



Published in final edited form as:

Am J Physiol Cell Physiol. 2025 March 01; 328(3): C939–C953. doi:10.1152/ajpcell.00786.2024.

Activated Cardiac Fibroblasts are a Primary Source of High Molecular Weight Hyaluronan Production

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Abstract

During acute myocardial infarction, the composition of the extracellular matrix changes remarkably. One of the most notable changes in the extracellular matrix is in the accumulation of collagen; however, hyaluronan rivals collagen in its abundance. Yet, the extent to which specific cells and enzymes may contribute to such accumulation has been largely unexplored. Here, we hypothesized that activated cardiac fibroblasts produce hyaluronan via hyaluronan synthase 2 (HAS2). We show that hyaluronan accumulates following myocardial infarction and persists through at least four weeks. Our analyses of failing heart RNA sequencing data suggest fibroblasts are the cells most changed in the expression of *HAS2*. Given these insights, we used HAS2 gain- and loss-of-function approaches to examine the extent to which activated cardiac fibroblasts produce hyaluronan. TGF β -induced activation of fibroblasts caused a significant increase in *Has2* mRNA and concomitant accumulation of hyaluronan greater than 1 MDa in size. Deletion of *Has2* abrogated TGF β -induced production of hyaluronan. In addition, overexpression of *Has2*

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Contributions:

DTL: Designed and performed experiments; analyzed and interpreted data; provided funding; wrote and revised manuscript

CMH: Performed experiments

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YY: Provided critical reagents

BGH: Provided funding

REB: Performed experiments

RS: Designed experiments; analyzed and interpreted data

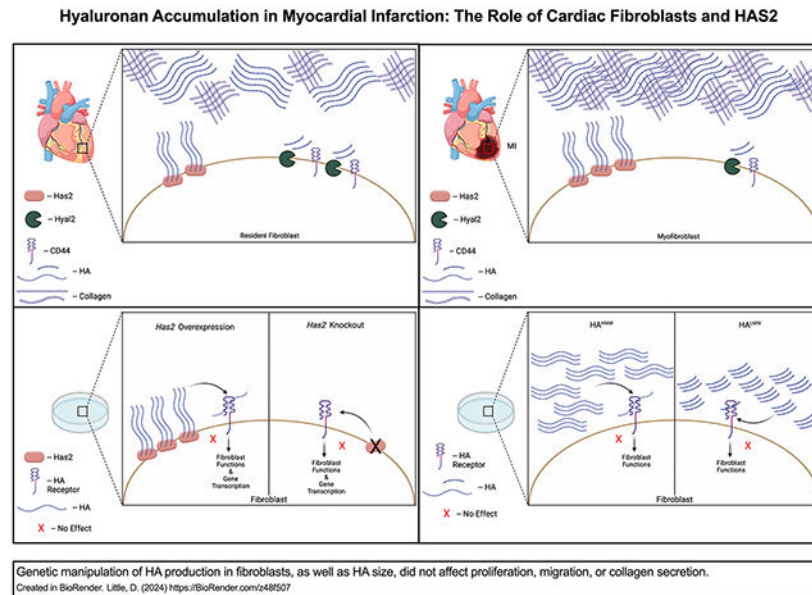
SPJ: Conceived project; designed experiments; interpreted data; provided funding; wrote and revised manuscript

DISCLOSURES

None of the authors has any relevant conflicts to declare.

was sufficient to cause an increase in hyaluronan accumulation in the absence of TGF β -induced activation. Our data indicated negligible impacts of *Has2* on proliferation, migration, and collagen production. Exposing fibroblasts to exogenous hyaluronan also had minimal impact on fibroblasts. We also assessed whether fibroblast-borne *Hyal2* plays a role in the degradation of hyaluronan, and our data indicated little impact of *Hyal2* on hyaluronan accumulation (or even any impacts on the transcriptional profile of fibroblasts). Activated fibroblasts produce high molecular weight hyaluronan via *Has2*, which occurs independent of other fibroblast functions.

Graphical Abstract



NEW & NOTEWORTHY

Activated cardiac fibroblasts produce copious quantities of collagen and much is known about this process. They also produce hyaluronan, which is abundant in the extracellular matrix, but less is known about hyaluronan. Here, we identify cardiac fibroblasts as major producers of hyaluronan, and specifically, that they produce high molecular weight hyaluronan via HAS2. This has important implications for ventricular remodeling and for metabolic regulation of activated fibroblasts as they produce this abundant matrix component.

Keywords

Fibrosis; Extracellular Matrix; Heart Failure; Myocardial Infarction

Introduction

The extracellular matrix (ECM) is a complex network of polysaccharides, proteins, and glycoproteins, which provides structural support and biochemical cues to cells within an organ. In the heart, the ECM surrounds and supports cardiomyocytes and the vasculature and plays crucial roles in tissue organization, mechanical strength, and cell signaling. The ECM

serves as a reservoir for growth factors and cytokines that influence cell behavior and tissue homeostasis. Fibroblasts, which are key arbiters of the ECM, also support homeostasis in the heart by maintaining the ECM through synthesis and degradation of collagens, elastin, proteoglycans, and other components of the ECM. Yet, many specific aspects of fibroblast function in health and disease remain unexplored.

In addition to supporting healthy tissue, fibroblasts support tissue repair during wound healing. Following acute myocardial infarction, fibroblasts play major roles in the three phases of cardiac repair that occur after injury. This wound healing response results in a scar that prevents cardiac rupture. Unfortunately, this phenotype, if prolonged, can lead to adverse ventricular remodeling and heart failure. Most assessments of ventricular remodeling focus on the contribution of collagen, which is clearly important. Yet, collagen is only one of the two most abundant components of the extracellular matrix. Hyaluronan (HA) is the other abundant component; however, it has been disproportionately under-studied.

HA is an abundant, non-sulfated, unbranched glycosaminoglycan in the extracellular matrix. It consists of repeating disaccharide units of N-acetylglucosamine and glucuronic acid and can be as large as 10^7 Da. HA can be synthesized by the three isoforms of hyaluronan synthase (HAS1, HAS2, HAS3), along the plasma membrane. Each isoform is known to synthesize hyaluronan of different sizes and chain size has shown to be important in many disease conditions.(1-4) In mammals, HA is degraded by several enzymes classified as hyaluronidases. Three hyaluronidase genes (*Hyal1*, *Hyal2*, *Hyal3*) (5), are found in different areas of a cell, and each degrades different size chains of HA. Classically, *Hyal2* has been thought to be a primary extracellular hyaluronidase (catabolizing high molecular weight HA into low molecular weight HA) and *Hyal1* has been considered an intracellular hyaluronidase, which presumably cleaves internalized low molecular weight HA into oligomeric and monomeric units. More recently, HYBID (CEMIP) and TMEM2 (CEMIP2) were identified as enzymes that share similar gene sequences and may have important functions as hyaluronidases.(6-9)

It has long been known that HA accumulates after MI or other damage to the heart (10-12). Yet, the cellular and enzymatic source of HA has not been definitively shown. Along with other ECM components they make, fibroblasts likely produce HA concomitant with their production of collagen. Yet, little is known about fibroblast-dependent HA production, whether it changes after activation, and which synthase is responsible for such production. The present study identifies a primary cell type and specific enzyme responsible for HA production by fibroblasts. These findings have important implications for ventricular remodeling in disease and general maintenance of myocardial structure in health.

Methods

Myocardial Infarction:

C57BL/6J mice, 12–20 wk old, (both sexes) were subjected to non-reperfused myocardial infarction (MI) as described previously.(13-17) Briefly, mice were anesthetized with intraperitoneal injections of ketamine hydrochloride (50 mg/kg) and sodium pentobarbital (50 mg/kg). Mice were orally intubated and ventilated; the ventilator's room-air port was

supplemented with oxygen. A 7–0 silk suture was passed under the left coronary artery and tied. The chest and skin were closed in layers. Mice were extubated upon recovery of spontaneous breathing. Analgesia (ketoprofen, 5 mg/kg) was provided prior to recovery and by 24 and 48 h post-surgery. The surgeon was blinded to mouse genotype. Seven days after MI, all mice were subjected to an echocardiogram to confirm sufficient depression of cardiac function (LVEF<60%). Mice were followed up through 4 wk.

Echocardiography:

Transthoracic echocardiography of the left ventricle was performed as previously described(13, 15, 16, 18, 19) with adjustments for the VisualSonics Vevo 3100 system. The sonographer was blinded to the treatment group.

