MECHANISMS WHEREBY INSULIN-LIKE GROWTH FACTOR-1
PROMOTES ATHEROSCLEROTIC PLAQUE STABILITY

AN ABSTRACT
SUBMITTED ON THE 13TH DAY OF MARCH 2014
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OF THE SCHOOL OF MEDICINE
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FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
BY

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ABSTRACT

Rupture of atherosclerotic plaques can cause acute life-threatening events such as myocardial infarction and ischemic stroke; therefore, there is much interest in developing therapies aimed at increasing plaque stability. More stable lesions are characterized as having high collagen content and containing a large number of vascular smooth muscle cells (SMCs) of contractile/differentiated phenotype. In our previous studies using an apolipoprotein E-deficient (Apoe<sup>-/-</sup>) mouse model of atherosclerosis, we found that insulin-like growth factor-1 (IGF-1)-infusion not only reduced total plaque burden, but also increased collagen expression and the number of alpha-smooth muscle actin (αSMA)-positive cells in plaque. In this study, we identify cellular mechanisms responsible for these observations. We found that in human aortic smooth muscle cells (HASMCs) grown in culture, IGF-1 post-transcriptionally upregulated expression of the procollagen type I α1(I) as well as contractile proteins, αSMA and smooth muscle 22-alpha (SM22α), via a PI3K-dependent but Erk1/2- and mTOR-independent signaling mechanism. Furthermore, experiments using an inhibitor of collagen synthesis (prolyl-hydroxylase inhibitor) or a blocking antibody against the alpha2beta1-integrin (α2β1) suggested that interaction with collagen type I promotes HASMC contractile phenotype. To elucidate mechanisms underlying IGF-1 upregulation of collagen synthesis we investigated the effect of IGF-1 on the mRNA-binding protein, la ribonucleoprotein domain family member 6 (LARP6), which had been shown to bind a conserved stem-loop secondary motif in the 5’UTR of COL1a1 and COL1a2
mRNA. IGF-1 rapidly increased LARP6 expression in HASMCs leading to increased
*COL1a1* and *COL1a2* mRNA bound to LARP6 and increased synthesis of collagen
type I. Mutation of the 5’stem-loop of *Col1a1* mRNA (that inhibited binding by
LARP6) or overexpression of a 5’stem-loop RNA molecular decoy (that sequesters
LARP6) both prevented the ability of IGF-1 to increase pro-α1(I) synthesis as well as
mature α1(I) expression in cultured medium. Furthermore, IGF-1-infusion in Apoe^{−/−}
mice increased LARP6 and pro-α1(I) expression in aortic lysates, and SMC-specific
IGF-1-overexpression in transgenic mice robustly increased collagen fibrillogenesis
in atherosclerotic plaque. In conclusion, this work identifies LARP6 as a critical
mediator by which IGF-1 augments synthesis of collagen type I in vascular smooth
muscle, and uncovers key mechanisms whereby IGF-1 promotes atherosclerotic
plaque stability.
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# Table of Contents

## I. Introduction

- Atherosclerosis ................................................................. 1
  - Prevalence and risk factors
  - Primary treatments
  - Etiology of disease

- Insulin-like Growth Factor -1............................................ 5
  - Endocrine and autocrine/paracrine hormone

- Original Studies ................................................................. 7
  - Protective role of IGF-1 in atherosclerosis

- Atherosclerotic Plaque Stability........................................ 12
  - Scope and overall goal

## II. Materials and Methods

- Animals ............................................................................. 15

- Atherosclerosis Burden Quantification ................................. 16

- Immunohistochemistry (IHC) ............................................... 17

- Quantification of Cell Apoptosis .......................................... 17

- Cell Culture ......................................................................... 18

- Western Blot ......................................................................... 19

- Real-time Reverse Transcriptase-Polymerase Chain Reaction 20

- Exogenous Collagen Assembly Assay................................. 20

- Collagen Synthesis and Extracellular Accumulation ............ 22

- Pharmacological Inhibitors .................................................. 23
III. **Aim 1: Identify Mechanisms Whereby IGF-1 Induces Vascular Smooth Muscle Cell Contractile Phenotype and Collagen Expression**

- **Rationale** ................................................................. 27  
  - VSMC role in plaque stability  
  - Contractile phenotype and collagen expression  
  - IGF-1-signal transduction  

- **Results** ........................................................................ 31  
  - Pro-\(\alpha_1(I)\), \(\alpha\)SMA, and SM22\(\alpha\) expression  
  - Posttranscriptional regulation of pro-\(\alpha_1(I)\)  
  - Posttranscriptional regulation of \(\alpha\)SMA and SM22\(\alpha\)  
  - Translation-dependent regulation of pro-\(\alpha_1(I)\) and \(\alpha\)SMA  
  - PI3K-dependent and Erk1/2-independent signaling  
  - mTOR-independent regulation  
  - Effect of collagen on VSMC contractile phenotype  
  - Exogenous collagen type I polymerization and degradation  

- **Summary** ........................................................................ 47  

IV. **Aim 2: Determine The Role of the Collagen Type I mRNA-Binding Protein, LARP6, in the Ability of IGF-1 to Enhance Collagen Synthesis**

- **Rationale** ................................................................. 49  
  - Collagen synthesis and structure  
  - LARP6 and 5’Stem-loop of collagen type I mRNA  

- **Results** ........................................................................ 56  
  - Collagen type I synthesis and extracellular accumulation  
  - Expression of the mRNA-binding protein, LARP6  
  - Pro-\(\alpha_1(I)\) expression correlates with LARP6 expression  
  - LARP6 expression in cytosolic and nuclear fractions.
o Association of LARP6 with COL1a1 and COL1a2 mRNA
o Posttranscriptional and translation-dependent regulation of LARP6 expression
o PI3K/Akt-mediated LARP6 and proc1(I) expression
o Collagen type I synthesis in 5’stem-loop mutant MEFs
o 5’Stem-loop RNA molecular decoy for LARP6
o LARP6 expression in vivo
o Collagen fibrillogenesis in atherosclerotic plaque

- Summary………………………………………………………………………………82

V.  DISCUSSION………………………………………………………………………………84
    o Interpretation and Conclusions
    o Implications and Significance

VI. LIST OF REFERENCES ……………………………………………………………….93
LIST OF FIGURES

Figure 1. Development and Progression of Atherosclerosis ..................... 4
Figure 2. GH/IGF-1 Axis ................................................................. 6
Figure 3. Roles of IGF-1 in Atherogenesis ....................................... 8
Figure 4. En Face Analysis of Atherosclerosis In Vivo ......................... 10
Figure 5. VSMC Number and Apoptosis in Plaque .............................. 11
Figure 6. IGF-1 Increases Collagen Content in Aortic Plaque ............... 13
Figure 7. IGF-1 Increases SMC Contractile Protein Expression in Plaque .. 14
Figure 8. Modes of IGF-1 Signal Transduction .................................. 30
Figure 9. Collagen Type I, αSMA, and SM22α Expression in HASMCs ....... 32
Figure 10. Posttranscriptional Regulation of Pro-α1(I) ......................... 34
Figure 11. Posttranscriptional Regulation of αSMA and SM22α .............. 35
Figure 12. Translation-dependent Regulation of Pro-α1(I) and αSMA .......... 36
Figure 13. PI3K-dependent and Erk-1/2-Independent Signaling ............... 38
Figure 14. mTOR-independent Upregulation of Protein Synthesis .......... 39
Figure 15. Effect of Collagen on HASMC Contractile Phenotype .......... 42
Figure 16. Effect of Prolyl-Hydroxylase Inhibitor on Expression .......... 43
Figure 17. Exogenous Collagen Synthesis and Degradation in HASMCs .... 46
Figure 18. Schematic of Procollagen Type I Subunit Molecule ............... 51
Figure 19. COL1a1 mRNA 5’Stem-loop Structure and Sequence Homology ... 53
Figure 20. Schematic of LARP6 Protein Domains ............................... 55
Figure 21. Time-dependent Pro-α1(I) Synthesis in HASMCs .................. 57
Figure 22. Extracellular Accumulation of Collagen Type I ...................... 59
Figure 23. Dose-dependent Increase in LARP6 Protein Expression............. 61
Figure 24. Correlation in Expression of LARP6 and Pro-α1(I).................... 63
Figure 25. LARP6 Expression in Cytosolic and Nuclear Fractions..............65
Figure 26. LARP6 Association with COL1a1 and COL1a2 mRNA .............. 67
Figure 27. IGF-1 Modes of Regulating of LARP6 Expression....................69
Figure 28. 3H-Proline Accumulation in 5’Stem-loop Mutant MEFs..............71
Figure 29. Pro-α1(I) and LARP6 Expression in 5’Stem-loop Mutant MEFs……73
Figure 30. 5’Stem-loop mRNA Decoy Constructs.....................................76
Figure 31. Collagen Type I Expression In Presence of 5’Stem-loop Decoy.....77
Figure 32. Aortic LARP6 and Pro-α1(I) Expression In Vivo....................... 79
Figure 33. Collagen Fibrillogenesis in Atherosclerotic Plaque of
SMC-specific IGF-1-overexpressing Transgenic Mice..................... 81
INTRODUCTION

Atherosclerosis

Atherosclerosis is the deadliest disease in the Western world and the primary cause of cardiovascular events, including myocardial infarction and ischemic stroke \(^1\). Atherosclerosis is an inflammatory disease characterized by the accumulation of cholesterol-rich calcified plaque along the vascular wall causing “hardening of the arteries.” Plaque build-up, such as in the coronary or carotid arteries, can result in arterial stenosis and restriction of vital blood supply. Plaque rupture can cause a thrombosis or embolus resulting in an acute ischemic emergency. Major risk factors for developing atherosclerosis include diabetes mellitus, cigarette smoking, family history of cardiovascular disease, and hyperlipidemia \(^2\). Current preventative methods include lifestyle changes such as exercise and dietary restriction, and pharmacological treatments such as antithrombotic and anti-cholesterol therapies. The primary treatments for atherosclerosis-caused ischemia include angioplasty with stenting, coronary artery bypass graft (CABG), or carotid endarterectomy \(^3,4\).
Although much of the underlying pathology remains obscure, atherosclerosis is generally understood as an inflammatory disease that involves the oxidation of lipids in the circulation, which become incorporated into the vessel wall and perpetuate further inflammation. Figure 1 provides a simplified view of the development and progression of atherosclerosis.
Figure 1. Cartoon depicts sequential steps in the progression of atherosclerosis. Modified from Cho, Y. et al. J Am Coll Cardiol 2011;57:99-10. 1-3. High circulating lipids and reactive oxygen species (ROS) cause oxidation of low-density lipoproteins (LDL → oxLDL) and stimulate endothelial cells, smooth muscle cells, and macrophages to secrete various inflammatory cytokines/chemokines including TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-18, MCP-1 and CXCL-1,10,11,12. 4-5. These paracrine/autocrine mediators act on vascular smooth muscle cells (VSMCs) and endothelial cells to activate their expression of vascular cell adhesion molecule (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1), resulting in the recruitment of monocytes and their invasion into the vascular wall. 6-8. Uptake of oxLDL results in activation of monocyte/macrophage foam cell formation, thereby causing retention of macrophages in the plaque and secretion of cytokines, chemokines, lipid mediators, and matrix metalloproteinases (MMPs). 9. These inflammatory mediators trigger VSMC dedifferentiation, characterized by a loss of contractile protein expression and increased proliferation and migration. 10. Sustained inflammation and ROS generation in plaque causes macrophages and VSMCs to undergo apoptosis and addition to necrotic core. Furthermore, activation of MMPs causes degradation of collagen and extracellular matrix (ECM) structural proteins leading to plaque instability.
Insulin-like Growth Factor-1

