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ORIGINAL RESEARCH

# SMC-Derived Hyaluronan Modulates Vascular SMC Phenotype in Murine Atherosclerosis

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**RATIONALE:** Plaque instability remains poorly understood and new therapeutic approaches to reduce plaque rupture and subsequent clinical events are of great interest. Recent studies revealed an important role of phenotypic switching of smooth muscle cells (SMC) in controlling plaque stability, including ECM (extracellular matrix) deposition.

**OBJECTIVE:** The aim of this study was to elucidate the role of hyaluronan derived from SMC–hyaluronan synthase 3 (*Has3*), in phenotypic switching and plaque stability in an animal model of atherosclerosis.

**METHODS AND RESULTS:** A mouse line with SMC-specific deletion of *Has3* and simultaneous SMC-lineage tracing (*e*YFP [enhanced yellow fluorescent protein]) on an *Apoe*$^{-/-}$ background was used. Lineage tracing of SMC with *e*YFP revealed that SMC-specific deletion of *Has3* significantly increased the number of LGALS3$^{+}$ (galectin-3) transition state SMC and decreased ACTA2$^{+}$ (alpha-smooth muscle actin) SMC. Notably, SMC-*Has3* deletion led to significantly increased collagen deposition and maturation within the fibrous cap and the whole lesion, as evidenced by picrosirius red staining and LC-PolScope analysis. Single-cell RNA sequencing of brachiocephalic artery lesions demonstrated that the loss of SMC-*Has3* enhanced the transition of SMC to a *Lgals3*$^{+}$, ECM-producing phenotype with elevated acute-phase response gene expression. Experiments using cultured murine aortic SMC revealed that blocking CD44 (cluster of differentiation-44), an important hyaluronan binding receptor, recapitulated the enhanced acute-phase response, and synthesis of fibrous ECM.

**CONCLUSIONS:** These studies provide evidence that the deletion of SMC-*Has3* results in an ECM-producing transition state SMC phenotype (characterized by LGALS3$^{+}$ expression), likely via reduced CD44 signaling, resulting in increased collagen formation and maturation, an index consistent with increased plaque stability.

**GRAPHIC ABSTRACT:** An online graphic abstract is available for this article.

**Key Words:** atherosclerosis ■ extracellular matrix ■ galectin-3 ■ mice ■ phenotype

**In This Issue, see p 971 | Meet the First Author, see p 972**

Atherosclerosis and subsequent thromboembolic complications of ruptured or eroded lesions result in myocardial infarction and stroke. A collagen-rich fibrous cap (FC) is thought to play a crucial role in stabilizing atherosclerotic lesions, whereas unstable lesions, characterized by a thinner FC, are more prone to rupture.[1–3] Newman et al[4] recently showed that multiple cell types give rise to ACTA2$^{+}$ (alpha-smooth muscle actin) FC cells. However, smooth muscle cells (SMC) are the most abundant source and appear to be required for ECM (extracellular matrix) deposition and long-term durable plaque stability. To form the FC, SMC undergo phenotypic modulation in response to atherogenic stimuli and migrate from the media into atherosclerotic lesions.[5–7] Recent lineage tracing studies in mice revealed that SMC can be beneficial or detrimental in atherosclerotic lesions, depending on the specific SMC phenotypic transitions.[7–13] For example, KLF4 (Kruppel-like factor 4)-dependent transitions appear to be detrimental,

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## Novelty and Significance

### What Is Known?

- Phenotypic modulation of smooth muscle cells (SMC) can be beneficial or detrimental to atherosclerotic lesion stability.
- Recent studies revealed an important role of LGALS3 (galectin-3)-expressing modulated SMC in controlling plaque stability, including inducing ECM (extracellular matrix) deposition.
- Hyaluronan, an important ECM component, is increased during atherosclerotic lesion progression and can directly influence SMC function by promoting SMC activation and migration.