Echocardiography Analysis:

The parasternal long axis (PLAX) view was used to obtain M-mode images for the measurement of the thickness of the left ventricle anterior wall (LVAW) and the left ventricular posterior wall (LVPW), and the left ventricular interior diameter (LVID). Fractional shortening (FS) was calculated by using the formula $[(LVID;d - LVID;s)/LVID;d] \times 100$. Four measurements were taken and averaged for each parameter. The parasternal long axis (PLAX) view, in B-Mode, was used to obtain two-dimensional images for the measurement of LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), stroke volume (SV), ejection fraction (EF), cardiac output (CO), and heart rate, with six measurements taken and averaged. An apical four chamber view in Pulsed Wave (PW) Doppler was used to obtain two-dimensional images for the measurement of the isovolumic relaxation time (IVRT), with nine measurements taken and averaged.

RNA Sequencing:

Total RNA was isolated from cardiac fibroblasts as previously described for cardiac mesenchymal cells.(13) RNA quality was evaluated using NanoDrop ONEC (Thermo Scientific) and at least 2 µg of RNA was sent to the University of Louisville Sequencing Technology Center (*Hyal2* and *Has2* over-expression) and Admera Health (*Has2* deletion) for poly-A RNA sequencing. At the University of Louisville Sequencing Technology Center, the TruSeq Stranded mRNA Library Prep Kit (Illumina Cat# 20020594) and TruSeq RNA CD Index Plate (Illumina Cat# 20019792) were used to generate a sequencing library. Size, purity, and semi-quantitation were performed on an Agilent Bioanalyzer 2100 system using the Agilent DNA HS Kit. Single-end sequencing was performed on an Illumina NextSeq 2000, using a P3 100 cycle cartridge with a P3 flow cell. Libraries were sequenced with a single 101 cycle read length with two index reads of 8 bp each. At Admera Health, isolated RNA sample quality was assessed by RNA TapeStation (Agilent Technologies Inc., California, USA) and quantified by Qubit 2.0 RNA HS assay (ThermoFisher, Massachusetts, USA). For library construction, the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® was utilized (New England BioLabs Inc., Massachusetts, USA). Final libraries quantity was assessed by Qubit 2.0, and quality was evaluated by TapeStation HSD1000 ScreenTape (Agilent Technologies Inc., California, USA). Equimolar pooling of libraries was performed based on QC values, and paired-end sequencing was performed on

an Illumina® NovaSeq X Plus (Illumina, California, USA). FASTQ files were generated utilizing BaseSpace DRAGEN analysis Version 1.2.1. (20-24)

Bioinformatics Analysis:

Quality control (QC) of the raw sequence data was performed using FastQC (version 0.10.0) for each sequencing sample(25). The sequences were aligned to the mm10 mouse reference genome using STAR version 2.6.(26). Differential expression of ENSEMBL protein-coding transcripts was performed using DESeq2 (3), and raw counts were obtained from the STAR-aligned bam format files using HTSeq version 0.10.0.(27). The raw counts were normalized using the Relative Log Expression (RLE) method and then filtered to exclude genes with fewer than ten counts across the samples. DESeq2 guidelines were used to identify differentially expressed genes (sex was added as a covariate in analysis pipeline), and all P values were adjusted for testing multiple genes (Benjamini–Hochberg procedure; $p \leq 0.05$). Functional enrichment analysis was performed using the clusterProfiler R package to identify enriched Gene Ontology biological processes and KEGG pathways for each set of differentially expressed genes (DEGs)(28).

HA ELISA:

Heart tissue from infarct zone from 1 wk and 4 wk MI and from left ventricle from 1 wk and 4 wk sham hearts was powdered and weighed. 500 μ L of Cell Lysis Buffer 2 (R&D Systems: 895347) was added to each sample and tissue was lysed for 30 min at room temperature with gentle agitation. Samples were centrifuged to remove debris. Samples were diluted to a 1:500 dilution. Quantikine ELISA: Hyaluronan Immunoassay (R&D Systems: DHYAL0) was used to assess HA concentration in each sample.

Adult Mouse Cardiac Fibroblast Isolation:

Adult mouse cardiac fibroblasts were isolated using collagenase II. The mice were anesthetized with 5% isoflurane and the hearts were quickly excised and washed with ice-cold phosphate buffered saline (PBS; pH: 7.4, Invitrogen 10010023). Excess fatty tissue was subsequently removed. The heart was then perfused with 5 mL of PBS, the aorta was removed, and the heart was finely chopped using a razor blade before being placed into a collagenase II solution (Worthington Biochemical, CLS2 LS004177) for 45 min. After digestion, the collagenase was neutralized using DMEM/F-12 containing 10% fetal bovine serum (VWR 97068-085), 1% penicillin-streptomycin (10,000 U/mL) (Invitrogen 15140122), 1% ITS-G (100 $\times$) (Invitrogen 41400-045), and 20 ng/mL human bFGF (Peprotech 100-18B). The collagenase-CFB Media solution was centrifuged, supernatant was aspirated, and the fibroblasts were resuspended in 5 mL of CFB media and filtered through a 70 μ m cell strainer into a sterile 50 mL centrifuge tube. Fibroblasts were plated at a ratio of 1 heart per 75 cm² (10 mL/flask) and allowed to sediment for 2 h. Cardiac fibroblast media was changed following 2 h, 24 h, and subsequently every 2 d until reaching 70–80% confluency.

Fibroblast Activation:

Cardiac fibroblasts were treated at passage 2. Cells (5×10^5) were plated in 60 mm dishes (Corning 353002) and allowed to adhere to the bottom of the dish overnight. Cells were then serum starved for 24 h. Serum-containing media was added back to cells and 20 ng/mL of bFGF or TGF β (Peprotech 100-21C) was added to reflect control conditions (bFGF) or activation (TGF β). Conditioned media was collected from cells 24 h after treatment and hyaluronan was isolated from media.

Has2 Deletion and Overexpression in Fibroblasts:

Cardiac fibroblasts from *Has2^{fl/fl}* mice(29) were treated at P2. Cells (7×10^5) were plated in 60 mm dishes and allowed to adhere to the bottom of the dish overnight. Cells were then serum starved for 24 h. To treat, fibroblasts were given Cre adenovirus (Vector Biolabs 1045N), a *Has2* overexpression adenovirus (Vector Biolabs), or GFP adenovirus as the control. After 5 h, cells were given fresh media with 20 ng/mL of bFGF and TGF β , respectively. After 48 h of stimulation, the conditioned cell media was collected, and HA was isolated from it. After isolation, the HA was sized using gel electrophoresis.

Hyal2 Deletion and Overexpression in Fibroblasts:

Cardiac fibroblasts from homozygous *Hyal2^{fl/fl}* mice were treated in the exact same manner as *Has2^{fl/fl}* fibroblasts. An adenovirus harboring *Hyal2* (Vector Biolabs) was used to overexpress *Hyal2*.

Reverse Transcriptase PCR and Real-Time PCR:

Total cardiac RNA was extracted and used to make cDNA. The relative levels of mRNA transcripts were quantified by real-time PCR using Power SYBR Green (Thermo Fisher Scientific) on a real-time PCR system (QuantStudio5). Most primers were made using NCBI Primer Blast except HPRT primers (PPM03559E-200, QIAGEN). The data were normalized to mouse HPRT mRNA threshold cycle (CT) values by using the $\Delta\Delta CT$ comparative method. All primer sequences are listed in the (Supplemental Table 1).

Protein Isolation:

Total cardiac protein was isolated, and protein concentration was determined by the Bradford assay with a protein assay dye (Bio-Rad, Cat. No. 5000006) using different concentrations (0, 1, 2, 4, and 8 mg/mL) of bovine serum albumin as standards. We measured protein concentrations using a Thermo Multiskan Spectrum spectrophotometer.

Western Blot Analysis:

Following experimental treatments, conditioned medium was collected, centrifuged and supernatant mixed with Laemmli sample buffer (125 mM Tris-HCl, 10% SDS, 50% Glycerol, 0.05% Bromophenol Blue, pH 6.8) and incubated at 95°C for 5 min. Each sample was then loaded on a 7.5% acrylamide/bis SDS-PAGE gel for electrophoresis at 120 V. SDS-PAGE-resolved proteins were then transferred overnight onto a PVDF membrane (Cytiva, 10600021) at 4°C at 20 V for 16 h. Subsequently, membranes were blocked for 1 h at room temperature with Tris-buffered saline containing Tween-20 (TBS-T) and 5% bovine

serum albumin (Sigma-Aldrich, A7906). Membranes were probed with primary antibodies overnight at 4°C. The following day, membranes were washed three times using TBS-T and then incubated with secondary antibody for 1.5 h. Membranes were then washed three times with TBS-T before exposure to Pierce™ ECL Plus Western Blotting Substrate (Thermo Scientific, 32132) and imaging on a ChemiDoc imager (BioRad). Protein was normalized to total cell protein and parallel blots were normalized to an anchor protein. Antibodies used include primary anti-COL1A1 (1:20,000; Thermo Fisher, PA5-29569) and secondary anti-rabbit IgG HRP-linked (1:2,500; Cell Signaling, 7074). Because of potential promiscuity of collagen antibodies, we referred to immunopositivity with the COL1A1 antibody simply as ‘collagen’.