In efforts to find a treatment or prevention of the atherosclerotic vascular disease, our laboratory has extensively studied the role of the endogenous peptide hormone, insulin-like growth factor-1 (IGF-1), throughout the cardiovascular system. IGF-1 acts as an endocrine hormone, produced by the liver in response to growth hormone (GH), to mediate most of the effects of GH throughout the body via signaling through the IGF-1-receptor. IGF-binding proteins (IGFBP-1 through IGFBP-6) are present in the circulation and regulate the molecular stability of IGF-1 and its affinity for binding and activating the IGF-1-receptor. IGF-1 is also synthesized locally by most cell types and exerts autocrine and paracrine effects on surrounding tissue. Overall, IGF-1 has essential roles in growth, metabolism, survival, and homeostasis.
**Figure 2. GH/IGF-1 axis.** Diagram indicates sources of growth hormone (GH) and insulin-like growth factor (IGF-1) and their modes of action.
Original Studies

While IGF-1 serum levels help regulate IGF-1-signaling, the relative expression density of IGF-1 receptor (IGF-1R) on the cell surface is important in mediating sensitivity to IGF-1. Macrophages, VSMCs, and endothelial cells all express the IGF-1R and thus exhibit responses to IGF-1. A previous study suggested an inverse relationship between IGF-1-signaling and disease; namely, both early and advanced-stage atherosclerotic lesions from patients undergoing aortic, carotid, or femoral artery surgery showed reduced expression of IGF-1R and IGF-1. Intriguingly, IGF-1 levels can be elevated in obesity, a major risk factor for developing atherosclerosis. However, models of diet-induced obesity and type II diabetes mellitus have also been shown to display reduced sensitivity to the glucose-lowering and vasodilatory effects of IGF-1, thus correlating diminished IGF-1-signaling with the pathogenesis of cardiovascular disease. Therefore an elevation in circulating IGF-1 in obesity states may be a compensatory effect to counteract IGF-1-resistance. Also, data from the Framingham Heart Study show an inverse relation between the incidence of metabolic syndrome and IGF-1 levels. Our laboratory has explored the role of IGF-1 in the vasculature and found IGF-1 to have a broad range of protective effects. Figure 3 summarizes our findings of the effects of IGF-1 in atherogenesis.
Figure 3. Effects of insulin-like growth factor-1 (IGF-1) on cell types involved in atherogenesis. IGF-1 exerts cell type specific effects and also alters cell–cell interactions, leading to a reduction in atherosclerosis and a more stable atherosclerotic plaque phenotype.
In our studies using an apolipoprotein E-deficient (Apoe<sup>−/−</sup>) mouse model of atherosclerosis, we determined that IGF-1-infusion increased endothelial nitric oxide synthase (eNOS) expression, decreased vascular oxidative stress, and reduced total plaque surface area<sup>13</sup>. Because endothelial dysfunction is highly associated with cardiovascular disease, the ability of IGF-1 to increase eNOS expression, and therefore nitric oxide (NO) production, seemed likely to be a key mechanism of IGF-1-induced atheroprotection. Further in vivo studies from our group using a pan-NOS inhibitor, L-arginine methyl ester hydrochloride (L-NAME), determined that the ability of IGF-1 to decrease oxidative stress and suppress TNF-α levels was dependent on induction of nitric oxide production. However, while NOS-inhibition worsened atherosclerotic burden, IGF-1 reduced total plaque surface area, suppressed apoptosis, and increased αSMA-positive cells in plaque in both the L-NAME and D-NAME (control) groups, therefore demonstrating that IGF-1 provides NO-independent protection against atherosclerosis<sup>14</sup>. 
Figure 4. IGF-1 reduced total atherosclerotic burden in a nitric oxide-independent manner. Apoe-null mice were given continuous infusion with saline or IGF-1 and fed a Western-type diet in presence of L-NAME (pan NOS-inhibitor) or D-NAME (control) for 12-weeks. Aortas were stained en face with Oil Red O and lesion surface area was quantified by manually outlining stain using Image Pro software.
Figure 5. IGF-1 increases contractile protein expression and reduces apoptosis within plaque in an nitric oxide-independent manner. Immunohistochemistry was performed on aortic valve cross-sections, in which terminal dUTP nick end labeling (TUNEL)-stain was used to identify apoptotic cells, αSMA was used as SMC contractile protein marker, and total cell number in the plaque was determined using DAPI nuclear stain. Representative images are shown for each group (n=12), and p-values are indicated.
Atherosclerotic Plaque Stability

In humans, acute cardiovascular events such as myocardial infarction or ischemic stroke most often result from a thrombus or embolus due to plaque rupture. Physical disruption to the plaque’s fibrous cap exposes the thrombogenic elements of the plaque’s core to the coagulation proteins in the blood, which can trigger formation of thrombus or embolus. Therefore, there is much interest in developing therapies aimed at increasing plaque stability. Plaque rupture generally occurs in lesions that have thin, collagen-poor fibrous caps with few contractile smooth muscle cells and abundant macrophages. Likewise, stable plaques are characterized as having abundant collagen content and many smooth muscle cells of contractile phenotype. Our previous studies in-vivo have shown that smooth muscle cell-specific IGF-1-overexpression increased contractile protein expression and collagen content in atherosclerotic plaque, suggesting that IGF-1 promotes plaque stability. The overall goal of this dissertation is to identify the molecular mechanisms whereby IGF-1 induces these features of plaque stability.
Figure 6. IGF-1-infusion increases collagen content in plaque. Shown is aortic root cross-sections from Apoe \( ^{-/-} \) mice given continuous infusion of either IGF-1 (1.5 mg/kg/day) or saline control. Immunohistochemistry (IHC) using Gomori trichrome staining was used to detect collagen (green) surface area within atherosclerotic plaques.

**Figure 7.** Smooth muscle cell-specific IGF-1-overexpression increases contractile protein expression in plaque. Immunohistochemistry was performed on cross-sections of aortic valve atherosclerotic plaque from Apo E deficient (Apoe<sup>−/−</sup>) mice or smooth muscle cell-specific IGF-1-overexpressing Apoe<sup>−/−</sup> transgenic mice (SMP8/Apoe<sup>−/−</sup>) to detect expression of αSMA, SM22α, and calponin.


MATeRIALS AND METhODS

Animals

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee. Apoe−/− mice of C57BL/6 background (Jackson Lab) were infused with saline or human recombinant IGF-1 (1.5 mg/kg/day) using subcutaneously implanted osmotic minipumps (ALZET, Cupertino, CA). In this model of atherosclerosis, Apoe−/− mice were fed a high fat, Western diet (42% of total calories from fat, 0.15% cholesterol, Harlan, Indianapolis, IN) for 12 weeks while given continuous infusion of either IGF-1 (1.5 mg/kg/day) or saline (control). For analysis of LARP6 expression in vivo, mice were sacrificed after 5-day continuous IGF-1-infusion and whole aorta was isolated and cleaned, followed by mechanical homogenization in RIPA cell-lysis buffer. Human and mouse IGF-1 levels in serum at time of sacrifice were determined via ELISA (Diagnostic Systems Laboratories, Webster, TX).

SMP8-IGF-1 transgenic mice (in an FVB background) were obtained from Dr. James A. Fagin (University of Cincinnati, Cincinnati, Ohio) 18. SMP8-IGF-1 transgene–
positive mice (SMP8) were identified by polymerase chain reaction (PCR) and bred to Apoe<sup>−/−</sup> (C57BL/6 background) mice for 8 generations before this line was used for experiments. SMP8/Apo<sub>e</sub><sup>−/−</sup> and Apo<sub>e</sub><sup>−/−</sup> mice 8 weeks of age were fed a Western-type diet as described above for 12 weeks to generate atherosclerosis.

**Atherosclerosis Burden Quantification**

Atherosclerosis burden was quantified by measuring surface area of Oil Red O–positive lesions on en face preparations of whole aortas. At sacrifice each mouse was perfused with saline then 4% buffered paraformaldehyde plus 5% sucrose through the left ventricle and then the entire aorta was dissected and placed overnight in formaldehyde. The adventitial fat was dissected, the aorta was stained with Oil Red O and opened longitudinally, pinned en face, and photographed. The total arterial surface area and total lesion area were determined using Image-Pro Plus. The extent of lesion development was defined as percentage of the total area of a given artery that was occupied by Oil Red O–positive atherosclerotic lesions. Additionally, heart was dissected and serial sections (6 μm) were taken throughout the entire aortic valve area as per Paigen et al.,<sup>19</sup> and stained with H&E for quantification of plaque cross-sectional area.
**Immunohistochemistry (IHC)**

Serial aortic valve paraffin-embedded cross sections were stained for smooth muscle cells using an anti-αSMA antibody (Millipore). 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen) was used as a nuclear stain to quantify cell number. Sections were also stained with Gomori’s Trichrome stain to visualize collagen (Richard-Allan Scientific, Kalamazoo, MI). Antibody specificity was verified by staining of serial sections with “normal” IgG (obtained from an unimmunized animal of the same species as primary antibody) and/or by staining with blocked primary antibody.

**Quantification of Cell Apoptosis**

Cell apoptosis was quantified in paraffin-embedded aortic valve cross sections with the Apoptosis TUNEL detection kit from Roche as per manufacturer’s instructions. To identify apoptotic smooth muscle cells (SMC) in the atherosclerotic plaque, TUNEL-stained sections were co-stained with αSMA antibody (1:1000) followed by incubation with biotinylated secondary antibody and streptavidin-Alexa 594 complex (Invitrogen). Sections were mounted with DAPI-contained mounting media (Vector Laboratories Inc). Total cell apoptosis was defined as TUNEL-positive cell number per 1000 plaque cells.
and SMC apoptosis was measured as the number of αSMA/TUNEL-double positive cells per 1000 αSMA-positive cells.

**Cell Culture**

Human aortic smooth muscle cells (Lonza) were cultured in SmBM medium (Lonza) supplemented with 5% fetal calf serum (FCS), antibiotics, human recombinant epidermal growth factor, insulin, and human recombinant fibroblast growth factor. Experiments were performed at cell passages 4 to 9 under serum-free conditions using a 1:1 mixture of Dulbecco’s modified essential medium (DMEM) and F-12 nutrient solution (Gibco).