### What New Information Does This Article Contribute?

- SMC-hyaluronan synthase *(Has) 3* deficiency leads to more LGALS3$^+$ modulated SMC within the lesion, as well as increases collagen deposition and maturation, which are indices consistent with increased plaque stability.
- In-depth single-cell RNA sequencing demonstrates that SMC-*Has3* deletion–driven expansion of phenotypically modulated SMC drives increases in acute-phase response gene expression.
- Blocking the important hyaluronan binding receptor, CD44 (cluster of differentiation-44), recapitulates the enhanced acute-phase response and synthesis of fibrous ECM.

The present studies provide evidence that SMC-*Has3* knockout plays a crucial role for lesion stability indices by enhancing an ECM-producing transition state SMC phenotype which is characterized by LGALS3$^+$ expression, likely via reduced hyaluronan/CD44 signaling.

## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **ACTA2** | alpha-smooth muscle actin |
| **BCA** | brachiocephalic artery |
| **CD** | cluster of differentiation |
| **ECM** | extracellular matrix |
| **eYFP** | enhanced yellow fluorescent protein |
| **FC** | fibrous cap |
| **GO** | gene ontology |
| **HAS** | hyaluronan synthase |
| **IL** | interleukin |
| **LGALS3** | galectin-3 |
| **NF-κB** | nuclear factor κB |
| **scRNA-seq** | single-cell RNA sequencing |
| **siHas3** | siRNA targeting Has3 |
| **SMC** | smooth muscle cells |
| **SMC-Con** | contractile SMC |
| **SMC-Mod** | modified SMC |
| **WT** | wild-type |

as SMC-*Klf4* knockout resulted in a 50% decrease in lesion size but a near doubling of the thickness of the FC per lesion area.[9] In contrast, SMC-*Oct4* knockout resulted in larger lesions that had multiple features of decreased plaque stability including reduced numbers of ACTA2$^+$ SMC within the FC.[10] Up to 80% of phenotypically modulated SMC within the lesion lose their typical marker genes like ACTA2 and often express LGALS3 (galectin-3), initially thought to mark the transition of SMC to a terminal macrophage-like state, especially within the lesion core.[9,11,14,15] However, subsequent reports and combined lineage tracing and single-cell RNA sequencing (scRNA-seq) studies[7,8,12,13] showed that the majority of LGALS3$^+$ SMC represent unique transition state phenotypes. Indeed, using microdissected lesions isolated from a novel *Myh11*-DreER$^{T2}$-*Lgals3*-Cre dual recombinase mouse model system, Alencar et al[7] showed that *Lgals3* mRNA expression marks this SMC transition state that gives rise to at least 4 to 5 distinct transcriptomic clusters. Similarly, Wirka et al[8] recently defined a single putative beneficial ECM-producing SMC phenotype, so-called a fibromyocyte, that expresses *Lgals3*. Additionally, Pan et al[12] revealed multiple *Lgals3* expressing cell types derived from SMC in human and mouse lesions, including an intermediate cell state positive for stem cells, endothelial cells, and macrophage markers. They further showed that transitions back to a contractile SMC state might occur. Our aim, here, was to better understand the ECM-SMC cross-talk that regulates SMC populations and their functions within the lesion to promote beneficial (plaque stabilizing) changes in SMC phenotype or inhibit detrimental changes.

An interesting candidate for ECM-mediated phenotypic modulation is hyaluronan. It has indeed been shown that hyaluronan is important in atherosclerosis and in controlling lesion characteristics. Hyaluronan is a linear unbranched glycosaminoglycan that is synthesized by SMC and other cells during atherosclerotic lesion development.[16] It is directly extruded into the extracellular space by 3 transmembrane HAS (hyaluronan synthases) 1 to 3, where it contributes to the matrix microenvironment of cells and acts as a direct signaling agent via hyaluronan-binding receptors. One of its most important receptors is CD44 (cluster of differentiation-44), which is thought to contribute to macrophage-induced inflammation during development of atherosclerosis.[17,18]