Pathology:

Following final echocardiography, hearts were excised and arrested in diastole with 2% KCl. Hearts were then sectioned into 1-mm short-axis sections. A midventricular section was fixed with 10% formalin; the samples were subsequently embedded, cut, and mounted. MATLAB version 9.7.01216025 (R2019b) was used to compile data files exported from the Keyence analysis software to generate a summary file for data analysis, and the microscopist was blinded to group assignment.

Transverse heart sections from sham, 1-week, and 4-week post MI mice were fixed in neutral-buffered 10% formalin and embedded in paraffin wax. The hearts were sliced into 4 µm sections, deparaffinized in an oven, dehydrated in xylene, and rehydrated with ethanol and water. All stained tissue slides were imaged with a Keyence BZX-810 microscope and analysis was performed with the Keyence software couple with customized Matlab macros.

To visualize fibrosis accumulation, the heart sections were stained with Picro-Sirius Red made from Direct Red 80 and saturated Picric Acid (Sigma-Aldrich 365548-5G, P6744-1GA). The right ventricle was removed from the image and the total fibrosis percentage was quantified in the remote zone, infarct zone, and left ventricle.

To examine HA accumulation, the tissue was incubated in hyaluronic acid binding protein (Sigma-Aldrich 385911-50µg) with 1× PBS and stained with Alexa Fluor 568 (ThermoFisher Scientific S11226). The nuclei were stained with DAPI. HA percentage was quantified using the same methods above.

Cardiomyocyte hypertrophy and capillary density were assessed. The borders of the cardiomyocytes were stained with wheat germ agglutinin (WGA CF™ 488A conjugate, Biotium) and the capillaries with Isolectin B4 DyLight 649 (Vector Labs DL-1208-.5) in 10 mM HEPES solution with calcium chloride dihydrate. DAPI was used to visualize the nuclei. Only myocytes with centrally located nuclei and a circularity of 50–100 were used for quantification. Capillary density was calculated by dividing the number of capillaries by the total tissue area in the image (mm²).

Hyaluronan Isolation, Sizing, and Analysis:

Hyaluronan was isolated as described(13, 30), with slight modifications. For cultured fibroblast media, hyaluronan (HA) was isolated from conditioned media of treated

fibroblasts. Proteins were digested using 1× proteinase K (Invitrogen 25530015) at 60°C for 4 h and first precipitation was done with 200 proof ethanol at −20°C (Day 1). Samples are then washed with 75% ethanol, resuspended in 100 mM ammonium acetate (Sigma Aldrich A1542) and nucleic acids were digested with Benzonase (EMD Millipore 70664-3 25 U/μl) at 37°C (Day 2). Second precipitation was done with 200 proof ethanol and samples were washed with 75% ethanol. Samples were air-dried, resuspended in 100 mM ammonium acetate (Sigma Aldrich A1542) and incubated at 4°C (Day 3). Samples were lyophilized and resuspended in 10 M formamide (Sigma Aldrich F9037) and incubated at 4°C (Day 4). A 1% agarose gel was made with 1× TAE was used to size HA. After casting, gel was pre-run in 1× TAE for 6 h at 80 V to remove impurities. Loading buffer (0.2% bromophenol blue (Sigma Aldrich B0126) in 10 M formamide) was added to each sample and HA^{HMW} (R&D Systems GLR002) positive control. Samples were run on gel in 1× TAE at 100 V for 2.5–3.0 hours and equilibrated in 30% ethanol for 1 hour before being stained with 1 mL of 400× stock stains-all solution (12.5 mg of stains-all (Sigma Aldrich E9379) to 5 mL of 200 proof ethanol) in 400 mL of 30% ethanol in the dark, overnight (Day 5). Gels were equilibrated in dd-water for 1 h in the dark and destained under ambient light on the bench for 15–20 min. Color images were taken on an EPSON flatbed scanner for presentation purposes. Image for quantitation was taken using a Bio-Rad ChemiDoc using the Stain-Free setting. Stain-Free images were analyzed using Fiji-Image J. Confirmation of Hyaluronan isolation are shown in (Supplemental Figure 1).

For heart tissue, mouse hearts were chopped into small pieces using a razor blade and suspended in 5 ml of 100 mM ammonium acetate (Sigma Aldrich A1542). Proteins were digested using 1× proteinase K (Invitrogen 25530015), shaken at 60°C for 4 h, and first precipitation was done with 200 proof ethanol. Isolation from this point was done as “Cultured fibroblast media” (*vide supra*).

Glucose Treatments:

Cardiac fibroblasts from wild-type mice were treated at P2. Cells (5×10^5) were plated in 60 mm dishes and allowed to adhere to the bottom of the dish for 24 h and then serum starved overnight. To treat, fibroblasts were given various concentrations of glucose, mannitol, and stimulant for various time points. The media was changed and collected at various time points and HA was isolated.

Glucose Deprivation Experiments: Fibroblasts were treated with 0 mM glucose media, 17.5 mM glucose media, and 17.5 mM mannitol media. Each group of treatments were treated with either bFGF or TGFβ to simulate naïve or activated cells, respectively. The first group of treatments were collected at 48 h and the second group was collected at 72 h. The third group was treated, and media was changed at 48 h with new treated media and then collected at 72 h. The 4th group was treated, and media was collected at 24 h, 48 h, and 72 h.

Low and High Glucose Experiments: Fibroblasts were treated with 5.5 mM glucose media, 17.5 mM glucose media, 25mM glucose media, and 7.5 mM mannitol + 17.5 mM glucose media. Each group of treatments were treated with either bFGF or TGFβ to simulate naïve or activated cells, respectively. Media was collected at 48 h.

Proliferation:

Cardiac fibroblasts from *Has2*-floxed mice were treated at P2. Cells (6×10^4) were plated in 6 well plates and allowed to adhere to the bottom of the dish for 24 h and then serum starved overnight. Fibroblasts were given Cre adenovirus (Vector Biolabs 1045N), a *Has2* overexpression adenovirus (Vector Biolabs), or GFP adenovirus as the control. After 5 h, cells were given fresh media. 24 h and 48 h later, cells were lifted from the plate and counted using a flow cytometer. Media was collected from cells before lifting were imaged and cells were kept for RNA isolation after flow. bFGF treatment (naïve cells) was used as a control and TGF β was used to stimulate cells into activation.

Fibroblasts were given HA^{LMW} and HA^{HMW} for 24 h and 48 h, then harvested, and counted using a flow cytometer. Cells were kept for RNA isolation after flow. bFGF was used a naïve control and TGF β was used to stimulate cells into activation.

Scratch Assay:

Cardiac fibroblasts from *Has2*-floxed mice were treated at P2. Cells (2×10^5) were plated in 6 well plates and allowed to adhere to the bottom of the dish overnight. Cells were then serum starved for 24 h. Fibroblasts were given HA^{LMW} or HA^{HMW} for 24 h. Afterwards, cells were scratched for 6 h and 12 h time periods. Images were taken after timepoints with a Keyence imager. 0 h scratch images were taken immediately after 24 h HA treatment. Cells were kept for RNA isolation. BFGF was used a naïve control and TGF β was used to stimulate cells into activation.

Fibroblasts were transduced with Cre- (Vector Biolabs 1045N), a *Has2* overexpression adenovirus (Vector Biolabs), or GFP adenovirus (Vector Biolabs 1060) as the control. After 5 hours, cells were given fresh media for 24 h. After 24 h, cells were scratched, then followed for 0 h, 6 h, and 12 h. Images were taken after timepoints with a Keyence imager. 0 h scratch images were taken immediately after 24 h virus treatment. Media was collected from cells after scratches were imaged and cells were kept for RNA isolation. bFGF was used a naïve control and TGF β was used to stimulate cells into activation.