Mouse Embryonic Fibroblasts (MEFs) from 5’stem-loop mutant (5’SLL/−) or wildtype (WT) C57/BL6 mice were obtained as previously described 20. The 5’SLL/− MEFs contained a 21-nt mutation in the 5’UTR of the COL1a1 gene which disrupted formation of the stem-loop structure, inhibited LARP6 binding, but did not affect the coding region of the COL1a1 gene 20,21. MEFs were cultured in 10% FCS, DMEM, and experiments were performed at passages 4 to 8.
Western Blot

In brief, cells were washed with PBS and lysed in RIPA buffer, containing 150 mM NaCl, 20 mM Tris–HCl, pH 7.2, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1 M okadaic acid, 0.1 μM aprotinin, 10 μg/mL leupeptin, and 10 mM NaF. Lysates were quantified via bicinchoninic acid (BCA) protein assay (Thermo-Scientific) and subjected to 10% SDS-PAGE, after addition of loading buffer containing β-mercaptoethanol. β-mercaptoethanol was not included in sample buffer for non-reducing Western blot. Polyclonal antibodies were used for detection of collagen type I (Rockland, 1:1000) αSMA (Abcam, 1:300), SM22α (1:1000), LARP6 (Abnova, 1:500), lamin A (Abcam, 1:700), phospho-Thr308-Akt (Cell Signaling Technology, 1:1000), Collagen I alpha 1 telopeptide (Rockland, 1:1000), GFP (Abcam, 1:4000). Antibodies against β-actin (Abcam, 1:700) and β-tubulin (Abcam, 1:500) were used as control for equal loading. Immunopositive bands were visualized by enhanced chemiluminescence (ECL, Amersham), and images were captured using a Biorad GelDoc imager.
**Real-time Reverse Transcriptase-Polymerase Chain Reaction**

Total RNA extraction and quantitative PCR were performed as previously described\(^{22}\). Briefly, total RNA was isolated using the TriPure Isolation Reagent (Roche) followed by purification with the RNeasy mini-kit (Qiagen). cDNA was synthesized using the First Strand cDNA Synthesis kit (SA Biosciences) and amplified using 40-cycle 2-step PCR with sequence-specific primer pairs (SA Biosciences) in the iCycler IQ Real-Time Detection System (Bio-Rad). Relative \(COL1a1\) and \(COL1a2\) mRNA expression were determined via normalizing to \(\beta\)-actin mRNA expression, and LARP6 mRNA expression was normalized to \(\beta\)-tubulin mRNA expression.

**Exogenous Collagen Assembly Assay**

The protocol was adopted from Pickering et al.\(^{23}\). Solubilized collagen obtained from bovine skin after pepsin digestion and solubilization in HCl (Vitrogen; Cohesion Technologies, Palo Alto, CA), was diluted in acetic acid. The collagen preparations were dialyzed against borate-buffered saline (170 mmol/L boric acid, 170 mmol/L sodium tetraborate, 75 mmol/L NaCl, pH 9.3) at 4°C overnight and then labeled with fluorescein isothiocyanate (FITC) by transferring the dialysate to a solution of borate-buffered saline
containing 30 mg/ml of the fluorochrome and mixing in the dark at 4°C overnight. Acidic pH was restored and unbound FITC was removed by dialysis against 0.1% acetic acid at 4°C for 4 days. Labeled soluble collagen was diluted with ice-cold F-12 serum-free medium (+/- 100ng/mL IGF-1) and was added to SMC cultures that were maintained on ice for 2 minutes, at room temperature for 2 minutes, and then at 37°C for designated intervals. After treatments, cultures were then washed 2 times in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Cells were then incubated with Texas Red-labeled phalloidin (1:100 dilution, Molecular Probes) for 1 hour, stained with Hoechst 33258 (Molecular Probes) in glycerol/PBS (9:1), and collagen fibril formation was visualized with fluorescence microscopy. Quantification of FITC-collagen and cell number were determined spectrophotometrically via measuring FITC and Hoechst signal intensities, at M 485/528 and M 360/460 wavelengths, respectively. To assess for collagen self-assembly in absence of cells, the identical procedure was performed without cells. Collagen assembly assays were normalized to cell number by dividing FITC-signal by Hoechst signal intensity.
**Collagen Synthesis and Extracellular Accumulation**

Synthesis and extracellular accumulation of intact collagen was measured using the method described by Zhang et al. 24, with slight modifications. Cells were grown to ~80% confluence, washed twice with serum-free medium, and incubated for 0-30 hours in serum-free medium containing 2 mCi/mL L-[2,3,4,5-3H(N)]-proline (Perkin Elmer, USA) +/- 100ng/mL IGF-1. At the designated time intervals, the cultured medium was removed and the cells were washed twice with ice-cold PBS. The separated cells and cultured medium were then digested with 1 mg/mL pepsin (in 0.1M acetic acid) overnight at 4°C with gentle rocking. Following pepsin-digestion, the remaining triple-helical core of intact collagen was precipitated in 30% trichloroacetic acid (TCA). Samples were then centrifuged and the pellet was resuspended and precipitated in 10% TCA, two times. Precipitant was then resuspended in 500 μL of 0.5 M NaOH, 0.1% Triton X-100, and radioactivity (CPM) was measured using Packard Tri-Carb Liquid Scintillation Counter (1600 CA, USA).
Pharmacological Inhibitors

The following chemical inhibitors were dissolved in DMSO and added to culture medium 1 h prior to treatment with IGF-1: actinomycin D (1 μg/mL), cycloheximide (1 μg/mL), LY29004 (50 μM), Akt VIII (250 nM), rapamycin (100 nM), ethyl-3,4-dihydroxybenzoate (EDHB, 50μM), or 0.1% DMSO (vehicle). To inhibit the α2β1-integrin, an α2β1-specific blocking antibody (20 μg/mL) (BHA2.1, Upstate Biotechnology Inc., Lake Placid, NY) was added to culture medium for 3 h prior to stimulation with IGF-1.

LARP6 siRNA

siRNA targeting exon 1 of LARP6 (Sigma-Aldrich) were custom designed using the D2 siRNA sequence (5’-UCCAACUCGUCCACGUCCU-3’), previously shown to knock down LARP6 protein expression in human lung fibroblasts. scrRNA (5’-GGAGGGCUUCGAGUUA-GGA-3’) was used as control. siRNA or scrRNA were transfected into HASMCs via electroporation followed by overnight incubation in 5% serum-containing SmBM (Lonza) culture medium. Cells were then placed in serum-free medium for an additional 48 hours before treatment with IGF-1, and efficacy of the siRNA to knockdown LARP6 was assessed by Western blot.
Immunoprecipitation of Ribonucleoprotein Complex

Messenger RNA–protein complexes were immunoprecipitated as described\textsuperscript{26,27}. Briefly, mRNA–protein complexes were extracted from the cells using polysome lysis buffer (100 mM KCl, 5 mM MgCl\textsubscript{2}, 10 mM HEPES pH 7.0, 0.5% IGEPAL CA-630 (Sigma-Aldrich), 1 mM dithiothreitol, 100 units/mL RNase OUT (Invitrogen), and 0.2% Ribonucleoside Vanadyl Complex, protease inhibitor cocktail (Halt Protease Inhibitor Cocktails, Thermo Scientific). Protein contents in the extract were determined using RC DC Protein Assay kit (Bio-Rad), and the equal amount of protein from each sample was subjected to immunoprecipitation. Immunoprecipitation reaction was achieved by mixing the extract with anti-LARP6 antibody (Abnova) in 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 0.05% IGEPAL CA-630, 15 mM EDTA, 1 mM dithiothreitol, 100 units/mL RNase OUT (Invitrogen), and 0.2% Ribonucleoside Vanadyl Complex containing protease inhibitor cocktail for 18 h at 4 \textdegree C. The antibody-LARP6-mRNA complexes were isolated via \textmu MACS Protein G magnetic beads/columns kit (Miltenyi Biotec Inc.) according to the manufacturer’s protocol. The collected immunoprecipitants were extracted for RNAs using Tripure reagent (Roche) and further purified using RNeasy kit (Qiagen). \textit{COL1a1} and \textit{COL1a2} mRNA levels were determined as described using quantitative realtime-PCR. Mouse non-immune IgG was used to replace the anti-
LARP6 antibody in the immunoprecipitation procedure to confirm specific precipitation of LARP6–mRNA complexes.

5’Sterm-loop Molecular Decoy for LARP6

p74WT and p74MUT decoys were designed as previously described \textsuperscript{28}. Briefly, double-stranded oligonucleotide constructs contained the identical sequence as the 5’SIL structure of the COL1a1 gene (or a mutated sequence as control, MUT) and the optimal Sm binding site (p74) from the mouse U7 small nuclear RNA-derived gene \textsuperscript{29}, which allowed for accumulation in both the nucleus and cytoplasm. It was previously shown that LARP6 binds to the wild-type 5’SIL sequence but does not bind to the mutated sequence \textsuperscript{25}. Decoy constructs were cloned into the pAdTrack vector containing green fluorescent protein (GFP) expression cassette under independent control of the cytomegalovirus promoter, as control for infection. Resulting adenoviruses express both GFP and the p74WT or p74MUT decoy, and were amplified by ViraQuest Inc. Cells were incubated in presence of viruses at a multiplicity of infection of 1000 for 3 days prior to treatment. Between ~95-100% transduction efficiency was achieved, as judged by GFP fluorescence viewed under fluorescent microscope.
**Histochemical Assessment of Collagen Fibers in Atherosclerotic Plaque**

After 12 weeks on Western diet, SMP8/ Apoe^{−/−} and Apoe^{−/−} animals were sacrificed and serial 6 μm paraffin-embedded cross-sections were made through the aortic valve area. Sections were stained with picrosirius red, examined under polarized light with a Motic BA300 Pol polarized microscope, and collagen fibers in plaque were assessed as described in detail by Whittaker et al. 30. Picrosirius red dye stains thick, tightly packed collagen fibrils red/orange, intermediate fibrils yellow, and thin, loosely packed fibrils green. 31,32,33 A hue selection method, performed via Image-pro software (Media Cybernetics), was used to select and quantify the proportions of different fibril color.

**Statistical Evaluation**

All statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc. San Diego, CA), and the data is presented as the group mean +/- the standard error (Mean±SEM). Differences between groups were determined by student t-test, one-way ANOVA, or two-way ANOVA, as appropriate, followed by Tukey or Bonferroni post-tests. P values of less than 0.05 (P<0.05) were considered statistically significant. Sample number (N) is represented per group and is indicated.
AIM 1: Identify mechanisms whereby IGF-1 induces vascular smooth muscle cell contractile phenotype and collagen expression

Rationale

Vascular smooth muscle cells (VSMCs) play a major role in maintaining vessel homeostasis and in the pathogenesis of atherosclerosis. Unlike cardiac and skeletal muscle cells, smooth muscle cells do not terminally differentiate and display unique plasticity in which they can undergo rapid change to diverse phenotypes in response to local environmental cues\(^{34}\). In the normal healthy artery, VSMCs exhibit a differentiated phenotype and express contractile proteins such as alpha-smooth muscle actin (αSMA), smooth muscle 22-alpha (SM22α), myosin-heavy chain (MHC), and calponin. In the setting of atherosclerosis, VSMCs are exposed to inflammatory cytokines, reactive oxygen species (ROS), and lipid molecules, which alter their normal contractile phenotype. The VSMCs undergo dedifferentiation characterized by increased rates of proliferation and migration and loss of expression of contractile proteins/markers.\(^{35}\). While this compensatory change in phenotype may have critical function in wound
healing, excessive VSMC proliferation and a deficiency in contractile protein expression can result in increased neointima formation and contribute to vascular pathogenesis and plaque instability. Thus, preservation of a differentiated contractile VSMC phenotype represents an important feature of plaque stability.