Although hyaluronan is found and synthesized mainly in the adventitia in the healthy arterial vessel wall by fibroblasts and SMC, it has become clear that many different cells such as T cells, macrophages, and endothelial cells contribute to the vascular hyaluronan matrix. Adding to the complex and highly variable hyaluronan-microenvironment, hyaluronan receptors including CD44 and hyaluronan-binding molecules are expressed by a variety of cell types[19] often found within the atherosclerotic lesion milieu. Both in the injured or atherosclerotic vessel and in vitro, it has been shown that hyaluronan directly influences the behavior of SMC by promoting their migration and proliferation.[20] This activation of hyaluronan synthesis-induced expansion of SMC is suggested to contribute to re-stenosis and development of atherosclerotic lesions.[20,21] In contrast, endothelial hyaluronan, a part of the glycocalyx, contributes to the protective function of the endothelium with loss of endothelial hyaluronan leading to endothelial dysfunction.[22] Given the physiological relevance of hyaluronan and HAS in atherosclerotic development, it is important to define the role of the different HAS-isoenzymes and the cellular origin in lesion development. *Has3* drives neointimal hyperplasia[23] and has been shown to be the most strongly induced HAS-isoenzyme during early atherosclerotic lesion development in mice.[24] Using global *Has3*$^{-/-}$ mice on an *Apoe*$^{-/-}$ background, we previously showed that global loss of *Has3* decreased Th1-cell polarization and subsequently, macrophage-driven plaque inflammation, resulting in an overall beneficial plaque phenotype.[24]

In this study, we used a combinatorial SMC-lineage tracing and SMC-specific *Has3* knockout approach to investigate the role of SMC-derived *Has3* expression and hyaluronan on SMC phenotypic modulation during development and progression of atherosclerosis in mice.

# METHODS

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Mice, Lineage Tracing, and Study Design

*Myh11*-CreER$^{T2}$ (myosin heavy chain 11-Cre estrogen receptor) ROSA26-STOP floxed *e*YFP (enhanced yellow fluorescent protein) *Apoe*$^{-/-}$ *Has3*$^{fl/fl}$ mice (henceforth: SMC-*Has3* wild-type (WT)/SMC-*Has3* knockout, Figure IA in the Data Supplement) were generated by crossing *Myh11*-CreER$^{T2}$ ROSA26-STOP floxed *e*YFP *Apoe*$^{-/-}$ mice[7,9,10,25] with *Has3*$^{fl/fl}$ mice[23,24] to create a SMC-specific *Has3* deficiency and simultaneous lineage tracing in SMC by *e*YFP expression. To induce the CreER$^{T2}$ recombinase under the control of the *Myh11* promoter, expressed exclusively by SMC and a subset of microvascular pericytes,[25,26] 5- to 7-week old mice were given a series of 10 intraperitoneal tamoxifen injections (1 mg/day per mouse) over 2 weeks as previously described.[7,9,10,25] At the time of labeling within the healthy vessel, the activation of the YFP reporter gene and the *Has3* excision corresponds exclusively to the vascular SMC population in the media; these events are permanent and independent of *Myh11* expression levels at later time points. Three days after the treatment, the mice were fed a western-type diet for 15 weeks, containing 21% butterfat and 0.15% cholesterol (Ssniff, S8200-E010, Figure IB in the Data Supplement). This system allows the identification of all progeny of medial SMC by *e*YFP expression even after accumulating within the lesion and losing their specific marker genes under pathological conditions. Hypercholesterolemia was defined as plasma cholesterol levels >500 g/dL. All animal protocols were approved by the University of Virginia Animal Care and Use Committee and the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Bezirksregierung Düsseldorf.