Hydroxyproline Assay:

Cardiac fibroblasts from *Has2*-floxed mice were treated at P2. Cells (7×10^5) were plated in 60 mm dishes and allowed to adhere to the bottom of the dish overnight. Cells were then serum starved for 24 h. Fibroblasts were transduced with Cre- (Vector Biolabs 1045N), a *Has2* overexpression adenovirus (Vector Biolabs), or GFP adenovirus (Vector Biolabs 1060) as the control. After 5 hours, cells were given fresh media for 24 h. After 24 h media was collected, and cells were left to produced ECM for 2 wk. Media was changed every 2 days. After 2 wk, dishes were decellularized using decellularizing solution (1 $\times$ PBS containing 50 μ M ammonium hydroxide [NH₄OH, 0.05% Triton X-100] to lyse cells and DNase solution (20 U/mL DNase 1, 20 μ L/mL DNase 1+MgCl₂ buffer, in sterile H₂O)] to degrade the DNA. The ECM left was removed from the dish and hydrolyzed using 12 N NaOH and boiling for 1 h at 120°C. Hydrolyzed ECM was neutralized using 12 N HCl and hydroxyproline concentration in ECMs were quantified using a commercial hydroxyproline assay (Abcam Ab222941). Confirmation of decellularization is shown in **Supplemental Figure 2**.

Results

Hyaluronan accumulates post-MI.

To confirm that HA accumulates in our mouse MI model (Figure 1F and 1G, **Supplemental Table 2**), we investigated hyaluronan accumulation in the heart acutely and chronically after myocardial infarction (MI). Our data indicate that HA accumulates in the left ventricle by 7 d post-MI (Figure 1A and 1B) and persists through 28 d post-MI (Figure 1C and 1D), and this was corroborated via HA ELISA (Figure 1E). This, coupled with our(13) and others'(31) prior observations indicate that HA accumulation is an early and durable occurrence following MI. This has important but largely unexplored implications for ventricular remodeling. From a spatial perspective, accumulation of HA was most notable in the infarct region and generally coincided with collagen, which was highly abundant at both 7 d (Figure 2A and 2B) and 28 d after MI (Figure 2C and 2D). HA did not accumulate significantly in the remote zone at either time point. We did not observe any sex differences in both HA accumulation and collagen in the infarct zone.

HA exhibits polydispersity (i.e., a range of sizes) (32) and is most often categorized into two main groups: high molecular weight (HMW; low MDa range) and low molecular weight (LMW; below 0.5 MDa). Because hyaluronan accumulates in the heart acutely and chronically post MI, we determined the relative size of the accumulating HA. These analyses indicate that HA^{HMW} accumulates in the infarct zone at 7 d and at 28 d (Figure 3A and 3B), with accumulation of HA^{HMW} being most notable at 28 d. Given the spatial coincidence of hyaluronan accumulation and scar formation, we next queried whether fibroblasts produce HA.

Activated cardiac fibroblasts produce hyaluronan.

Fibroblasts can be activated by a variety of cues. The cytokine, TGF β , is one of the commonest inducers of fibroblast activation *in vitro*. We used this well described activator in cardiac fibroblasts to assess the impact of fibroblast activation on HA levels. We show that after 24 h of TGF β , there was a significant increase in HA accumulation compared to naïve (bFGF) fibroblasts (Figure 4A and 4B). We also show that the accumulated HA was HA^{HMW}. This is consistent with the observations depicted in Figure 3. Given that activated fibroblasts produce HA, we next determined whether the likeliest enzyme, HAS2 (encoded by *Has2*), was responsible for TGF β -induced accumulation of HA.

Has2 drives HA production in cardiac fibroblasts.

There are three known hyaluronan synthases. Based on the literature and our prior observations of increased expression, it was likely that HAS2 was a source. When we evaluate RNA sequencing data from activated fibroblasts, we notice that the *Has2* gene is the only one of the three hyaluronan synthases significantly increased compared to naïve fibroblasts (Figure 4C). Further supporting this idea, HAS2 is considered the most important isoform because it is finely regulated(33, 34) and its deletion is embryonic lethal.(35) For loss-of-function studies (Figure 5A and 5B), *Has2* deletion significantly decreased HA^{HMW} accumulation in the media; this observation was true in naïve fibroblasts and TGF β -activated fibroblasts. For gain-of-function experiments (Figure 5A and 5B), overexpression

of *Has2* (in the absence of TGF β stimulation) increased HA^{HMW} accumulation in the media compared to naïve control, which effectively recapitulated the effect of TGF β stimulation in terms of HA^{HMW} accumulation. These proof-of-concept studies definitively show that *Has2* is the primary source of HA^{HMW} production by fibroblasts. To determine the extent to which deletion or overexpression of *Has2* impacts the expression of relevant elements of relevant regulatory elements of the matrix, we also performed RNA-Seq.

Substrate level regulation.

To understand the substrate-level regulation of HA production, naïve and TGF β -activated fibroblasts were treated with varying concentrations of glucose. In naïve fibroblasts, low glucose showed no significant change compared to basal glucose with high glucose showing the same (Figure 6A and 6B). Of the TGF β -induced activated fibroblasts both low and high glucose showed no significant differences compared to basal glucose (Figure 6C and 6D). Interestingly, zero glucose shows significant decrease in HA^{HMW} accumulation in TGF β -induced activated fibroblasts compared to normal glucose treatments. This effect was significant only after 72 h post treatment (Figure 6J), with the 48 h post treatment trending towards a decrease (Figure 6G). In naïve fibroblasts, the zero glucose treatment appears to be slightly fainter after both 48 h (Figure 6E) and 72 h (Figure 6H). Overall, these data suggest that extracellular glucose concentrations affect the amount of HA^{HMW} accumulated by activated cardiac fibroblasts.

Transcriptional profiling.

To determine the extent to which changing HA production influences gene expression in fibroblasts, we used loss- and gain-of-function approaches as described above, isolated RNA, and performed bulk RNA-Seq.

In samples in which we forced expression of *Has2*, there were limited significant changes (only 13 genes downregulated) in gene expression (other than an increase in *Has2*). Of these 13 genes, four genes (*Gbp3*, *Herc6*, *Ddx60*, and *Iigp1*) were also downregulated in the context of TGF β treatment. Whether these four downregulated genes are part of a HAS2-dependent suppression of expression, which also occurs in TGF-induced fibroblast activation, could be speculated. There is not, however, a clear pathway or role of these genes in fibroblast function. Although TGF β changed the expression of thousands of genes, deletion of *Has2* had limited impact on the response to TGF β . When we deleted *Has2*, we observed upregulation of only five genes in naïve fibroblasts and zero gene changes after deletion of *Has2* in activated fibroblasts (**Supplemental Figure 3**).

Initially, we were surprised to note that the total counts of *Has2* mRNA did not significantly decrease after Cre adenoviral transduction; however, differential exon usage RNA sequencing (DEXseq) analysis revealed that the sequencing counts for the non-deleted region of *Has2* were disproportionately higher than for the deleted region of *Has2* (**Supplemental Fig 4**). To further confirm deletion of *Has2*, we performed qRT-PCR for the recombined exon, and generated UCSC Genome Browser sample expression tracks positioned at the *Has2* gene and Integrative Genome Browser Sashimi plots depicting exon 2 skipping in *Has2* deleted samples (**Supplemental Fig 4**). This is an important technical note

for deletion studies, i.e., that the RNA-Seq data may not fully reflect artificial interventions into living systems.

In terms of manipulation of *Hyal2*, overexpression of *Hyal2* in naïve fibroblasts changed the expression of no genes except *Hyal2*. In the context of TGF-activation, deletion of *Hyal2* had little impact on the fibroblast transcriptional profile. Overall, 66 genes changed after deletion of *Hyal2* in naïve cardiac fibroblasts. None of those genes were related to hyaluronan metabolism, hyaluronan receptors, or ECM proteins and when evaluating the Gene Ontology visualizations, most of the biological processes with the most changed genes involved were related to inflammation. Understanding HA's role in inflammation is important in understanding HA's role in cardiac wound healing (**Supplemental Figure 5**).

Hyal2 alone does not degrade HA derived from cardiac fibroblasts.