In addition to maintaining a stable contractile phenotype, VSMCs help promote plaque stability by secreting extracellular matrix (ECM) proteins, mainly collagens type I and III. The collagen forms a fibrous cap that acts to contain the thrombogenic elements in the necrotic core of plaque. A deficit in collagen expression results in plaque vulnerability and significantly increases likelihood of plaque rupture. Hence, collagen content in plaque is established as a major hallmark of plaque stability.

Collagen may also play a major role in modulating VSMC phenotype. Aortic smooth muscle cells grown on fibrillar collagen have been shown to express a more differentiated (contractile) phenotype than cells grown on denatured collagen, which express a more proliferative and less differentiated phenotype. Polymerized collagen type I interacts with cell-surface receptors such as the α2β1-integrin, which may influence cell phenotype and responsiveness to growth factors. Because we had found that IGF-1 increased collagen expression, we investigated whether cellular interaction with collagen affects VSMC contractile phenotype and/or response to IGF-1.
Vascular homeostasis and responses to IGF-1 are mediated through intracellular signal transduction. Figure 8 summarizes some of the established IGF-1-signal transduction pathways and their downstream effects. Of particular interest is the PI3K / Akt pathway, which is known to be important in maintaining cardiac and vascular smooth muscle cell contractility and physiological response to stress. Interestingly, Sessa et al. showed in Apoe<sup>−/−</sup> mice that deletion of the serine/threonine kinase, Akt1, resulted in increased atherosclerosis with larger necrotic core size, diminished collagen content, and decreased αSMA expression<sup>45</sup>. In aim 1 of this study, we determined the signal transduction pathways that mediate the ability of IGF-1 to increase collagen and contractile protein expression.
Insulin-like growth factor (IGF)-1 signal transduction.

**Figure. 8 Insulin-like growth factor-1 (IGF-1) signal transduction.** IGF-1 binds to IGF-1R and IGF-1/Insulin hybrid receptor, whereas insulin binds only to the insulin receptor (Insulin-R), and triggers several signaling pathways. IGF binding proteins modulate IGF-1 signaling by altering IGF-1 binding to the receptors. The IGF-1R is a tyrosine kinase that undergoes autophosphorylation and catalyzes the phosphorylation of multiple cellular proteins, including members of the insulin receptor substrate (IRS) family. On phosphorylation, IRSs interact with signaling molecules, including Akt, Ras/Raf, and Rac. Activation of the phosphoinositide 3-kinase and Akt pathway supports cell survival and enhances endothelial nitric oxide synthase activity, thereby inducing vasodilation. The Ras/Raf pathway is critical for proliferative responses, whereas activation of Rac is important for cell migration.\(^{12}\)
RESULTS

IGF-1 increases pro-α1(I) and contractile protein expression in human aortic smooth muscle- To identify mechanisms responsible for the IGF-1-induced increase in collagen expression seen in vivo, human aortic smooth muscle cells (HASMCs) were grown in primary culture and treated with IGF-1 for 18 hours (h). IGF-1 induced a dose-dependent increase in expression of procollagen type I alpha-1 (pro-α1(I)), and contractile proteins, alpha-smooth muscle actin (αSMA), and smooth muscle22-alpha (SM22-α) (Fig. 9). Of note, the same response to IGF-1 was observed with and without overnight serum starvation before IGF-1-treatment. With 100ng/mL, IGF-1 induced a 2.4-fold increase in expression of pro-α1(I), and ~2.2-fold increase in expression of αSMA and SM22-α.
Figure 9. HASMCs were treated with 0, 10, 50, or 100ng/mL IGF-1 for 18 hours (h) and expression of procollagen-α1(I) (pro-α1(I)), alpha-smooth muscle actin (αSMA), and smooth muscle-alpha (SM22-α) was determined by Western blot and normalized to β-tubulin expression. Quantification is shown; Mean±SEM, N=6, *p<0.05, **p<0.01, ***p<0.001
**IGF-1 increases pro-α1(I) and contractile protein expression via a posttranscriptional and translation-dependent mechanism.** To determine if IGF-1 upregulated expression of pro-α1(I), αSMA, and SM22α at the transcriptional level, time-course gene and protein expression levels in response to IGF-1 were measured from 0-24 h. Real-time RT-PCR showed that IGF-1 had no significant effect on COL1a1 or COL1a2 mRNA expression (Fig. 10), nor αSMA, and SM22α mRNA expression (Fig. 11). Additionally, actinomycin D, an inhibitor of RNA polymerase II-dependent transcription, did not prevent the IGF-1-induced increase in pro-α1(I), αSMA, and SM22α expression, indicating that upregulation by IGF-1 is posttranscriptional. Furthermore, cycloheximide, an inhibitor of protein synthesis, completely blocked the ability of IGF-1 to increase pro-α1(I) and αSMA expression (Fig. 12), suggesting that a translational mechanism mediated upregulation of expression by IGF-1.
Figure 10. (A) Real-time RT-PCR determined COL1a1 and COL1a2 time-dependent mRNA expression in response to IGF-1 (100 ng/mL) versus Con (serum-free medium); N= 6-12/time-point, Mean±SEM, p>0.05, not statistically significant. (B) Western blot of HASMCs treated with IGF-1 (100ng/mL) for 6 or 18 h in presence of 1 μg/mL actinomycin D (act D), or DMSO (vehicle). Act D significantly reduced basal pro-α1(I) protein expression but IGF-1 significantly increased pro-α1(I) protein expression both with and without the presence of act D. **p<0.01, N=8
Figure 11. (A) Real-time RT-PCR determined αSMA and SM22α mRNA expression in response to IGF-1 (100 ng/mL). Mean±SEM p>0.05 not statistically significant. (B) Western blot of HASMCs treated with IGF-1 (100ng/mL) for 6-18 h with or without presence of 1 μg/mL actinomycin D (act D). Act D reduced basal contractile protein expression; however, IGF-1 significantly increased αSMA and SM22α expression both with and without presence of act D. **p<0.01, N=6-8.
Figure 12. Western blot of HASMCs treated with IGF-1 (100ng/mL) for 6 hours with or without presence of cycloheximide (CH). Procollagen-α1(I) (pro-α1(I)) and α-smooth muscle actin (αSMA) expression were normalized to β-tubulin and quantification is shown; Mean±SEM, *p<0.05, ***p<0.001, N=6
IGF-1 increases pro-α1(I) and contractile protein expression via PI3K-dependent but Erk1/2- and mTOR-independent signaling. To gain insight into the signaling pathways involved in the IGF-1-induced increase in procollagen-α1(I) and contractile protein expression, we pretreated the cells with a PI3K-inhibitor (LY29004) or an Erk1/2-inhibitor (PD98059). The PI3K-inhibitor completely blocked the ability of IGF-1 to increase procollagen-α1(I) and αSMA expression, while the Erk1/2-inhibitor had no effect (Fig. 13). Therefore we concluded that IGF-1 increased procollagen-α1(I) and αSMA expression via a PI3K-dependent and Erk1/2-independent signaling mechanism. Because IGF-1 has been shown to increase general cap-dependent protein synthesis via the canonical PI3K / mTOR-pathway, we considered whether mTOR may mediate the ability of IGF-1 to increase procollagen-α1(I) and contractile protein expression. Rapamycin, an inhibitor of mTOR (inhibits both mTORC1 and mTORC2) did not block the IGF-1-induced increase in pro-α1(I) and contractile protein expression, indicating upregulation by IGF-1 to be independent of mTOR-signaling (Fig. 14). Interestingly, rapamycin showed a trend to enhance basal contractile protein expression as well as the IGF-1-induction of collagen expression.
Figure 13. Western blot of lysate from HASMCs treated for 18 h with IGF-1 (100 ng/mL) in presence of the indicated chemical inhibitor: 50 μM LY29004 (PI3K-inhibitor - LY) or 50 μM PD98059 (Erk1/2-inhibitor - PD). Mean±SEM, **p<0.01, N=4
**Figure 14.** Western blot of lysate from HASMCs treated with IGF-1 (100 ng/mL) for 18 h in presence of 0, 50, or 100 nM rapamycin (mTOR-inhibitor). Mean±SEM, N=4-6
Cellular interaction with collagen affects VSMC phenotype. To determine if collagen influences VSMC phenotype or response to IGF-1, HASMCs were cultured in presence of a collagen synthesis inhibitor, ethyl-3,4-dihydroxybenzoate (EDHB), or a blocking antibody against the alpha2beta1-integrin (α2β1). EDHB is a competitive inhibitor of prolyl 4-hydroxylase, the enzyme that hydroxylates proline residues on the procollagen molecule, which is critical for collagen folding into its triple helix [46]. The α2β1 integrin is a major cell-surface receptor for collagen which has been implemented in the orchestration of collagen assembly as well as regulation of cell phenotype [23,47]. Western blot shows that basal expression levels of αSMA and SM22α were greatly attenuated in the presence of the collagen synthesis inhibitor as well as with the α2β1-blocking antibody, thus indicating that collagen supports a contractile VSMC phenotype (Fig. 15). Still, IGF-1 significantly increased the expression of αSMA and SM22α under each condition, suggesting collagen-independent upregulation by IGF-1. However, Western blot showed that EDHB did not block the IGF-1-induced increase in pro-α1(I) expression (Fig. 16). Therefore, we could not rule out the possibility that the ability of IGF-1 to induce a contractile phenotype is related to the ability of IGF-1 to increase collagen expression. Interestingly, immunocytochemistry confirmed that IGF-1 increased total collagen positivity in presence of EDHB; however, the fluorescent signal in EDHB-
treated cells was much more diffuse, presumably because of improper collagen assembly and folding. The fact that IGF-1 increased expression of the procollagen-α1(I) polypeptide in presence of EDHB, but did not overcome the inhibition on collagen folding and polymerization, suggests that IGF-1 regulates collagen expression at the translational level, rather than posttranslationally.
Figure 15. HASMCs were treated with 0, 50, or 100 ng/mL IGF-1 in serum free media either with or without the presence of a prolyl-hydroxylase inhibitor, EDHB, or a blocking antibody for the α2β1-integrin. Cell lysate was collected in RIPA buffer after 18 h and Western blot was performed to determine protein expression levels of alpha-smooth muscle actin (αSMA) and smooth muscle 22-alpha (SM22α). Quantification is shown. Mean±SEM, N=6-8
Figure 16. HASMCs were treated for 18 h with 0, 50, or 100 ng/mL IGF-1 in serum free media with or without the presence of a prolyl-hydroxylase inhibitor, EDHB. Immunocytochemistry was performed using a rabbit collagen-α1(I) polyclonal antibody or normal rabbit IgG as negative control. Additionally, cell lysate was collected in RIPA buffer at 18 hours time point and Western blot was performed using the same collagen-α1 (I) antibody. Western blot quantification is shown. Mean±SEM, *p<0.05, N=6
IGF-1 has no significant effect on SMC-mediated exogenous collagen type I polymerization. To assess whether IGF-1 had an effect on extracellular collagen assembly, we labeled bovine collagen type I with FITC dye, which allowed us to visualize and quantify exogenous collagen in our cell culture system using immunofluorescence and spectrometric emission. Although entropy allows for collagen self-polymerization \(^{48}\), we found that the presence of cells drastically increased collagen polymerization into a fibrous network, as observed both qualitatively using immunofluorescence and quantitatively by spectrophotometric detection of FITC emission. To visualize cells’ cytoskeleton, actin filaments were stained with Texas Red-labeled phalloidin. After merging immunoflourescent images, we found that the collagen fibrils seemed to align in parallel with the actin filaments, suggesting that cytoskeletal elements play a role in collagen fibril assembly (Fig. 17). To assess if IGF-1 affected extracellular collagen assembly, HASMCs were stimulated with IGF-1 and FITC-labeled collagen type I (FITC-COL) was added to the medium. After 24 h incubation, cells were washed and fixed for analysis. Spectrophotometric detection of FITC emission was modestly increased by IGF-1; however IGF-1 also increased total cell number, determined by DAPI spectrophotometric detection. Because the presence of cells augments the collagen fibril assembly, FITC-COL signal was normalized to DAPI signal
to account for differences in cell number. After normalizing to cell number, IGF-1 had no significant effect on FITC-COL assembly (Fig. 17).
A. FITC-COL Assembly

