical forces,[48] which alter activation patterns in the myocardium, promoting their differentiation to MFs.[49] MFs are a highly proliferative, migratory, and secretory cell type involved in the pathological remodeling of the myocardium on injury, promoting cardiac fibrosis, which ultimately leads to HF. Although MFs initially trigger a reparative wound healing response, prolonged persistence of MF activity is detrimental for organ compliance.

Production and polymerization of FN are both elevated in clinical and experimental models of HF.[37,38] Polymerization of FN tightly regulates the assembly of different ECM proteins, including collagen type I.[19,20,50] These processes are necessary for normal tissue repair but become exacerbated during fibrosis. However, the impact of inhibiting FN polymerization or targeting its gene expression in cardiac fibrosis and organ function after injury had not been elucidated.

In the present study, the functional role of FN matrix deposition in cardiac remodeling was evaluated with the adhesin-based peptide pUR4, which specifically binds to FN and inhibits its cell-mediated polymerization. Administration of pUR4 for 7 days after cardiac I/R preserved cardiac function, reduced FN and collagen deposition, and attenuated pathological cardiac remodeling, cardiac fibrosis, and myocardial neutrophil infiltration up to 4 weeks after I/R. Similar results were obtained in vitro, where pUR4 attenuated key properties of primary mouse and human failing cardiac MFs such as proliferation and migration. Our data demonstrate that pUR4 blocked FN polymerization in the extracellular space in primary mouse CFs isolated from unchallenged hearts. However, pUR4-treated cells did not show a decrease in FN transcript level or intracellular protein content. These observations are in agreement with previous studies[31,32] showing that pUR4 administration does not interfere with FN transcript level expression or intracellular protein content.

The ECM microenvironment modulates cell phenotype and function. FN is known to be required during tissue homeostasis and after injury wound healing, influencing a variety of crucial cellular processes, including proliferation and migration. The in vitro data demonstrate that pUR4 blocks the deposition of FN into ECM fibrils in primary cardiac MFs isolated from mouse hearts after I/R. In addition, decreasing FN polymerization rendered a concomitant reduction of collagen deposition and maturation into the ECM measured by immunocytochemistry and Western blotting. These data are consistent with several studies demonstrating the requirement of an established FN network to nucleate the elaboration of a collagen matrix into the ECM.[19,20,51] Mouse MFs exhibited a significant reduction in cell proliferation, likely mediated through c-myc signaling pathway downregulation. Previous in vitro studies have demonstrated the role of FN in cell proliferation through c-myc axis regulation,[23] pinpointing its central role as a master regulator of cellular proliferation and its essential requirement for cell cycle progression.[52,53] FN polymerization also promotes migration of a variety of cell types, including MFs.[19,24,25] Therefore, our data are consistent with previous reports that recognize a positive effect of FN polymerization on cell migration. Furthermore, depletion of FN deposition promoted an increase of β1 integrin internalization accompanied by a reduction of phosphorylated FAK protein level and a