Given the history of extracellular hyaluronidase function ascribed to HYAL2 (encoded by *Hyal2*), we determined the extent to which *Hyal2* may contribute to HA^{HMW} degradation. To address a potential role of fibroblast-mediated degradation of HA, we used a similar gain- and loss-of-function approach for *Hyal2*. The rationale for focusing on *Hyal2* was driven by several lines of evidence. First, several studies show that *Hyal2* has hyaluronidase activity. Second, *Hyal2* is expressed in fibroblasts. Third, *Hyal2* is reported to have hyaluronidase activity for HA^{HMW}. Fourth, HYAL2 is expressed at the surface of cells. Fifth, HYAL1 has only been shown to be expressed inside of cells (where it cannot access secreted HA). Sixth, the other expressed HYAL, *Hyal3*, has been shown to have activity only in sperm(36). Given this robust premise, we focused on *Hyal2*. Deletion of *Hyal2* did not cause an increase in HA accumulation (Figure 7), suggesting little constitutive *Hyal2*-dependent hyaluronidase activity. Likewise, overexpression of *Hyal2* did not decrease HA levels in the media, which calls into question its capacity to function as a universal hyaluronidase—though additional experiments would be needed to address this separate question. Nevertheless, these collective data indicate that fibroblast-borne *Hyal2* does not contribute significantly to degradation of HA.

HA has limited impact on proliferation, migration, or secretion.

To better understand how HA may play a role during wound healing, we assessed whether changing HA accumulation caused changes in fibroblast proliferation. We first deleted and overexpressed *Has2* in naïve fibroblasts but observed no significant changes in proliferation compared to control. We then deleted and overexpressed *Has2* in TGFβ-induced activated fibroblasts and saw similar results (i.e., no group differences) (**Supplemental Figure 6A**). Next, we determined whether different-sized exogenous HA influences fibroblast proliferation. We treated naïve fibroblasts with HA^{LMW} or HA^{HMW} and found that changing the size of the HA did not influence fibroblast proliferation compared to control. We then treated TGFβ-induced activated fibroblasts with HA^{LMW} or HA^{HMW} and obtained similar results (**Supplemental Figure 6B**).

To further examine the effect of HA on fibroblast function, we assessed the impact of HA on fibroblast migration. Neither deletion nor overexpression of *Has2* in naïve fibroblasts impacted migration compared to GFP. Even in the context of TGFβ-induced activation,

neither deletion nor overexpression of *Has2* differed from control (GFP). To determine whether different sizes of HA impact fibroblast migration, we treated naïve fibroblasts with HA^{LMW} or HA^{HMW} and observed no impact fibroblast migration compared to control. When treating TGFβ-induced activated fibroblasts in the same manner, no significant changes in migration were found (**Supplemental Figure 6C**).

Lastly, it is known that, after activation, fibroblasts increase collagen secretion. To determine if HA plays a role in the increased collagen secretion, we deleted *Has2* in TGFβ-induced activated fibroblasts, but we found no difference in soluble collagen levels in media from treated fibroblasts. We also assessed *Has2* deletion and overexpression in naïve fibroblasts and did not identify changes in soluble collagen secretion compared to naïve control. To test whether there were changes in collagen deposited into the ECM, we deleted and overexpressed *Has2* in naïve fibroblasts and quantified hydroxyproline concentration from decellularized and hydrolyzed ECMs. There were no significant changes in hydroxyproline either after *Has2* deletion or *Has2* overexpression compared to the control (Figure 8).

Discussion

Hyaluronan (HA) is a glycosaminoglycan, which is prominent in the extracellular matrix. HA accumulates in the heart after MI in humans(10-12) and mice.(37, 38) Yet, the extent to which and how HA accumulation influences ventricular remodeling is not known. To make progress in this area, clear insight into which cells produce HA is needed. Here, we assessed those two questions and hypothesized that activated fibroblasts are a primary source of HA production. Indeed, fibroblasts produce HA^{HMW} through the enzyme hyaluronan synthase 2 (*Has2*).

Despite our clear evidence that fibroblast-borne *Has2* drives production of HA^{HMW}, forcing *Has2* expression or exposing fibroblasts to HA^{HMW}—in the absence of TGFβ-activation—did little to impact fibroblast function. This suggests that although fibroblasts are a primary source of HA, they may not be significantly impacted by high levels of HA^{HMW}. We have shown that HA^{HMW} impairs macrophage function (39), which is consistent with the literature, (40, 41) and thus has clear implications in wound healing and ventricular remodeling. Hence, activated fibroblasts produce HA^{HMW}, which impairs macrophage function. Collectively, these insights could have important implications for ventricular remodeling.

At several points in the text, we have emphasized the size of HA (e.g., HA^{HMW}). We note that HA^{HMW} accumulates in the heart both acutely and chronically post-MI. The polydispersity of HA has been known for decades(32), and since then, studies have explored the biological impact the size of HA has on various diseases and cellular processes. HA^{LMW} can stimulate inflammatory cells by inducing the production of cytokines and chemokines. (3, 42-44) HA^{LMW} is also considered a proangiogenic factor and plays a role in the regulation of fibroblast, myofibroblasts proliferation and collagen production.(45, 46) On the other hand, HA^{HMW} is considered to be antiangiogenic and acts as a proinflammatory molecule specifically blocking inflammation effects stimulated by HA^{LMW}.(3, 47) HA^{HMW} is also thought to induce different signal cascades than HA^{LMW}—even while binding to

the same HA receptors. Although the literature discusses how the size of HA plays a role in the biological impact HA has(48), few have examined the size of HA produced by activated cardiac fibroblasts. Our data indicate that activated fibroblasts produce HA^{HMW}. The concept of polydispersity and its biologic impact has not been adequately studied in the cardiovascular realm; the present study renews the call to action in this area.

The fact that HA exhibits polydispersity—the presence of varying molecular weights(32)—is driven, in part, by the three isoforms of the HA synthesizing enzyme hyaluronan synthase: *Has1*, *Has2*, *Has3*. Both *Has1* and *Has2* produce HA^{HMW}, while *Has3* produces HA^{LMW}.(2, 4, 49) For these (and other) reasons, we focused on *Has2* in the present study. Deletion of hyaluronan synthase 2 (*Has2*) expression has been used in both cardiac ischemia-reperfusion and liver fibrosis models.(31, 50) In the cardiac study, *Has2* was deleted in all cells. Although the insights are interesting, additional studies are warranted because loss of *Has2* expression in macrophages led to their death. The reasons for such premature demise are not known. This study(38) also supports the notion that perhaps some of the HA produced after MI may confer benefit; however, many intriguing questions remain.

HA synthesis depends on the cellular availability of UDP-GlcNAc and UDP-GlcA(51, 52), which are derived from glucose. To determine the acute requirement for extracellular glucose, we performed glucose deprivation experiments (Figure 6) and were surprised that it took three days of glucose deprivation before we observed a reduction in HA production in the media. We could speculate extensively as to how this may have occurred. Instead, we were excited by these surprising findings and are currently focused on studies of the metabolic regulation of HA production. Others have shown that in hyperglycemic conditions, HA production is increased along with *HAS* expression in several different cell types(53-56). In contrast, others have shown that diabetic cardiac fibroblasts do not increase HA secretion(57). Our study showed similar results with HA^{HMW} with both naïve and activated cardiac fibroblasts—that is, excess glucose does not have a significant impact on HA production in cultured cardiac fibroblasts. Again, we are currently addressing the metabolic regulation of HA production by fibroblasts.

The primary focus of this study was on the production of HA. We also attempted to address potential degradation of HA. We tested whether deletion or overexpression of *Hyal2* changed gene expression in both naïve and activated fibroblasts. Although we confirmed changes in *Hyal2* expression, we saw no significant changes in the gene expression relating to fibroblast function—frankly, almost nothing changed. At this point, we had presumed that fibroblast-borne *Hyal2* would degrade hyaluronan; however, when we tested whether deletion or overexpression of *Hyal2* in fibroblasts caused changes in HA, we found it did not. *Hyal2*(58), but not *Hyal3*(59), is a known hyaluronidase in other systems. The reason for the refractoriness of fibroblasts to degrade HA may relate to a cryptic set of conditions needed to support its enzymatic activity, which is why we are currently addressing *Hyal2* *in vivo* and in endothelial cells, which are known to have hyaluronidase activity. Transmembrane protein 2 (TMEM2) is another hyaluronidase that has been shown to be a more potent hyaluronidase than HYAL2(6-8, 60). Similar to *Hyal2*, deletion or overexpression of *Has2* had minimal impact on the transcriptional profile of both naïve and activated fibroblasts. While we expected little to no changes in

(myo)fibroblast-related genes as a function of *Hyal2* expression, the minimal transcriptional changes observed were unexpected. As mentioned above, *Has2* plays a critical role in cardiac development, with whole-body deletion of the gene being embryonically lethal(35). Despite its key developmental role, manipulating *Has2* expression specifically in fibroblasts did not significantly affect their gene transcription.

Such response could be driven by the fact that fibroblasts, like most cells, express receptors for hyaluronan. And, if changes in hyaluronan abundance occur, the cells' response may be reflected, in part, at the transcriptional level. It is also conceivable that changing the energetically demanding process of producing HA could have broader transcriptional effects. The metabolic regulation of HA production is certainly worthy of additional examination, as mentioned above.