![Image of SF and IGF-1 treated cells]

B. FITC-COL Assembly

![Image of SFM and IGF-1 treated cells]

**Figure 17. SMC-mediated assembly of exogenous collagen type I.** Fluorescein isothiocyanate-labeled Bovine collagen type I (FITC-COL) was added to cultured HASMCs and treated +/- 100ng/mL IGF-1. After 24 h treatment, cells were washed thoroughly, fixed in 4% paraformaldehyde, incubated with Texas Red-labeled phalloidin (to stain actin filaments) and Hoescht nuclear stain, and visualized under fluorescent microscope. Total FITC- and total Hoechst- emission intensities were measured spectrophotometrically and collagen assembly was assessed and normalized to cell number via dividing FITC-emission by Hoechst-emission intensity. IGF-1 had no significant effect on exogenous collagen assembly. N=4-6
Summary of Findings

In summary, these findings help elucidate the cellular mechanisms by which IGF-1 induces HASMCs to acquire a contractile/differentiated phenotype and to increase collagen deposition. IGF-1 caused a dose- and time-dependent increase in expression of procollagen-α1(I), and contractile proteins, αSMA and SM22α. IGF-1 had no significant effect on COL1a1, COL1a2, αSMA, or SM22α mRNA expression. Additionally, actinomycin D, an inhibitor of transcription, did not prevent the IGF-1-induced increase in procollagen-α1(I), αSMA, and SM22α protein expression; thus, demonstrating posttranscriptional upregulation by IGF-1. Conversely, an inhibitor of protein synthesis, cycloheximide, did prevent the ability of IGF-1 to increase procollagen-α1(I) and αSMA, indicating a translational mechanism mediated the upregulation by IGF-1. The use of cell-signaling inhibitors, LY29004 or PD98059, suggested that IGF-1 induced protein expression of pro-α1(I) and αSMA via PI3K-dependent and ERK1/2-independent signaling. Although PI3K is known to stimulate cap-dependent translation via activating mTOR, rapamycin did not inhibit the ability of IGF-1 to increase procollagen-α1(I), αSMA, and SM22α, indicating an alternate mechanism.
Experiments using a prolyl-hydroxylase inhibitor (inhibitor of collagen synthesis) or a blocking antibody against the α2β1-integrin (cell-surface receptor for collagen) showed a reduction in basal αSMA and SM22α expression in presence of these molecules, suggesting that cellular interaction with type I collagen is important for maintaining VSMC contractile phenotype. However, EDHB or the α2β1-integrin antibody did not block IGF-1-induced upregulation of αSMA and SM22α expression. Interestingly, IGF-1 increased procollagen-α1(I) expression in presence of the prolyl-hydroxylase inhibitor, suggesting a translational, rather than posttranslational mechanism by IGF-1 to upregulate collagen. Also in agreement, IGF-1 was found to have no significant effect on exogenous collagen assembly. Taken together, we concluded that cellular interaction with collagen through the α2β1-integrin has significant influence on maintaining VSMC contractile phenotype, and that IGF-1 upregulates collagen type I expression via a translational mechanism.
Aim 2: Determine the involvement of the mRNA-binding protein, LARP6, in the ability of IGF-1 to enhance collagen type I synthesis

Rationale

Because we found that collagen, itself, promotes a contractile VSMC phenotype, we refined our focus to understand the mechanisms whereby IGF-1 upregulates collagen synthesis. Our experiments indicated that IGF-1 increased collagen expression via a posttranscriptional and PI3K-dependent translational mechanism that did not involve the canonical mTOR-mediated protein synthesis pathway. Therefore we searched the scientific literature to see what had been shown about posttranscriptional regulation of collagen type I.

Collagen type I is the most abundantly expressed protein in the human body and has crucial functions in growth, development, and disease [49]. The collagen type I subunit molecule is a fibril-forming heterotrimeric protein composed of two alpha-1 (α1) chains and one α2 chain, which undergo a complex series of posttranslational modifications to fold into a stable and highly ordered triple-helix. Proper assembly of these subunit
molecules is important for collagen’s macroscopic fibril structure and is required for its tensile strength, stability, and biological function $^{50,51}$. Mutations within the collagen type I genes can result in formation of collagen species with compromised structural integrity and are associated with various connective tissue disorders including osteogenesis imperfecta types I-IV, Ehlers-Danlos syndrome (type VIIA, VIIB), Caffey Disease, and osteoporosis $^{49,52-54}$. Collagen type I assembly into its heterotrimeric structure requires co-translational processing which begins in the endoplasmic reticulum during translational elongation of the $\alpha$-polypeptide chains $^{55,56-58}$. After triple-helical formation in the ER, procollagen molecules then undergo complex post-transcriptional modifications and processing that allow for secretion and fibril assembly. One key step in fibril formation is the enzymatic cleavage of the procollagen C-propeptide, which exposes the C-telopeptide region and markedly decreases the threshold concentration for collagen fibril assembly $^{48,57}$. Figure 18 provides a schematic of the procollagen type I subunit molecule.
Figure 18. Procollagen type I molecule. Schematic depicts major domains of subunit molecule. Note, after enzymatic cleavage of the N- and C-propeptides, the N- and C
telopeptide regions are exposed. Diagram adopted from:

Marko Marcius¹, Nada Vrkić², Biserka Getaldić-Švarc².
Analytical evaluation of commercial P1NP assay.
Biochemia Medica 2006;16(2):178-90.
Within the 5’UTR of the mRNA encoding the α chains, \textit{COL1a1} and \textit{COL1a2}, there is an evolutionarily conserved sequence that forms a stem-loop secondary structure encompassing the translation start codon. This 5’stem-loop (5’SL) motif has been shown to regulate collagen type I mRNA expression and translation \cite{25,59,60}. Mutation of the \textit{COL1a1} 5’UTR, which abolishes the stem-loop structure but does not affect the coding region of \textit{COL1a1}, resulted in reduced collagen type I expression \textit{in vivo} \cite{20}. In addition to promoting translation, the 5’SL has also been shown to be inhibitory of translation \textit{in vitro} and in quiescent hepatic stellate cells in which there is an absence of protein binding to the 5’SL \cite{59,60}. The physical constraints of the 5’SL motif dictate accessibility to the start codon and prevent uncontrolled translation \cite{25,61}. Thus, the 5’SL allows for a tight regulation of initiation of collagen type I translation.
Figure 19. **COL1a1 mRNA 5’Sem-loop (5’SL).** (A) The mRNA 5’stem-loop secondary structure ($\Delta G=25-30$ kcal/mol) is located 75-85 nt from 5’cap and contains the translation start codon, indicated in the box. (B) The 5’SL is present only in collagen $\alpha1(I)$, $\alpha2(I)$, and $\alpha1(III)$ mRNAs and is evolutionarily conserved from distantly related vertebrates. S1 - bottom stem; S2 - top stem; B1 - 5’ side of the bulge; B2 - 3’ side of the bulge $^{25}$. 
The 5’SL secondary structure is only present in the collagen type I and III (fibril-forming) mRNAs, and the la ribonucleoprotein domain family member 6 (LARP6) is the only protein that has been identified to directly bind the 5’SL. LARP6 is a member of the La-related protein family of RNA-binding proteins (LARPs) and has been implemented in coordinating efficient translation of the COL1a1 and COL1a2 mRNAs. For example, suppressing LARP6 expression via targeted siRNA significantly reduced collagen type I synthesis and secretion. Additionally, LARP6 has been shown to recruit and tether other molecular chaperones, including RNA-helicase A and a peptidylprolyl cis-trans-isomerase (FKBP65), to allow for translation and maturation of the collagen-α1(I) heterotrimer. The complex co-translational processing and assembly of collagen requires a coordinated mechanism to select and colocalize the three α-chains for formation of the heterotrimer. It is proposed that LARP6 initiates configuration of the collagen type I heterotrimer via binding the 5’-SL of the COL1a1 and COL1a2 mRNA and recruiting the molecular chaperones to ensure correct alignment and maturation. Because LARP6 is important for posttranscriptional regulation of collagen synthesis, we investigated the role of LARP6 in the ability of IGF-1 to upregulate collagen type I expression.
Figure 20. Schematic representation of the protein domains of la ribonucleoprotein domain family member 6 (LARP6). LARP6 has a distinctive bipartite RNA binding domain consisting of the conserved La domain (amino acids 85–183) found in the members of La-related protein super family (LARPs) \(^{62,65}\) and the RNA recognition motif (RRM, amino acids 183–296) that is similar to a generic RNA binding domain \(^{66}\). The other regions of LARP6 have no homology to known proteins. Mutant constructs of LARP6 indicated that the RRM and the several amino acids adjacent to the N-terminus of the La domain are necessary for interaction with the 5’ stem-loop. These amino acids are not present in the other La containing proteins. The C-terminal domain (amino acids 300–491) allows for recruitment of molecular chaperones including the serine-threonine kinase receptor associated protein (STRAP), RNA-helicase A, and FKBP65 (a peptidylprolyl cis-trans-isomerase), which may play essential roles in collagen assembly \(^{63,67,68}\).
Results