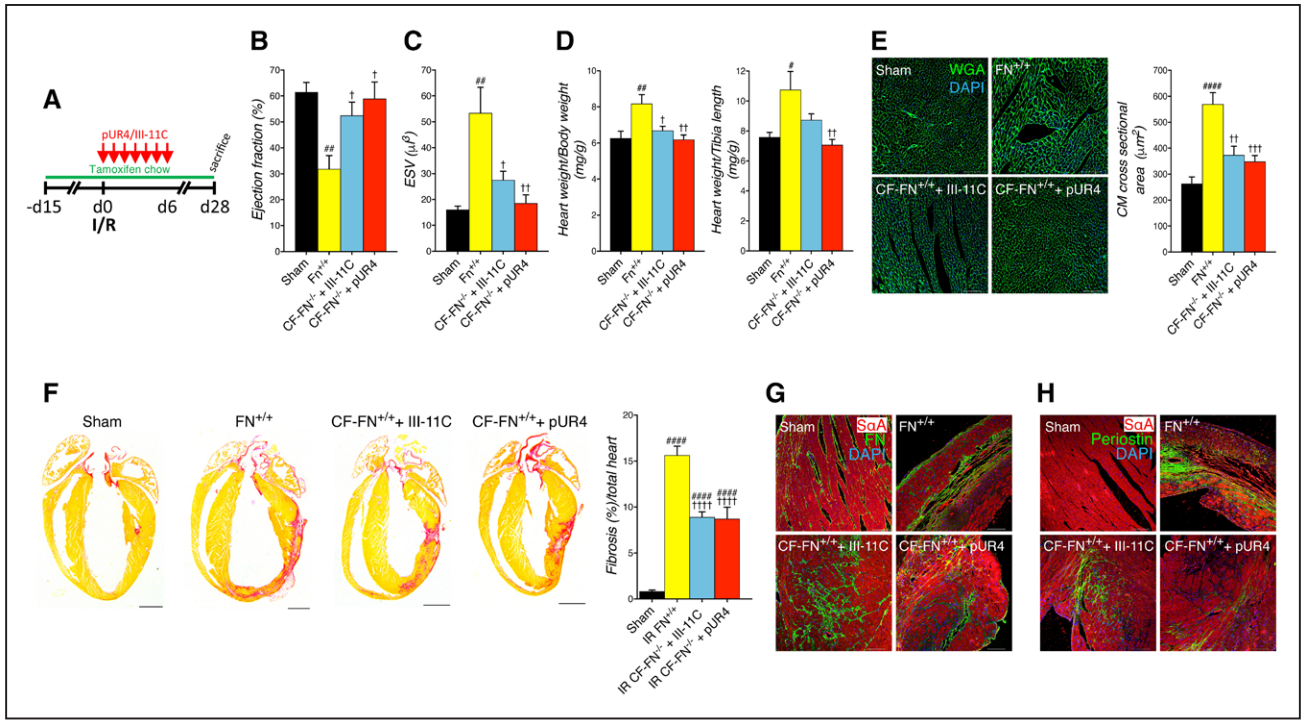

Figure 7. Effect of fibronectin (FN) genetic ablation in cardiac fibroblasts (CFs).
A, Animals were fed tamoxifen chow diet 15 days before cardiac surgery, continued on it for 4 weeks after the injury, and received daily intraperitoneal peptide injection for 7 days immediately after ischemia/reperfusion (I/R). Animals were followed up for 4 weeks total. **B**, Cardiac function evaluated by echocardiography shown by percent ejection fraction at 4 weeks after I/R. **C**, Cardiac dilation assessed by echocardiography shown by end-systolic volume (ESV) 4 weeks after I/R. **D**, Cardiac morphometry represented as heart weight to body weight ratio (**left**) and heart weight to tibia length ratio (**right**). **E**, Representative cardiomyocyte (CM) cross-sectional images of wheat germ agglutinin (WGA) staining (green) and DAPI (blue) in the infarct border zone of the indicated treatment (**left**) and quantification (**right**). Scale bars, 100 μm. **F**, Fibrotic scar formation was evaluated by Picrosirius Red staining (representative pictures, **left**; quantification, **right**). Scale bars, 1000 μm. **G**, Representative images for FN staining from the infarcted area of the mentioned experimental groups. Scale bars, 100 μm. **H**, Representative images for periostin staining from the infarcted area of the mentioned experimental groups. Scale bars, 100 μm. *Fn*$^{+/+}$: *Fn*$^{flox/flox}$; CF-FN$^{-/-}$: Tcf21^{mERCremER};*Fn*$^{flox/flox}$. Sarcomeric α-actinin (SαA). Statistical significance was determined with 1-way ANOVA with Tukey post hoc analysis. Data are presented as mean±SEM. n=6 to 7 per group. #*P*<0.05 vs sham. ##*P*<0.01 vs sham. ####*P*<0.0001 vs sham. †*P*<0.05 vs *Fn*$^{+/+}$. ††*P*<0.01 vs *Fn*$^{+/+}$. †††*P*<0.001 vs *Fn*$^{+/+}$. ††††*P*<0.0001 vs *Fn*$^{+/+}$.