In terms of our present sequencing data, it should be interpreted in an appropriate framework. Our *in vitro* data was derived from cultured cardiac fibroblasts. We do not know whether we have differential (by chance) enrichment of populations of fibroblasts. Indeed, scRNA-Seq of mouse hearts has indicated at least five types of fibroblasts depending on their location in the heart; estimates from human hearts suggest twice as many.(20, 61) It is also conceivable that, despite our present observation of limited responses to HA manipulations by our *in vitro* fibroblasts, certain subpopulations may be quite responsive to changes in HA conditions. And, as is standard for any *in vitro* study of fibroblasts, it is always possible that the *in vivo* conditions differ substantively enough to influence the response of cells, which goes well beyond perfunctory presumptions are criticisms related to the artificiality of using polystyrene plates. Although not the focus of this study, there are indications of robust sex differences (which were adjusted for in our present RNA-seq data).

Collectively, we demonstrate that activated fibroblasts produce significant amounts of high molecular weight hyaluronan in a *Has2*-dependent fashion. Our data also indicate that producing large amounts of hyaluronan (via forced expression of *Has2*) causes surprisingly few changes to gene expression in fibroblasts *in vitro*. It is conceivable, however, that changes in hyaluronan production in fibroblasts challenged with multiple stressors and perhaps limited substrate availability could exhibit changes in expression or functional activity. We also cannot know the extent to which changes in hyaluronan production alters the organization of collagen in living tissue. Such questions require investigation *in vivo*. Ultimately, this study shines a light on a significant function of cardiac fibroblasts—as bioreactors for hyaluronan production, not simply collagen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We acknowledge helpful discussions and the expert technical assistance of members of the Jones Lab and others in the Center for Cardiometabolic Science. Biorender was used to make at least one figure.

FUNDING SOURCES

DL was supported by an NIH NRSA Predoctoral Fellowship (F31 HL162518). Grants from the NIH support research in the Center (P30 GM127607, S10 OD025178, and R01 GM127495) and in the laboratory of SPJ (R01 HL 163272 and R01 HL147844). SPJ has also been funded by the Jewish Heritage Fund for Excellence (University of Louisville).

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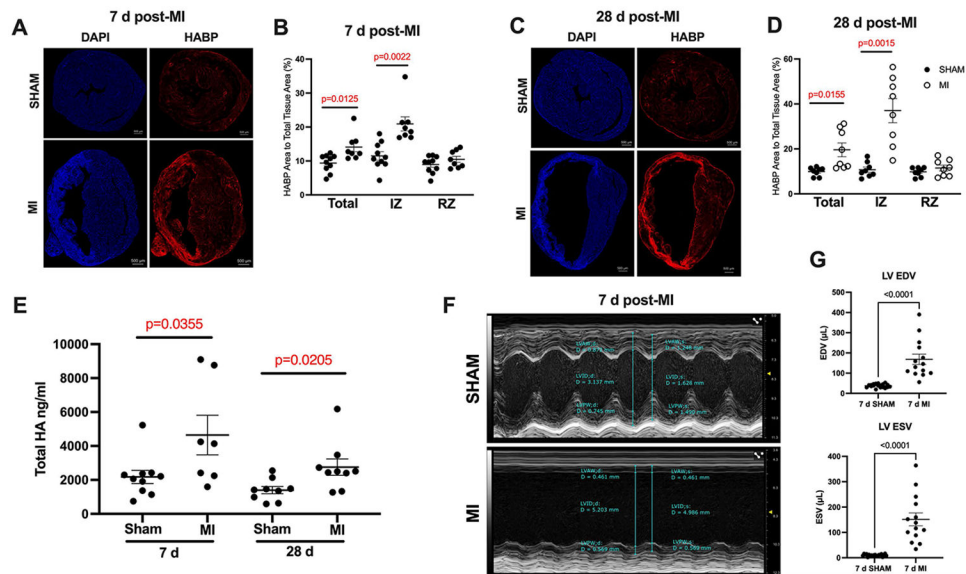


Figure 1. Hyaluronan accumulates in the heart 7 d and 28 d post-MI.

Cardiac tissue from male and female mice were sectioned and stained with hyaluronan binding protein (HABP) 7 d after MI or sham and imaged with epifluorescence microscopy. (A,C) Representative images taken after staining with DAPI (blue), which stains for nuclei and HABP (red), which is hyaluronan binding protein. (B) Quantification of stained tissue shows a significant increase in hyaluronan accumulation in the total left ventricle and especially infarct zone (IZ) but not remote zone (RZ), 7 d post-MI. Sham $n=10$ (5F, 5M), MI $n=8$ (4F, 4M). unpaired t-test with Welch's correction. (D) Quantification of stained tissue shows a significant increase in hyaluronan accumulation in the total left ventricle and especially infarct zone (IZ) but not remote zone (RZ), 28 days post MI. Sham $n=8$ (4F, 4M), MI $n=8$ (6F, 2M). t-test with Welch's correction. (E) HA ELISA shows an increase in HA in MI tissue in both 7d and 28 MI hearts. $n=7-10$ Unpaired t-test (F) Representative images taken after echocardiographs were done and analyzed. (G) Quantification of measured echocardiogram showing a significant decrease in end-diastolic volume and end-systolic volume in the left ventricle 7 d post-MI. Sham $n=20$, MI $n=14$.

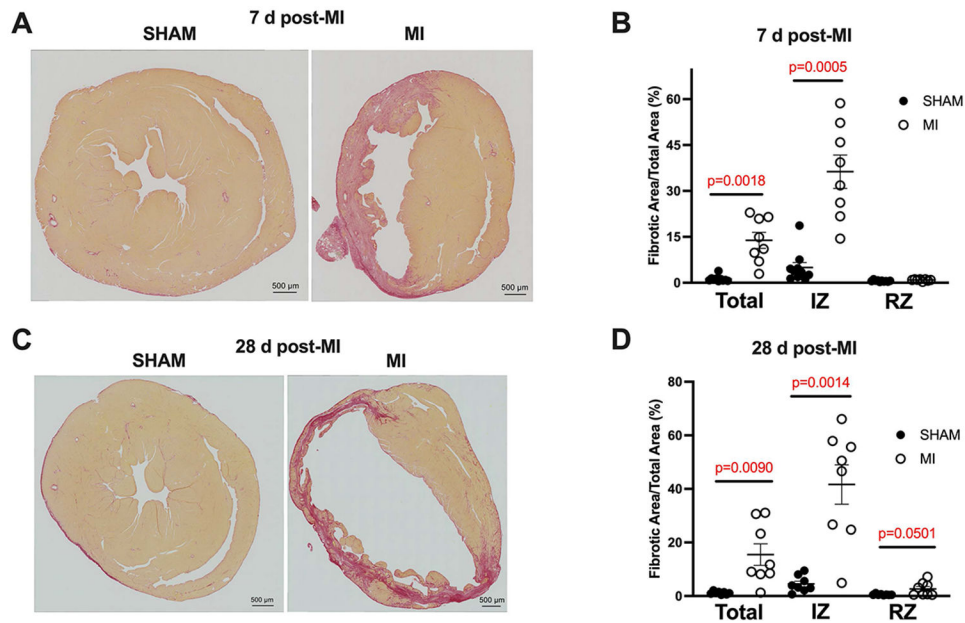


Figure 2. Collagen accumulates in the heart 7 d and 28 d post-MI.

Cardiac tissue from male and female mice were sectioned and stained with picrosirius red 7 days after MI. (A,C) Representative images are shown; reddish color indicates collagen. (B) Quantification of stained tissue shows a significant increase in collagen accumulation in the total left ventricle and especially infarct zone (IZ) but not remote zone (RZ) 7 days post MI. Sham n= 10 (5F, 5M), MI n=8 (4F, 4M). t-test with Welch's correction. (D) Quantification of stained tissue shows a significant increase in collagen accumulation in the total left ventricle, especially infarct zone (IZ) 28 days post MI. An increase in collagen was also seen in the remote zone. Sham n=8 (4F, 4M), MI n=8 (6F, 2M). t-test with Welch's correction.