## Single-Cell RNA Sequencing

Single-cell capture and library preparation were performed at the Genome Analysis and Technology Core, RRID:SCR_018883 of the University of Virginia, as previously described.[7] Cells were isolated and pooled from advanced lesions by microdissection of the BCA plaque from 5 SMC-*Has3* WT and 6 SMC-*Has3* knockout mice, after 15 weeks of western diet feeding. Samples were then processed using the 10x Genomics Chromium platform. Sequencing was performed on an Illumina NextSeqTM, 150 cycle high output. Quality control was conducted by Qubit and Agilent DNA high sensitivity tape stations after 10x library prep and Next Generation Sequencing (NGS) library prep. Data analysis was performed in R version 3.6.1, using the Seurat package version 3.1.5.[27] Integration and normalization were performed using the combined SCTransform and integration workflow of Seurat as described before.[28,29] GO term and Reactome pathway overrepresentation analysis were performed using a PANTHER powered web service, http://geneontology.org/.[30] Interaction networks were generated and exported using the STRING v11 web service, https://string-db.org/.[31] The figures, including enrichment plots, were generated with ggplot2.[32,33] Full details can be found in the Data Supplement. Please see the Major Resources Table in the Data Supplement.

# RESULTS

## SMC-Has3 Regulates SMC Phenotype and Modulates Plaque Composition During Atherosclerosis

To determine the effect of SMC-specific *Has3* knockout on atherosclerotic lesion development, we generated a mouse model combining tamoxifen-inducible SMC-specific *Has3* deletion and simultaneous SMC-lineage tracing by *e*YFP expression under the control of the SMC-specific *Myh11* promoter (see Methods, Figure IA in the Data Supplement). SMC-*Has3* WT and SMC-*Has3* knockout mice were fed a western-type diet for 15 weeks to induce advanced atherosclerotic lesions (Figure IB in the Data Supplement). All mice were genotyped, and a sufficient knockdown of

*Has3* as well as induction of *e*YFP expression in SMC was validated (Figure II in the Data Supplement). Body weight, plasma cholesterol, and plasma triglycerides were not affected by the SMC-*Has3* knockout (Figure III in the Data Supplement). To test if SMC-*Has3* knockout reduced overall hyaluronan abundance within lesions, we performed hyaluronan affinity-histochemistry and observed a significant reduction of hyaluronan in SMC-*Has3* knockout mice (Figure IV in the Data Supplement). To elucidate the effects of SMC-*Has3* knockout, the lesion cell composition was analyzed by high-resolution z-stack confocal microscopy using *e*YFP as a lineage tracing marker for SMC origin, as well as ACTA2 and LGALS3 (Figure 1). Whereas the latter 2 markers do not rigorously define distinct SMC-derived lesion phenotypes,[7] they represent critical SMC-derived subsets and enable us to compare our results to those described in previous publications in the field.[7–9,12] SMC-*Has3* knockout mice did not show statistically significant differences in the percentage of *e*YFP$^+$ SMC compared with SMC-*Has3* WT within the lesion core or FC (defined as the 30 µm thick, subluminal portion of the lesion[4]). However, notably, lesions of SMC-*Has3* knockout mice contained significantly fewer ACTA2$^+$ SMC and showed marked increases in LGALS3$^+$ SMC within the lesion core (Figure 1C) and the FC (Figure 1D). These data indicate that SMC-*Has3* knockout has a major influence on SMC phenotypic transitions within advanced atherosclerotic lesions.