decrease in α-SMA expression. Numerous studies have demonstrated that FN typically binds to α5β1 integrins[54] and that cessation of FN polymerization triggers the internalization of β1 integrins in a caveolin-1–dependent manner.[55] In addition, FAKs plays a critical role in cell migration,[56] localizing with integrins at focal contact sites and activated by cell binding to ECM proteins such as FN.[57] Our results suggest that pUR4 treatment of primary mouse CFs promotes integrin internalization on inhibition of FN polymerization with the consequent disruption of p-FAK localization and expression, hence decreasing the mobility of pUR4-treated cells. Similar results were observed in pUR4-treated human failing CFs. As with murine CFs, FN remarkably attenuated the ability of the failing human CFs to proliferate and migrate, exhibiting a downregulation in c-myc and p-FAK pathways and α-SMA expression. Collectively, the findings in mouse and human HF MFs highlight the crucial role of FN in modulating cell behavior and the well-established relationship between FN and cell proliferation and migration. Our in vitro human data in failing MFs after pUR4 treatment suggest therapeutic promise for the treatment of HF.

The positive role that FN exerts in cell survival, thus inhibiting apoptosis, has been previously reported.[23,58] Our data demonstrate that inhibiting FN matrix formation does not significantly alter cell survival, suggesting that this process may rely on other mechanisms or that the low levels of FN deposited into the cell surface are sufficient to maintain cell survival. In addition, no differences in reactive oxygen species production or total mitochondria content were observed in MFs after pUR4 administration, suggesting that inhibiting FN polymerization does not interfere with normal mitochondrial functions. Plus, pUR4 did not alter cellular metabolism, as evidenced by unaffected ATP production, oxygen consumption, glycolysis, and fatty acid oxidation. Together, these data suggest that the attenuation of proliferation and migration detected in MFs on pUR4 administration is not attributed to survival and metabolic state, highlighting that blocking FN polymerization directly affects specific aspects of cellular behavior.

Although FN expression levels are low in unchallenged hearts,[59,60] its expression is elevated on cardiac injury in both mouse and human hearts.[60,61] Daily pUR4 peptide treatment, initiated the same day as I/R injury,

decreased fibrosis-related genes, including FN and collagens, in the LV of hearts 7 days after I/R. pUR4 was sufficient to decrease FN expression and accumulation in the heart. Concurrently, in vivo EdU pulse-chase experiments revealed a significant decrease of proliferative fibroblasts in both remote and infarcted areas 7 days after cardiac insult. These in vivo data are consistent with many of our in vitro results demonstrating the role of pUR4 in attenuating cell proliferation.

The tight balance between MMPs and TIMPs regulates ECM homeostasis.[62] However, after cardiac injury, several MMPs and TIMPs are significantly increased in the myocardium,[63] contributing to the development and progression of pathological ventricular remodeling. pUR4 treatment displayed a downward trend in MMP-9 activity and expression and a significant downregulation of TIMP-1, suggesting that blocking FN deposition may attenuate the imbalance of MMPs and TIMPS that occurs after cardiac injury.

Inflammation plays a critical role in adverse ventricular remodeling and decreased cardiac function after injury.[42] In this context, TLRs are proposed to be one of the main targets for endogenous ligands released after cardiac insult to promote the recruitment of inflammatory cells into the injured myocardium.[64] FN acts as a ligand for TLR-2 and TLR-4. We found TLR-2 expression to be significantly reduced in the LV of pUR4-treated animals 7 days after I/R. Furthermore, the in vitro adhesion studies indicated that pUR4 significantly reduced neutrophil adhesion to tumor necrosis factor-α–activated MHECs, which may explain the decreased myocardial neutrophil recruitment observed in vivo after I/R cardiac injury. The observation that pUR4 selectively affects neutrophil arrest, but not neutrophil rolling, suggests that pUR4 regulates the expression of heart endothelial cell integrin ligands, but not selectins, involved in the firm arrest and rolling steps of the leukocyte recruitment cascade, respectively. These data are consistent with published data showing that targeting FN expression or polymerization resulted in a suppressed inflammatory response.[31,32,65] In fact, the data demonstrate that inhibiting FN polymerization does not affect neutrophil integrins involved in cell recruitment, suggesting that the observed effects of pUR4 in neutrophil recruitment in vivo and in neutrophil arrest in vitro are mediated by pUR4 effects on heart endothelial cell integrin ligands (VCAM-1 and ICAM-1). Although flow cytometry data show that overall pUR4 does not significantly decrease ICAM-1 and VCAM-1 surface expression, immunocytochemistry analysis revealed decreased total expression of both ligands. Several reports have demonstrated that activated neutrophils trigger an intense oxidative response[66,67] and produce proteases and collagenases[68] to further contribute to the pathological remodeling and the detrimental organ dysfunction exhibited after cardiac injury. Our observations that pUR4 specifically prevents neutrophil myocardial infiltration after I/R in vivo suggest a significant role of FN in recruiting neutrophils to the injured myocardium, potentially through a regulation of TLRs and heart endothelial cell adhesion molecules involved in neutrophil recruitment.