*IGF-1 increases the rate of collagen type I synthesis and extracellular accumulation*—Time-course expression of pro-α1(I) in response to IGF-1 was assessed by Western blot and showed that IGF-1 induced a fairly constant increase in pro-α1(I) expression from 6-48 h (Fig. 21A). As a means to precisely measure rapid induction of collagen synthesis by IGF-1, ³H-proline incorporation assays were performed to determine accumulation of intact triple-helical collagen from 0-6 h. IGF-1 increased the rate of intracellular pepsin-resistant collagen accumulation by 2.2-fold within 4 h (p<0.0001, n=6, Fig. 21B). This rapid increase indicates that IGF-1 increased the rate of collagen translation. We also measured the rate of ³H-proline accumulation (p<0.0001, Fig 21C) in the presence of rapamycin (an inhibitor of mTOR-mediated general cap-dependent protein synthesis), which confirmed that IGF-1 increased collagen synthesis via an mTOR-independent mechanism.
Figure 21. (A) Western blot of HASMCs treated with IGF-1 (100 ng/mL) for 6, 24, or 48 h, quantification is shown to right (B) Collagen accumulation in lysate from HASMCs stimulated with IGF-1 (100 ng/mL) or serum-free medium (SFM) for 1-6 h. Collagen was determined via $^3$H-proline-recovery after overnight pepsin-digestion (1 mg/mL) followed by 10% TCA-precipitation. (C) same as in B, except IGF-1-stimulation was given in presence of 100nM rapamycin (rap). Asterisks (*) indicate statistically significant difference between IGF-1 and SFM at specified time point. Mean±SEM, **p<0.01, ***p<0.001, ****p<0.0001. N=4-6
Between 6 h and 30 h, IGF-1 steadily increased the rate of intracellular collagen accumulation by 41% (p<0.0001, Fig. 22A), and increased total secreted collagen accumulation (3.1-fold at 24 h, p<0.001, Fig. 22B). To confirm this increase in secreted collagen, non-reducing Western blot was performed to detect covalently cross-linked collagen type I in cellular medium after 24 h treatment (Fig. 22C). IGF-1 increased expression of both pro-α1(I) and disulfide-bonded collagen type I (of high molecular weight - HMW) (2.9-fold and 3.8-fold, p<0.01 and p<0.05, respectively), consistent with an increase in secretion of intact collagen type I. Taken together, these results demonstrate that IGF-1 increases the rate of collagen type I synthesis, leading to enhanced extracellular collagen type I accumulation.
Figure 22. (A) Collagen accumulation in lysate from HASMCs stimulated with IGF-1 (100 ng/mL) or serum-free medium (SFM) from 6 - 30 h, determined via $^3$H-proline-recovery after overnight pepsin-digestion (1 mg/mL) followed by 10% TCA-precipitation. (B) same conditions as in A except $^3$H-proline recovery was measured in cultured medium, representing secreted collagen. (C) Non-reducing Western blot detecting covalently cross-linked collagen of high molecular weight (HMW) and procollagen-α1(I) in cultured medium from HASMCs treated with IGF-1 for 24 h. Molecular weight is indicated to right of blot. Asterisks (*) indicate statistically significant difference between IGF-1 and SFM at specified time point. Mean±SEM, *p<0.05, ****p<0.0001, N=4-6
IGF-1 increases the expression of the mRNA-binding protein, LARP6 - Given our findings that IGF-1 posttranscriptionally regulates synthesis of collagen type I, we postulated that the mRNA-binding protein, LARP6, could play a role in the ability of IGF-1 to increase collagen type I. LARP6 binds to the 5’stem-loop secondary structure present in COL1a1 and COL1a2 mRNAs and regulates efficient translation of the collagen type I heterotrimer. IGF-1 significantly increased expression of LARP6 within 3 h (p<0.01), and this increase was sustained at 18 h (p<0.01, Fig. 23A). IGF-1 also dose-dependently increased LARP6 expression, and this corresponded with a similar increase in pro-α1(I) expression (p<0.01, Fig. 23B).
Figure 23. Western blot from HASMCs treated with (A) 0 or 100ng/mL IGF-1 for 3 h, or (B) 0, 50, or 100 ng/mL IGF-1 for 18 h. Mean±SEM, **p<0.01, ***p<0.001. N=4-8
Pro-α1(I) expression correlates with LARP6 expression- In an effort to determine if induction of LARP6 expression is necessary for the IGF-1-induction of pro-α1(I) expression, HASMCs were transfected with siRNA targeting exon 1 of LARP6 or with scrambled RNA (scrRNA) as control, and treated with IGF-1 for 18 h (administered 3 days after transfection). Although the siRNA effectively suppressed LARP6 expression under non-stimulated conditions (reduced by 53 %, p<0.05, Fig 3C), IGF-1 increased expression of LARP6, even in the presence of siRNA (2.4-fold, p<0.01, Fig 24). Accordingly, the IGF-1-induction of LARP6 expression in presence of the siRNA corresponded with an IGF-1-induced increase in pro-α1(I) expression (p<0.05, Fig. 24). Thus, to identify a potential correlation between LARP6 expression and pro-α1(I) expression, we plotted the relative expression of LARP6 versus pro-α1(I) as the fold-change compared to basal levels (i.e. scrRNA) (Fig 24). There was a highly significant correlation between the expression of LARP6 and expression of pro-α1(I) (Spearman r=0.9432, p<0.0001, N=15).
Figure 24. (A) Western blot from HASMCs after transfection with siRNA targeting LARP6. 3 days after electroporation of 100 nM siRNA or scrRNA (control), cells were stimulated with IGF-1 (100 ng/mL) for an additional 24 h and lysate was collected for Western blot. (B) Correlation of LARP6 expression and procollagen-α1(I) expression, from the experiment described in A. The relative expressions of LARP6 and procollagen-α1(I) were determined as the fold-change in expression compared to control levels (i.e. scrRNA without IGF-1-stimulation) and normalized to β-tubulin expression. One data point was plotted for each sample using the relative LARP6 expression as the X-coordinate and the relative procollagen-α1(I) expression as the Y-coordinate. Samples of the control group (scrRNA without IGF-1) were not plotted. Spearman statistical test for correlation, r=0.9429, p<0.0001. N=15
IGF-1 rapidly increases LARP6 expression in both nuclear and cytosolic fractions. LARP6 has a functioning nuclear localization sequence, is able to bind \(COL1a1\) and \(COL1a2\) mRNA in the nucleus, and can shuttle between the nucleus and cytoplasm. To determine the cellular localization of LARP6 and identify a potential effect by IGF-1, cellular lysate was separated into cytosolic and nuclear-enriched fractions. Within 1 h, IGF-1 increased LARP6 expression by 2.8-fold in the nuclear fraction and by 1.6-fold in the cytosolic fraction (Fig. 25, n=4). At this time point, the IGF-1-induced increase in pro-\(\alpha1(I)\) expression has not yet occurred (Fig. 25). Therefore, these results reveal that the IGF-1 induction of LARP6 expression precedes the increase in pro-\(\alpha1(I)\) expression. At 3 h, IGF-1 increased LARP6 expression by ~2.8-fold within both the cytosolic and nuclear fractions, and this corresponded with a similar increase in pro-\(\alpha1(I)\) expression (Fig. 25, p<0.01, n=5). Taken together, these results support the hypothesis that IGF-1 upregulates pro-\(\alpha1(I)\) expression via increasing LARP6 expression.
**Figure 25.** Sub-cellular localization of LARP6 and induction of expression by IGF-1.

Western blot of equal proportions of nuclear (nuc) and cytosolic (cyt) fractions from HASMCs harvested at indicated times after stimulation with IGF-1. β-tubulin served as a control for the cytosolic fraction and lamin A served as a control for the nuclear-enriched fraction. N=4-6
IGF-1 increases association of LARP6 with COL1a1 and COL1a2 mRNA—

Because LARP6 has been shown to bind COL1a1 and COL1a2 mRNAs and enhance their translation, we analyzed whether IGF-1 regulated LARP6 association with COL1a1 and COL1a2 mRNAs. HASMCs were treated with IGF-1 for 3 h and immunoprecipitation of LARP6 was performed followed by measurement of COL1a1 and COL1a2 mRNA via real-time RT-PCR. Significantly more COL1a1 and COL1a2 mRNA was present in LARP6-immunoprecipitant from IGF-1-treated cells (Fig. 26A). Importantly, Western blot of input immunoprecipitant shows significantly higher pull-down of LARP6 from IGF-1 treated cells (Fig. 26B), which reflects the IGF-1-induced increase in LARP6 expression (Fig. 23A). These findings suggest that as a result of IGF-1 increasing LARP6 expression, there is increased association of COL1a1 and COL1a2 mRNA with LARP6, leading to increased synthesis of collagen type I.
Figure 26. HASMCs were treated with IGF-1 (100 ng/mL) for 3 h and LARP6 was immunoprecipitated from cellular lysate followed by RNA-extraction. (A) Relative amount of COL1a1 and COL1a2 mRNA were subsequently determined via real-time RT-PCR (qPCR). (B) Western blot of pull-down from immunoprecipitation using a anti-LARP6 antibody or normal IgG (negative control). Mean±SEM, *p<0.05, **p<0.01. N=4-6
IGF-1 increases LARP6 and pro-\(\alpha1(I)\) expression via a PI3K/Akt-mediated posttranscriptional and translation-dependent mechanism. To gain insight into mechanisms whereby IGF-1 increased LARP6 expression, we analyzed LARP6 mRNA and protein levels after IGF-1 treatment with pharmacological inhibitors. IGF-1 had no effect on LARP6 mRNA expression, and actinomycin D did not block the ability of IGF-1 to increase LARP6 protein expression (Fig. 27B, C, \(p<0.01\)), indicating that IGF-1 upregulated LARP6 via a posttranscriptional mechanism. Furthermore cycloheximide completely blocked the IGF-1-induced increase in LARP6 expression indicating a translational mode of regulation.