## SMC-Has3 Deletion Increases BCA Lesion Collagen Content and Maturation

We next investigated if the observed changes in proportion of SMC phenotypes resulted in changes in multiple indices of plaque stability. We investigated lesion morphology at 3 well-defined locations along the BCA. SMC-*Has3* knockout mice showed no statistically significant differences in lesion area, necrotic core area, or overall aortic plaque burden as compared to littermate WT control mice (Figure VA and VB in the Data Supplement). We then tested intraplaque hemorrhage by lymphocyte antigen 76 staining and similarly found no significant difference between SMC-*Has3* knockout and SMC-*Has3* WT mice (Figure VI in the Data Supplement). Because we observed a decreased percentage of ACTA2$^+$ SMC in the lesion core and FC, we next assessed collagen deposition by picrosirius red staining. Unexpectedly, we observed markedly increased collagen deposition in SMC-*Has3* knockout mice compared with SMC-*Has3* WT mice at 3 locations along the BCA (Figure 2A). Moreover, in-depth characterization of collagen maturation by LC-PolScope analysis,[34–36] which is defined as indicative of increased organization and cross-linking to form aligned collagen fibers, revealed that SMC-*Has3* knockout did not only influence collagen deposition and fibrillar collagen content but also increased the average birefringence retardance (Figure 2B). Collagen organization into thick, aligned fibers in a tissue results in birefringence that retards plane polarized light. Thus, an increase in retardance corresponds to increased organization and cross-linking to form aligned collagen fibers and is indicative of both increased collagen content and maturation in the SMC-*Has3* knockout. Further characterization of fiber thickness showed that SMC-*Has3* knockout lesions also had increased thick collagen fibers, with no change in thin fiber deposition (Figure VII in the Data Supplement) within the lesion core and FC. This more matured fibrillar collagen matrix is thought to contribute to greater plaque stability.[37] To further understand ECM maturation and organization status, we assessed deposition of the collagen assembly proteoglycan decorin and found it was also increased in SMC-*Has3* knockout atherosclerotic lesions (Figure VIII in the Data Supplement). Decorin is an SLRP (small leucine-rich proteoglycan) that connects collagen type I fibrils and plays a key role in collagen fibrillogenesis.[38,39] Taken together, the increased collagen deposition, fibrillar collagen thickness, maturation, and organization, suggest that SMC-*Has3* knockout is associated with changes in the extracellular matrix milieu consistent with increased plaque stability despite reductions in the percentage of ACTA2$^+$ SMC within the FC.[37]

## scRNA-seq Characterization of BCA Lesions Reveals 5 Distinct SMC Clusters

To gain further insight into the role of SMC-*Has3* in atherosclerotic lesion development, we performed scRNA-seq on plaques from 5 SMC-*Has3* WT and 6 SMC-*Has3* knockout mice after 15 weeks of western diet. Briefly, advanced lesions were microdissected from the BCA, sorted for *e*YFP$^+$ cells, and processed using a 10x Genomics Chromium platform. Transcriptomes from 1600 cells were used after quality control filtering. SMC-*Has3* WT and knockout data sets were integrated and the cell identities were profiled together by clustering using the *Seurat* package.[27] We found 7 distinct populations of lesion cells, including endothelial cells (*Pecam1*$^+$, *Cdh5*$^+$), macrophages (*Ptprc*$^+$, *Cd68*$^+$, *Adgre1*$^+$, *Fcgr1*$^+$, *Itgam*$^+$, *Mrc1*$^+$), contractile SMC (SMC-Con; *eyfp*$^+$, *Myh11*$^+$, *Acta2*$^+$, *Tagln*$^+$) and 4 modulated SMC clusters characterized by *Lgals3* expression (SMC modified [SMC-Mod] 1-4; *eyfp*$^+$, *Lgals3*$^+$, *Tnfrsf11b*$^+$, *Lum*$^+$, *Spp1*$^+$, *Myh11*$^-$, *Acta2*$^-$, *Tagln*$^-$). These observed clusters are consistent with a recently published larger dataset by our group.[7] SMC-Con and SMC-Mod1-4 clusters were confirmed to be from SMC lineage by mRNA *eyfp* expression, which was absent in the endothelial cell and macrophage clusters (Figure 3A-through 3C, Figure IX in the Data Supplement). The presence of *Lgals3* expression and absence of canonical macrophage markers (*Adgre1, Cd68, Fcgr1,* and *Itgam)* in modulated SMC clusters is in agreement with recent scRNA-seq reports[7,8,12,13] showing