As outlined earlier, exaggerated ECM remodeling accelerates the pathological functional decline associated with HF progression, eliciting the formation of a mature scar that severely compromises organ compliance. Therefore, experiments were performed to test whether 7 days of daily pUR4 to inhibit FN immediately after I/R would be salutary after myocardial injury up to 28 days after I/R. It is remarkable that 7 days of pUR4 treatment significantly reduced myocardial dysfunction up to 4 weeks after I/R as measured by echocardiography. In addition, pUR4 attenuated pathological changes in cardiac morphometry. Seven days of pUR4 peptide injections dramatically reduced pathological cardiac remodeling and fibrosis as observed up to 4 weeks after I/R by a decrease in FN and collagen deposition in the LV. The decrease of FN in the heart after pUR4 treatment after injury reduced the fibrotic scar in the myocardium. Furthermore, delayed pUR4 treatment 4 weeks after I/R indicated a favorable downward trend in cardiac hypertrophy and fibrosis with no significant change in cardiac function compared with III-11C treatment. Although we could not detect a significant reduction in cardiac function at this stage despite a trend toward a reduction of hypertrophy and fibrosis, this may be the result of the short period of time of peptide administration initiated 4 weeks after I/R and the cessation of therapy for 2 weeks before sample analysis. Future experiments will further establish the optimal dose and timing of treatment to potentially revert pathological aspects of the pre-established scar concomitant with improvement in cardiac function.

Because CFs are believed to be the most abundant contributors to FN production in the heart, we sought to validate the salutary effects observed with systemic pUR4 delivery in our novel, inducible, fibroblast-specific CF-FN-KO. To this end, FN expression was ablated in Tcf21^{+}-expressing cells (quiescent CFs before I/R and their activated MF progeny after cardiac injury).[33,34] FN ablation in Tcf21^{+}-derived CF lineage attenuated cardiac hypertrophy and pathological fibrosis, including reduced CM cross-sectional area and collagen content. Addition of the FN inhibitor peptide pUR4 did not further preserve cardiac function in CF-FN-KO compared with III-11C. These findings suggest that the cardioprotective effects proffered by ablating FN expression or attenuating FN polymerization may be mediated through effects on FN from the CF lineage.

Although FN has been extensively studied, the role of blocking its polymerization or genetic ablation in CF populations in regulating cardiac cell behavior and the development of cardiac fibrosis has not yet been eluci-

dated. Our data are the first to demonstrate that suppressing FN matrix deposition with a peptide to attenuate FN polymerization or ablating its gene expression specifically in CF populations preserves cardiac function, attenuates adverse LV remodeling, and limits cardiac fibrosis in a mouse model of HF. These findings reinforce the importance of understanding the role of FN and its polymerization in the heart, both during homeostasis and after cardiac injury, and suggest that targeting FN polymerization may be a new therapeutic strategy for treating cardiac fibrosis and HF.

ARTICLE INFORMATION

Received July 12, 2017; accepted March 23, 2018.

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCULATIONAHA.118.034609.

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Acknowledgments

The authors thank Evan Meyer and Matthew Kofron for microscopy assistance, Philip Khoury and Jareen Meinzen-Derr for statistical support, and Michelle Tallquist for generously sharing the Tcf21-inducible Cre mice.

Sources of Funding

This work was supported in part by National Institutes of Health grants R01 HL132551, R01 HL133695, R01 HL134312 (Dr Blaxall), P01 HL069779 (Dr Blaxall and Molkentin), an American Heart Association–Great Rivers Affiliate Post-Doctoral Fellowship (No. 16POST30180015, Dr Valiente-Alandi), R35 GM119458 (Dr DeFalco), and National Institutes of Health grant R01 HL123658 (Dr Alcaide).

Disclosures

None.

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