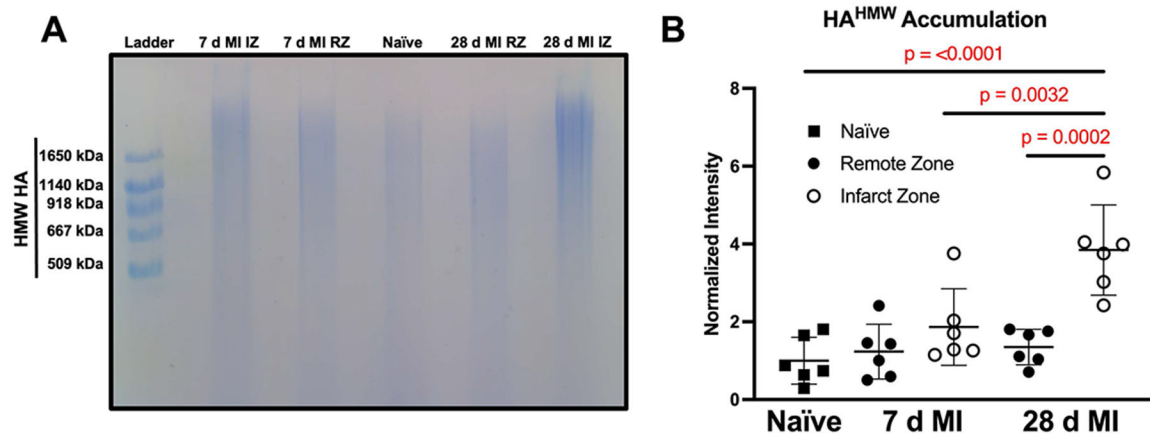


Figure 3. High molecular weight hyaluronan accumulates in the heart post-MI. Hyaluronan was isolated from male and female mouse hearts after 7 d MI or 28 d MI. Samples were electrophoresed and stained with Stains-All to indicate hyaluronan (blue). (A) Representative assessment of the size and abundance of hyaluronan isolated from mouse hearts. (B) Quantification of stained agarose gels (n=6 per group). HA^{HWM} increases chronically in the infarct zone (IZ) after myocardial infarction (MI) compared to naïve hearts, 7 d infarct zone (IZ), and 28 d remote zone (RZ). Quantification of stained agarose gels (n=6 per group). One-way ANOVA test with Tukey's multiple comparisons test.

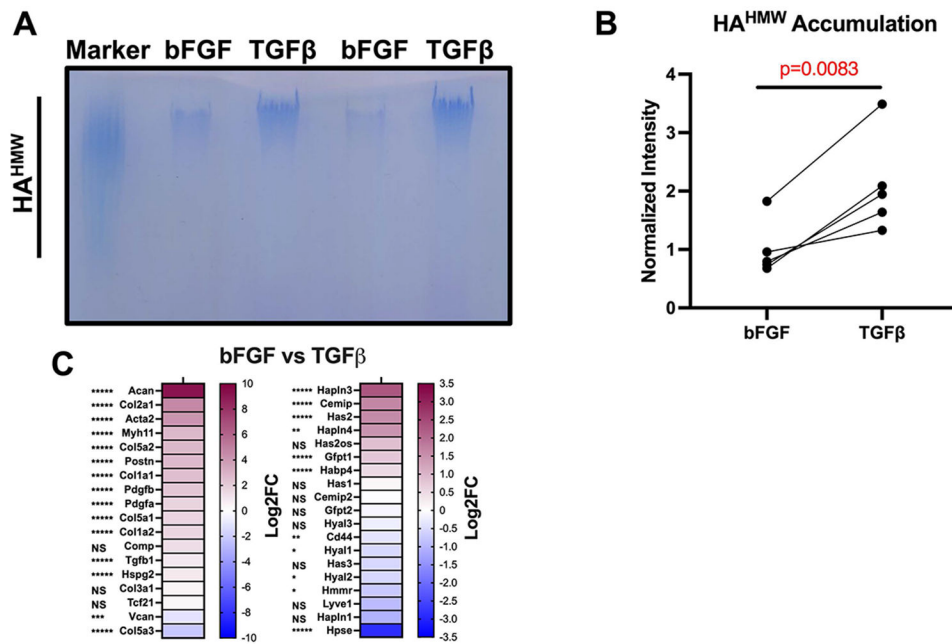


Figure 4. High molecular weight hyaluronan accumulation is increased in activated cardiac fibroblasts.

Hyaluronan was isolated from the media of cultured cardiac fibroblasts. Samples were electrophoresed and stained with Stains-All to indicate hyaluronan (blue). All fibroblasts were from WT mice. (A) Representative gel showing relative size and abundance of hyaluronan. High molecular weight hyaluronan (HA^{HWM}) was loaded as a molecular weight marker/positive control. (B) Quantification of stained agarose gels. Stimulation of fibroblasts with TGFβ for 24 h caused accumulation of HA^{HWM} compared to naïve fibroblasts n=5 (3F, 2M). Paired t-test. (C) Heat map illustrating Log2 fold changes of genes involved in hyaluronan metabolism, hyaluronan receptors, and ECM proteins from activated fibroblasts stimulated with TGFβ for 24 h compared to naïve fibroblasts. Quantification of Color: Blue color indicates downregulation of genes and maroon color indicates up-regulation of genes. Significance: p<0.05 = *, p<0.01 = **, p<0.001 = ***, p<0.0001 = ****, p<0.00001 = ***** and NS = not significant.

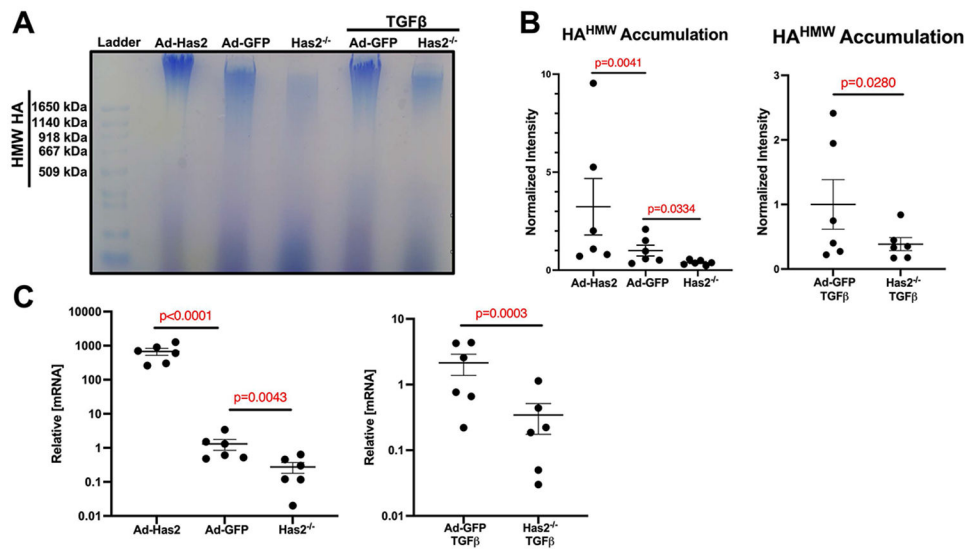


Figure 5. Has2 is necessary and sufficient for TGF-induced HA production.

Hyaluronan was isolated from the media of cultured cardiac fibroblasts. Samples were electrophoresed and stained with Stains-All to indicate hyaluronan (blue). All fibroblasts were from *Has2^{fl/fl}* mice. (A) Representative gel showing relative size and abundance of hyaluronan. Overexpressing *Has2* in fibroblasts via adenovirus (Ad-Has2) increased HA accumulation in the media compared to Ad-GFP. Deletion of *Has2* attenuated accumulation of HA in the media compared to Ad-GFP. Deletion of *Has2* attenuated TGF-induced accumulation of HA in the media compared to Ad-GFP TGFβ. (B) Quantification of stained agarose gels n=6 (3F, 3M). (C) Relative mRNA expression of *Has2* is significantly decreased in both naïve and activated fibroblasts with *Has2* deletion via adenovirus (Ad-Cre). *Has2* is significantly increased in fibroblasts with *Has2* overexpression via adenovirus (Ad-Has2). n=6 (3F, 3M). Ratio paired t-test, parametric w/ SEM.