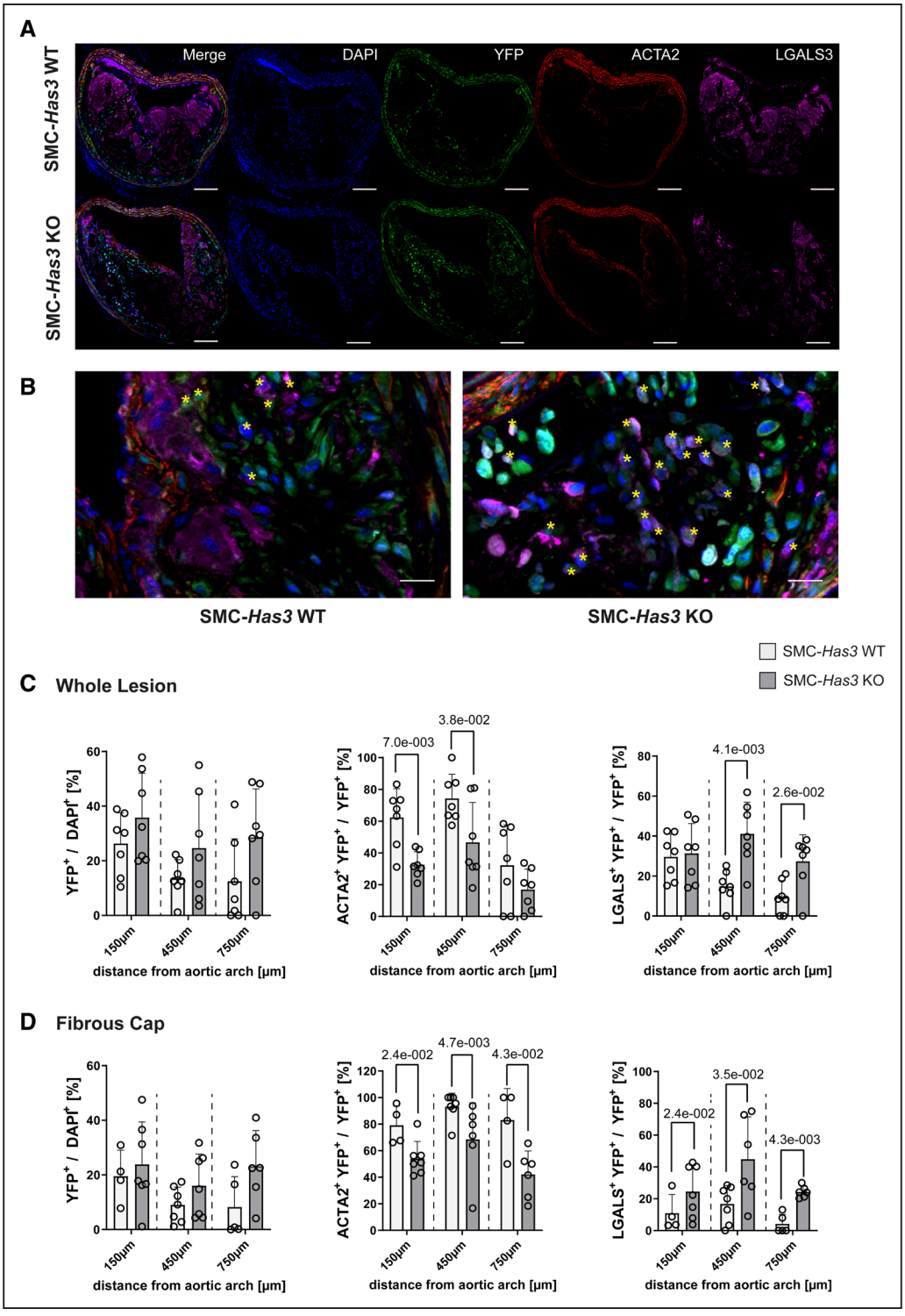


**Figure 1. Genetic deletion of smooth muscle cell (SMC)–hyaluronan synthase 3 (Has3) is associated with reduced SMC-derived ACTA2+ (alpha-smooth muscle actin) and increased LGALS3+ (galectin-3) lesion cells.**