Investigation of cell-signaling showed that both the PI3K-inhibitor (LY2940032) and Akt1/2-inhibitor (Akt VIII) prevented the ability of IGF-1 to increase LARP6 and pro-\(\alpha1(I)\) expression (Fig. 27D). Taken together these results indicate that IGF-1 increased LARP6 and pro-\(\alpha1(I)\) expression via a PI3K/Akt-mediated posttranscriptional and translation-dependent mechanism.
**Figure 27.** IGF-1 regulation of LARP6 expression. (A) Real-time RT-PCR determined expression of LARP6 mRNA after 6 h treatment with IGF-1. (B-C) Western blot of lysate from HASMCs treated with IGF-1 (100ng/mL) for (B) 6 h in presence of 1μg/mL actinomycin D (act d), (C) 18 h in presence of 1μg/mL cycloheximide (CH), or (D) 6 h in presence of the indicated chemical inhibitor: 50μM LY29004 (PI3K-inhibitor) or 250 nM Akt VIII (Akt1/2-inhibitor). Mean±SEM. N=4-6
IGF-1 stimulates the rate of collagen type I synthesis in a 5’stem-loop-dependent manner. To determine if the 5’SL is necessary for IGF-1 upregulation of collagen type I expression, we measured collagen type I production in response to IGF-1 in mouse embryonic fibroblasts (MEFs) harboring a mutation in the 5’SL of the COL1a1 gene (SL−/−). The 21-nt mutation in the 5’-UTR abolishes the 5’SL secondary structure but does not change the coding region of the COL1a1 gene (Fig. 28). This 5’SL mutation has previously been shown to prevent the binding of LARP6 to COL1a1 mRNA. In both SL−/− and wild-type (WT) cells, we determined the effect of IGF-1 on collagen accumulation after pepsin-digestion, which selects for only the triple-helical core region of intact collagen. IGF-1 had no effect on COL1a1 and COL1a2 mRNA expression in either cells (data not shown). Within 12 hours, IGF-1 induced a 2.0-fold increase in the rate of collagen accumulation in WT cells (p<0.0001), however, this increase was completely prevented in SL−/− cells (Fig. 28). Therefore, these findings indicate that the ability of IGF-1 to increase the rate of collagen synthesis is critically dependent on an intact 5’stem-loop.
**Figure 28.** 5'SL−/− mouse embryonic fibroblast contained a 21-nt mutation in 5’UTR, indicated by light blue text, which abolished formation of the 5’stem-loop. Graph on right shows collagen accumulation in response to IGF-1 (100 ng/mL) from 0-24 h in WT or SL−/− cells. Intact triple-helical collagen was measured via ^3^H-proline recovery after overnight pepsin-digestion (1 mg/mL) followed by 10% TCA-precipitation. Mean±SEM, *p<0.05, **p<0.01, ***p<0.001. N=6
Consistently, Western blot confirmed that IGF-1 increased expression of pro-α1(I) in WT cells (2.3-fold, 18 h p<0.01), while IGF-1 failed to increase collagen type I in SL⁻/⁻ cells (Fig. 29). Interestingly, the normal procollagen-α1(I) molecule of ~180 kDa was undetectable in SL⁻/⁻ cells and instead an altered collagen species of ~72 kDa (indicated with arrow, Fig. 29) was observed, suggesting that mutation of the 5’SL region resulted in abnormal processing and/or cleavage of the procollagen molecule. Also intriguingly, basal LARP6 expression was significantly lower in SL⁻/⁻ cells (26% compared to WT, p<0.05, Fig 29), and IGF-1 did not increase LARP6 expression in SL⁻/⁻, suggesting that LARP6 expression and its regulation by IGF-1 is dependent on LARP6 binding to the 5’stem-loop. Overall, these results highlight the significance of the 5’stem-loop for procollagen type I assembly and upregulation by IGF-1.
Figure 29. Western blot of lysate from wild-type (WT) or 5’stem-loop mutant (SL⁻⁻) mouse embryonic fibroblasts (MEFs) treated with IGF-1 (100 ng/mL) for 18 h. Arrow indicates an abnormal collagen species observed in SL⁻⁻. The two representative blots show collagen and LARP6 expression using different exposure times to visualize additional bands observed in SL⁻⁻. N=6-8.
5’Stem-loop RNA molecular decoy for LARP6 inhibits the IGF-1-induction of collagen type I expression– In order to inhibit binding of LARP6 to endogenous collagen type I mRNA in VSMCs, a 108-nt RNA containing the identical sequence as the 5’SLS structure of the COL1a1 gene was overexpressed in HASMCs using adenoviral delivery and served as a molecular decoy to sequester LARP6. The decoy RNA (p74WT) and a control RNA (p74MUT) containing a mutated stem-loop sequence unable to bind LARP6, were specially designed to accumulate in both the nucleus and the cytoplasm. Both adenoviral vectors additionally expressed GFP (independently of p74MUT or p74WT expression), which served as a control for infection (Fig. 30).

IGF-1 increased expression of LARP6 in presence of either decoy or control RNA (~2.5-fold, p<0.05, Fig. 31A). After 18 h treatment with IGF-1 (given 3 days after viral transduction), collagen type I expression was assessed in cellular lysate and in cultured medium. Because LARP6 is implicated in ensuring proper assembly of collagen type I, we also determined expression of mature collagen type I in the cultured medium via using an antibody which recognizes the α1(I) C-telopeptide. The α1(I) C-telopeptide is exposed after cleavage of the C’-terminal propeptide, serving as a crucial step for collagen cross-linking and fibril formation. IGF-1 increased intracellular pro-α1(I)
expression as well as pro-α1(I) and mature α1(I) expression in the cultured medium (Fig. 31A, B). However, in the presence of p74WT, the ability of IGF-1 to increase both the intracellular and extracellular expression of pro-α1(I) was precluded (Fig. 31A, B). The p74WT also inhibited the ability of IGF-1 to increase expression of mature collagen type I in the cultured medium (Fig. 31B). Therefore, these results demonstrate that sequestration of LARP6 prevents the ability of IGF-1 to increase synthesis of pro-α1(I) as well as expression of extracellular mature collagen type I.
Figure 30. (A) Schematic representation of the molecular decoys. The 108-nt RNA is shown as a line, eight core Sm proteins are indicated as circles, and the 5' stem-loop of collagen 1(I) mRNA is indicated. p74MUT decoy has a substitution of 15 nt, which abolishes formation of the 5' stem-loop. 7mG represents the monomethylated cap structure that the decoys initially acquire. The Sm protein region was derived from a snRNA construct that allows for nucleocytoplasmic trafficking. Decoy constructs were cloned into the pAdTrack vector containing green fluorescent protein (GFP) expression cassette under independent control of the cytomegalovirus promoter, as control for infection. (B) HASMCs were incubated in presence of adenoviruses (expressing both GFP and the p74WT or p74MUT decoy) at a multiplicity of infection of 1000 for 3 days prior to treatment. Between ~95-100% transduction efficiency was achieved, as judged by GFP fluorescence viewed under fluorescent microscope, shown above.
Figure 31. (A) Western blot of lysate from HASMCs treated with IGF-1 (100 ng/mL) in presence of stem-loop RNA decoy (p74WT) or a mutated stem-loop RNA (p74MUT) as control. Decoys were overexpressed using adenoviral delivery and cells were treated with IGF-1 3 days after transduction. B) Western blot of cultured medium from conditions described in A. Quantification of Western blot is shown. Mean±SEM, **p<0.01, N=6
IGF-1 increases LARP6 expression in vivo- To ascertain whether IGF-1 regulates LARP6 expression in vivo, we infused Apoe−/− mice with human recombinant IGF-1 (1.5 mg/kg/day) or saline (control) for 5 days and measured LARP6 expression in aortic lysates. Total IGF-1 (mouse + human) serum levels at time of sacrifice were measured via ELISA, which confirmed an approximate 2-fold increase in delivery of IGF-1 (Fig. 32A). After 5-day infusion, IGF-1 increased LARP6 by 3.1-fold, (Fig. 32B, p<0.05, n=6) and this increase correlated with a 2.3-fold-increase in pro-α1(I) (Fig. 32B, p<0.05, n=6).
Apoe<sup>−/−</sup> mice were given continuous infusion of either human recombinant IGF-1 (1.5 mg/kg/day) or saline (control) for 5 days via a subcutaneously implanted minipump. (A) Total IGF-1 (human IGF-1 + mouse IGF-1) levels in serum at time of sacrifice were determined via ELISA. (B) Western blot of the aortic lysate after 5-day infusion. Quantification is shown to right. Mean±SEM, *p<0.05, ***p<0.001. N=6
Smooth muscle cell-specific IGF-1 overexpression increases collagen fibrillogenesis in atherosclerotic lesions of Apoe<sup>−/−</sup> mice—During the progression of atherosclerosis, VSMCs migrate into the lesion and secrete collagen (mainly types I and III) as a means to prevent the necrotic core of the plaque from rupturing and triggering thrombosis. The secreted collagen fibrillar network can be examined histologically using picrosirius red stain, which distinguishes thick collagen fibers (orange-red), fibers of intermediate thickness (yellow), and loosely-packed thin collagen fibers (green), as described previously 71. To evaluate a potential effect of IGF-1 on collagen fibril composition in atherosclerotic plaque, we compared aortic valve cross-sections from Apoe<sup>−/−</sup> mice with sections from SMP8/Apoe<sup>−/−</sup> mice, which had an approximate 3-fold increase in aortic IGF-1 levels 18. SMP8/Apoe<sup>−/−</sup> mice had strikingly more total collagen fibrils within plaque compared to Apoe<sup>−/−</sup> control (Fig. 33, p<0.001), characteristic of having a more stable plaque phenotype. Furthermore, the ratio of thick (red) fibers to the total collagen fibers in plaque was significantly higher in SMP8/Apoe<sup>−/−</sup> mice than in Apoe<sup>−/−</sup> control mice (68% vs. 25%, p<0.0001, N=4-5, Fig. 8), indicating that IGF-1 enhanced collagen fibrillogenesis.
Figure 33. Cross-sections of aortic valve atherosclerotic plaque from Apo E deficient (Apoe\(^{-/-}\)) mice or smooth-muscle cell specific IGF-1-overexpressing Apoe\(^{-/-}\) transgenic mice (SMP8/Apoe\(^{-/-}\)). Cross-sections were stained with picrosirius red, which stains thick, tightly packed collagen fibrils red/orange, intermediate fibrils yellow, and thin, loosely packed fibrils green. A hue selection method (performed via Image-pro software) was used to analyze the proportions of different fibril color within plaque. The green arrowhead points to the thin fibrous cap present in Apoe\(^{-/-}\) animals, and the red arrowhead points to the thicker, more highly structured fibrous cap present in SMP8/Apoe\(^{-/-}\) mice. Quantification of total collagen content in plaque and composition of collagen fiber thickness in each group is shown. Mean±SEM, ***p<0.001, ****p<0.0001. N=4-5.
Summary of Findings

In summary, we show that in human aortic smooth muscle cells (HASMCs), IGF-1 rapidly increased LARP6 expression and the rate of collagen synthesis and extracellular accumulation. IGF-1 increased both LARP6 and collagen type I expression via a posttranscriptional and translation-dependent mechanism involving PI3K/Akt signaling. IGF-1 increased the expression of LARP6 in both nuclear and cytosolic fractions within one hour and this preceded the IGF-1-induced increase in pro-\(\alpha_1(I)\) expression. Immunoprecipitation of LARP6, followed by qPCR indicated that IGF-1 increased the association of COL1a1 and COL1a2 mRNA with LARP6. Mutation of the 5’stem-loop of Coll1 mRNA, which inhibits binding of LARP6, abolished the ability of IGF-1 to increase synthesis of collagen type I. Furthermore, overexpression of a 5’stem-loop RNA molecular decoy that sequesters LARP6, prevented the ability of IGF-1 to increase pro-\(\alpha_1(I)\) as well as mature \(\alpha_1(I)\) expression in cultured medium. IGF-1-infusion in Apoe\(^{-/-}\) mice increased expression of LARP6 and pro-\(\alpha_1(I)\) in aortic lysates, and SMC-specific IGF-1-overexpression robustly increased collagen fibrillogenesis in atherosclerotic plaque. In conclusion, we identify LARP6 as a critical mediator whereby IGF-1
augments synthesis of collagen type I in vascular smooth muscle, which likely plays an important role in promoting atherosclerotic plaque stability.
Overall, these findings reveal fundamental and novel mechanisms whereby IGF-1 induces vascular smooth muscle cell differentiation and orchestration of procollagen type I assembly. VSMC contractile phenotype and the structural integrity of the fibrous cap in atherosclerotic plaque are major determinants of plaque rupture\textsuperscript{72-74}. Therefore, the dynamic regulation of plaque phenotype by IGF-1 demonstrated in this study suggests that IGF-1 plays a protective role in atherosclerosis by enhancing plaque stability.