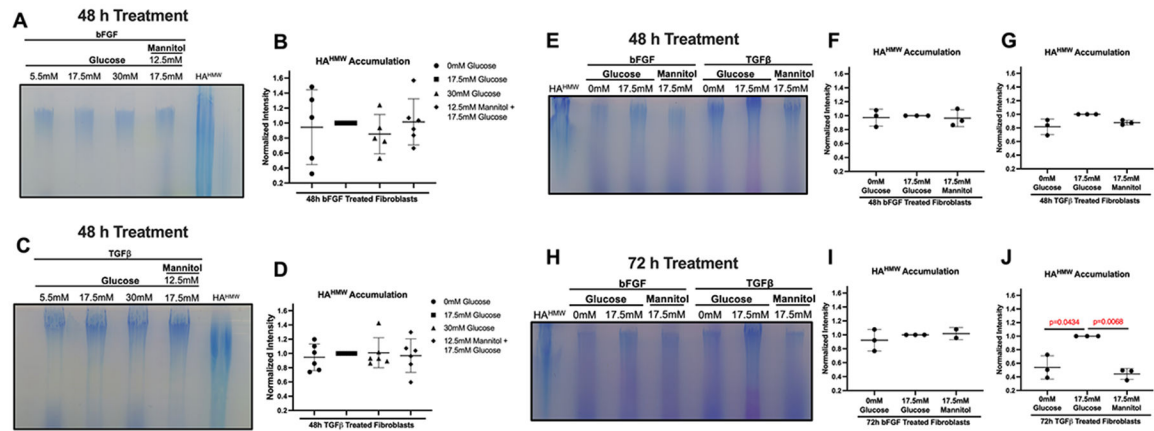


Figure 6. Depletion of glucose causes a decrease in hyaluronan accumulation in activated fibroblasts.

Hyaluronan was isolated from the media of cultured cardiac fibroblasts. Samples were electrophoresed and stained with Stains-All to indicate hyaluronan (blue). All fibroblasts were taken from WT mice. (A) Representative gel showing relative abundance of hyaluronan. Naïve fibroblasts treated with low or high glucose concentrations showed no significant differences compared to normal glucose concentrations. (B) Quantification of stained agarose gels. $n=5-6$ (2–3F, 2–3M) (C) Representative gel showing relative abundance of hyaluronan. TGF β -activated fibroblasts treated with low or high glucose concentrations showed no significant differences compared to normal glucose concentrations. (D) Quantification of stained agarose gels. $n=6$ (3F, 3M). Paired t-test between treatment and control. (E) Representative gel showing relative size and abundance of hyaluronan. Quantification of stained agarose gels (F) Naïve and (G) TGF β -activated fibroblasts treated with no glucose showed no significant differences compared to normal glucose concentrations. $n=3$ (3F). (H) Representative gel showing relative size and abundance of hyaluronan. Quantification of stained agarose gels (I) naïve fibroblasts treated with no glucose showed no significant differences compared to normal glucose concentrations. (J) TGF β -activated fibroblasts treated with no glucose showed a significant decrease in HA^{HMW} compared to normal glucose concentrations. $n=3$ (3F). Paired t-test between treatment and control.

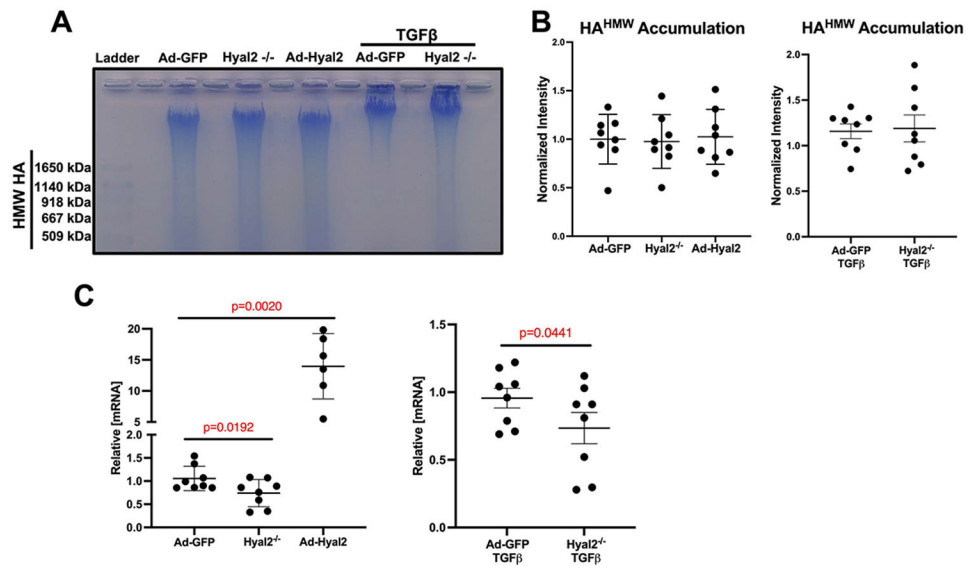


Figure 7. Hyal2 alone does not degrade HA derived from cardiac fibroblasts.

Hyaluronan was isolated from the media of cultured cardiac fibroblasts. Samples were electrophoresed and stained with Stains-All to indicate hyaluronan (blue). All fibroblasts were from *Hyal2^{fl/fl}* mice. (A) Representative gel showing relative abundance of hyaluronan. (B) Quantification of stained agarose gels. Overexpressing *Hyal2* in fibroblasts via adenovirus (Ad-Hyal2) does not change HA accumulation in the media compared to Ad-GFP. Deletion of *Hyal2* via adenovirus (Ad-Cre) attenuated TGF-induced accumulation of HA in the media. n=8 (4F, 4M). paired t-test. (C) Relative mRNA expression. *Hyal2* is significantly decreased in both naïve and activated fibroblasts with *Hyal2* deletion via adenovirus (Ad-Cre). *Hyal2* is significantly increased in fibroblasts with *Hyal2* overexpression via adenovirus (Ad-Hyal2). n=6–8 (3-5F, 3M). paired t-test

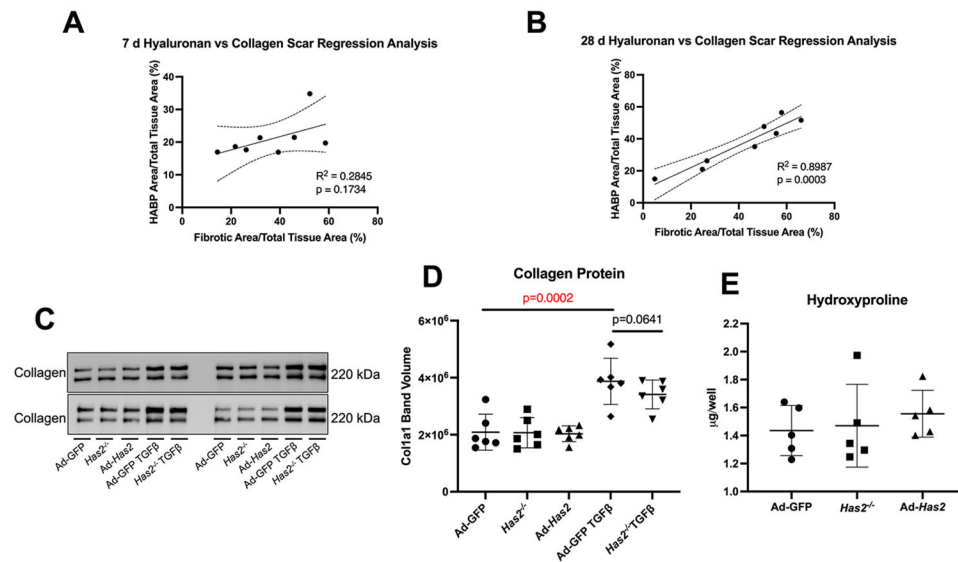


Figure 8. Little impact of HA on fibroblast collagen secretion.

(A) Linear regression analysis of the association between hyaluronan accumulation (HABP) and fibrotic area (Picro Sirius). 7 d MI hearts showed no significant relationship between an increase in HABP area and fibrotic area. (n=8, $p = 0.1734$, $R^2 = 0.2845$) (B) Linear regression analysis of the association between hyaluronan accumulation (HABP) and fibrotic area (Picro Sirius). 28 d MI hearts showed a significant relationship between an increase in HABP area and increase in fibrotic area. (n=8, $p = 0.0003$, $R^2 = 0.8987$) (C) Cardiac fibroblasts were treated and protein was isolated. All fibroblasts were from *Has2^{fl/fl}* mice. Representative Western Blot. (D) Quantification of Western blot showed that overexpression or deletion of *Has2* in naïve fibroblasts via adenovirus (Ad-Cre and Ad-Has2) showed no significant difference in collagen protein levels compared to GFP control. n=6 (3F, 3M) Paired t-test TGF-activated fibroblasts showed a significant increase in collagen protein levels compared to bFGF control. n=6 (3F, 3M) Paired t-test. (E) Quantification of hydroxyproline assay. There was no significant difference in hydroxyproline levels in *Has2* deleted and overexpressed naïve fibroblasts compared to GFP control. n=5 (3F, 2M) Paired t-test.