**A**, Immunostaining of representative brachiocephalic artery (BCA) sections (scale bars 100 µm) of SMC-*Has3* wild-type (WT) and SMC-*Has3* knockout (KO) mice fed a western diet for 15 wk shows a marked increase in SMC-derived LGALS3$^+$ cells and a decrease in the number of ACTA2$^+$ SMC within lesions of SMC-specific *Has3* KO mice, as compared to control mice. **B**, The yellow stars in higher magnification panel (scale bars 20 µm) indicates LGALS3$^+$ *e*YFP (enhanced yellow fluorescent protein)$^+$ cells in SMC-*Has3* KO mice. Quantification of *e*YFP$^+$ cells as part of total cell number (detected by DAPI signal), as well as percentages of LGALS3$^+$ *e*YFP$^+$ and ACTA2$^+$ *e*YFP$^+$ cells per *e*YFP$^+$ cells within the whole lesion (n=7/7) and (**C**) the fibrous cap (YFP$^+$/DAPI$^+$: n=4/7 (150 µm), n=7/7 (450 µm), n=5/6 (750 µm); ACTA2$^+$YFP$^+$/YFP$^+$: n=4/7 (150 µm), n=7/6 (450 µm), n=4/6 (750 µm); LGALS3$^+$YFP$^+$/YFP$^+$: n=4/7 (150 µm), n=7/6 (450 µm), n=5/6 (750 µm)). **D**, Statistical analysis was performed separately at three different locations along the BCA with Mann-Whitney tests. Error bars represent mean±SD.