Collagen type I is a major component of the vessel wall, and the capacity of VSMCs to synthesize collagen is important for maintaining vascular homeostasis and relevant to the pathophysiology of cardiovascular diseases. Collagen content in atherosclerotic plaque is a hallmark of plaque stability, and the physiological advantage of IGF-1 to upregulate collagen synthesis can be observed in the protected plaque phenotype of the SMP8/Apoe\textsuperscript{-/-} mice. The striking increase in collagen expression and fibril maturation observed in plaques from SMP8/Apoe\textsuperscript{-/-} mice suggests that IGF-1 protects against plaque rupture.
IGF-1 may also promote plaque stability by maintaining a contractile VSMC phenotype. Much evidence suggests that loss of VSMC contractile phenotype is associated with plaque instability and can contribute to further inflammation \(^5,75\). For example, in a carotid denudation arterial injury model in SM22\(\alpha\) knockout (SM22\(^{-}\)) mice, the loss of SM22\(\alpha\) expression correlated with increased ROS- and NFkB-activated arterial inflammation \(^76\). Thus, the effect of IGF-1 to induce contractile markers, \(\alpha\)SMA and SM22\(\alpha\), represents a potential mechanism by which IGF-1 suppresses the progression of atherosclerosis and reduces the propensity for plaque rupture.

RT-PCR showed that IGF-1 had no significant effect on mRNA levels of contractile protein and collagen type I genes. Additionally, actinomycin D did not block the IGF-1-induced increase in collagen type I, \(\alpha\)SMA, and SM22\(\alpha\) protein expression. Actinomycin D is a poly-peptide that intercalates at CpG sites in DNA, which inhibits RNA polymerase from carrying out mRNA transcriptional elongation \(^77,78\). Therefore, the capability of IGF-1 to increase collagen type I and contractile protein expression in presence of actinomycin D suggests a posttranscriptional mode of upregulation by IGF-1. Furthermore, cycloheximide, an inhibitor of translational elongation, completely blocked the IGF-1-induced increase in collagen type I and \(\alpha\)SMA expression, indicating that IGF-1 induces collagen type I and \(\alpha\)SMA expression via a translational mechanism.
The interaction of vascular smooth muscle cells (VSMCs) with the surrounding extracellular matrix (ECM) has been shown to play a crucial role in regulating cell differentiation, migration, proliferation, and in mediating growth factor signaling \cite{39,41,42,51,75}. Our experiments using an inhibitor of collagen synthesis or an α2β1-blocking antibody suggested that VSMC interaction with collagen promotes contractile phenotype. Therefore, the ability of IGF-1 to increase collagen content and fibrillogenesis in plaque may be a critical mechanism by which IGF-1 promotes a more contractile phenotype. For this reason we refined our focus to understand the mechanisms whereby IGF-1 upregulates collagen type I synthesis.

Given our findings that IGF-1 regulates synthesis of collagen type I via a posttranscriptional and translation-dependent mechanism, we investigated whether the mRNA-binding protein, LARP6, plays a role in the ability of IGF-1 to increase collagen type I expression. LARP6 binds to the 5’stem-loop secondary structure present in \textit{COL1a1} and \textit{COL1a2} mRNAs and has been implemented in regulating efficient translation of collagen type I. We observed a significant correlation between the expression levels of LARP6 and pro-α1(I) under conditions both with and without siRNA and IGF-1-stimulation (Fig. 24), suggesting that procollagen type I expression is dependent on LARP6 expression. This was consistent with previous findings in which
knockdown of LARP6 had been shown to cause a significant reduction in basal expression of collagen type I expression in human lung fibroblasts. Interestingly, we found that IGF-1 was able to overcome the “silencing effect” of the LARP6-targeted siRNA; and therefore, the IGF-1-induced increase in LARP6 expression correlated with a proportional increase in pro-α1(I) expression. To confirm that LARP6 was responsible for the IGF-1-induction of collagen expression, we used the 5’SL decoy to sequester LARP6, and this completely inhibited the IGF-1-induction of collagen type I expression (Fig 31). Therefore these experimental findings suggest that IGF-1 regulation of LARP6 is responsible for the ability of IGF-1 to increase collagen type I.

It has been shown that inter-chain bonding of the collagen type I α-chains occurs before their release from the polysome into the ER lumen. The co-translational processing of α-polypeptides requires a coordinated mechanism to select and colocalize the three α-chains in order to initiate heterotrimer formation and ensure proper assembly of the procollagen molecule. Our experiments suggest that IGF-1 augments heterotrimer formation by inducing LARP6 expression and its subsequent binding to the 5’SL, allowing for efficient procollagen assembly. We showed that IGF-1 increased LARP6 expression resulting in increased COL1a1 and COL1a2 mRNA bound to LARP6 (Fig. 25). In the SL−/− cells, which showed reduced expression of LARP6 and were non-
responsive to IGF-1, a collagen species of an altered molecular weight was produced (Fig. 29), suggesting that altered processing and/or cleavage of the procollagen molecule had occurred. This may be explained by the fact that in SL−/− cells LARP6 was unable to bind COL1a1 mRNA and recruit the appropriate molecular chaperones for maturation. While apparently collagen translation did occur independently of the LARP6/5′SL complex formation, mutation of the 5′SL resulted in substantially reduced production of intact-triple helical collagen and completely prevented regulation by IGF-1 (Fig 28). Therefore, these results signify the importance of the 5′SL and its binding by LARP6 for the proper assembly of procollagen type I and its regulation by IGF-1.

In addition to binding the 5′SL, LARP6 has been shown to recruit and tether molecular chaperones including RNA helicase A, FKBP65 (a peptidylprolyl cis-trans isomerase), and nonmuscle myosin 21,63,67, to coordinate translation and maturation of the collagen type I heterotrimer. Deletion of the C-terminal region of LARP6, which abolishes its interaction with these molecular chaperones but does not affect its binding to COL1a1 and COL1a2 mRNA, resulted in reduced collagen type I expression 21,25,63,67. Thus, LARP6 is proposed to orchestrate the formation of the collagen type I heterotrimer via binding to the 5′SL and recruiting the appropriate chaperones needed for alignment and maturation. Our experimental results are consistent with a role of LARP6 in coordi-
nating the synthesis of mature collagen type I. We found that IGF-1 increased LARP6 expression and induced accumulation of pepsin-resistant collagen (intact triple helical core) as well as covalently cross-linked collagen type I in cultured medium (Fig. 22). Covalent cross-linking of collagen and fibrillogenesis is highly dependent on the C-terminal telopeptide region of the collagen type I molecule, which is exposed after cleavage of the C-terminal propeptide in the process of maturation. Our experimental findings show that IGF-1 significantly increased expression of mature α1(I) (exposed C-telopeptide, Fig. 31) under control conditions, while in presence of the 5’stem-loop decoy, IGF-1 failed to increase mature α1(I) in cultured medium (Fig. 31). Therefore, this suggests that the ability of IGF-1 to increase extracellular collagen type I maturation is dependent on its ability to induce procollagen synthesis via LARP6.

LARP6 is a recently characterized RNA-binding protein and is expressed in various human tissues, however, little is known about what regulates LARP6 expression. In a model of wound healing, LARP6 expression was elevated after mechanical injury, although no specific mechanisms regulating LARP6 expression were identified. A different report showed that LARP6 (also known as Acheron) expression was increased in some human basal-like ductal carcinomas and that ectopic overexpression of LARP6 resulted in increased proliferation and angiogenesis.
Therefore, these reports suggest that increased expression of LARP6 is important for promoting growth and vascularization, however they do not address mechanisms by which LARP6 expression is regulated. Our experiments, in which we measured protein expression after treatment with actinomycin D and measured gene expression via quantitative PCR, demonstrate that IGF-1 regulates LARP6 expression posttranscriptionally (Fig. 27). Also similarly to IGF-induction of collagen, we show that IGF-1 regulation of LARP6 expression is dependent on PI3K/Akt-signaling (Fig. 27).

Furthermore, our studies show that IGF-1 increases expression of LARP6 in both cytosolic and nuclear fractions, which precedes the IGF-1 induction of pro-α1(I) expression (Fig 25). Hence, the rapid induction of LARP6 expression by IGF-1 seems to be critical for association of LARP6 with the 5’SL of Col1a1 and Col1a2 mRNAs and enhanced synthesis of collagen type I.

While our experiments show that the 5’SL positively regulates collagen type I expression via LARP6 and its induction by IGF-1, the 5’SL may be inhibitory of translation in the absence of LARP6. Experiments using 5’SL-reporter gene constructs have shown that the 5’SL inhibited expression of collagen type I in quiescent hepatic stellate cells (HSCs) but promoted expression in activated HSCs. Importantly, in quiescent HSCs, the 5’SL did not associate with LARP6, while in activated HSCs
association did occur and collagen type I expression was significantly increased. Similarly, our experiments showed that IGF-1 increased LARP6 expression and its subsequent binding to COL1a1 and COL1a2 mRNA leading to an increase in collagen type I synthesis, and when LARP6 was sequestered with the 5’SL decoy, the IGF-1-induction of collagen type I expression was completely inhibited. Thus, expression of LARP6 and its binding to the 5’SL is decisive for 5’SL-regulation of collagen synthesis. Therefore, the ability of IGF-1 to posttranscriptionally regulate LARP6 expression may be a critical mechanism by which rapid upregulation of collagen synthesis can be achieved. Furthermore, this capability of IGF-1 to upregulate LARP6 expression may have implications in various physiological settings such as development and wound healing, in which rapid upregulation of collagen type I synthesis is critical for growth and repair.

We have shown previously in Apoe<sup>−/−</sup> mice that in addition to reducing total atherosclerosis, IGF-1 increases collagen content and VSMC contractile protein expression in plaque. However, the mechanisms by which IGF-1 increases these features of plaque stability had not been identified. This study elucidates signal transduction and molecular mechanisms whereby IGF-1 induces a contractile phenotype in vascular smooth muscle. Furthermore, this study identifies LARP6 as a critical mediator by which
IGF-1 upregulates collagen type I synthesis and maturation. Overall, this work uncovers fundamental mechanisms whereby IGF-1 promotes stabilization of atherosclerotic plaque.
List of References