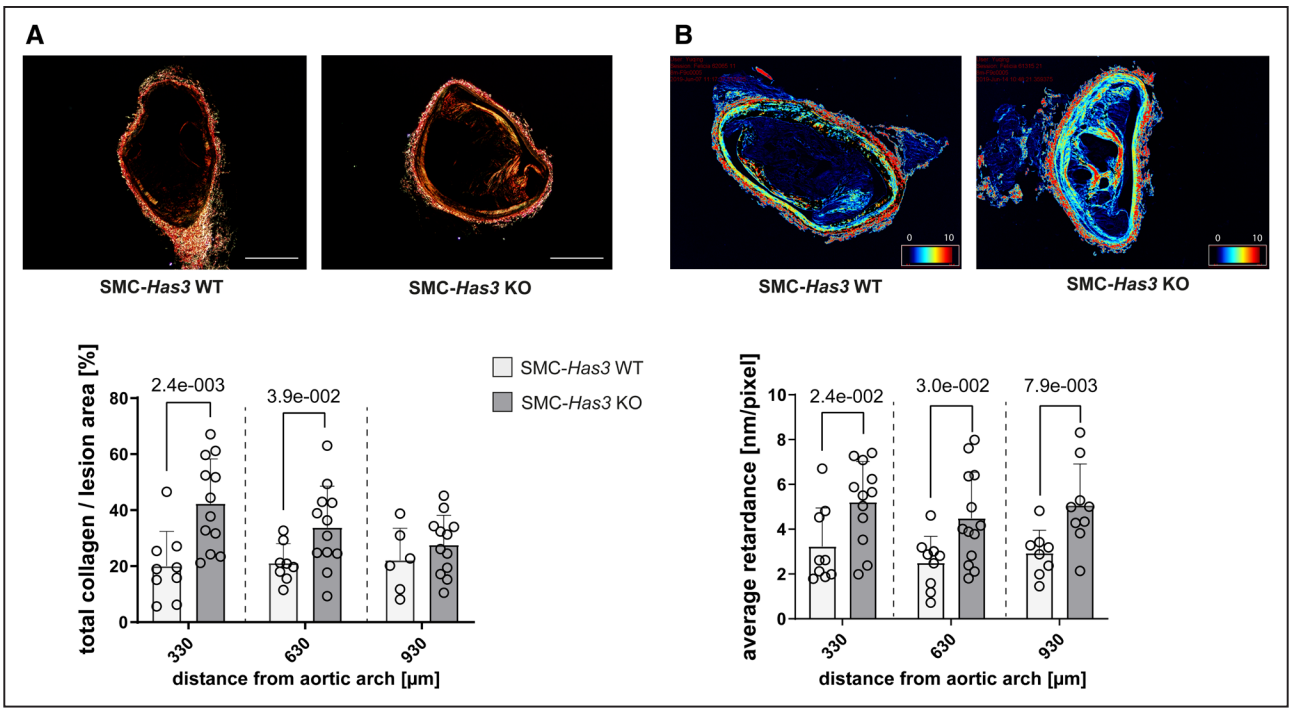


**Figure 2. Smooth muscle cell (SMC)–specific knockout (KO) of Has3 (hyaluronan synthase 3) results in increased collagen deposition and maturation in atherosclerotic lesions.**

**A**, Representative images of picrosirius red-stained brachiocephalic arteries (BCAs) under polarized light and analysis of total collagen deposition in atherosclerotic plaque lesions of SMC-*Has3* wild-type (WT) and SMC-*Has3* KO mice at three different locations along the BCA (n=9/12 [330 µm], n=8/12 [630 µm], n=6/12 [930 µm]). **B**, Measurement of collagen orientation and alignment using LC-PolScope analysis, where well-aligned collagen fibers are birefringent and retard plane polarized light. The heat map is proportional to retardance (nm/pixel) from low (black=0 nm) to high (red=10 nm) retardance (n=9/12 (330 µm), n=9/13 (630 µm), n=8/9 (930 µm)). Statistical analysis was performed separately at three different locations along the BCA with Mann-Whitney tests. Error bars represent mean±SD. Scale bars=500 µm.

that *Lgals3* expression marks an SMC transition state rather than a terminal macrophage-like state. The majority of SMC in this analysis had acquired a transition state, including cells in the SMC-Con cluster, which express both contractile marker genes (eg, *Acta2* and *Myh11*) as well as *Lgals3,* albeit at a lower level than SMC-Mod clusters. This gene expression profile may suggest that these SMC are poised to undergo phenotypic switching (Figure 3B) or have transitioned back to a semicontractile state, as they were distinct from the modulated state. Gene expression signatures of fibrillar collagens and SLRPs were elevated in the SMC-Mod clusters compared to SMC-Con (Figure 3B, Files I and VII in the Data Supplement), indicating that SMC-Mod express a higher amount of collagen and collagen fiber assembly machinery. Differential gene expression analysis also supported the observation that SMC-Mod clusters exhibited an ECM-synthesizing phenotype, as the ECM genes *Acan, Col2a1, Hapln1, Col27a1, Col11a2, Col6a1, Sparc, Fmod*, and *Dcn*, among others, were significantly upregulated compared with SMC-Con (Figure X, File III in the Data Supplement).

By identifying cluster marker genes (File IV in the Data Supplement) and analyzing their gene ontology (GO) enrichment (Figure XI in the Data Supplement) the 4 subpopulations of modulated SMC clusters can be generally described as collagen-producing (SMC-Mod1), osteochondrogenic (SMC-Mod2), inflammatory (SMC-Mod3), and growth factor responsive (SMC-Mod4). Specifically, SMC-Mod1 exhibited high expression of *Col1a1, Col3a1, Col5a2, Col6a1*, and *Col6a3* and had an overrepresentation of genes belonging to ECM-related GO terms like "extracellular matrix organization" and "endochondral bone growth" (Figures IX and XI in the Data Supplement). SMC-Mod2 expressed chondrogenic marker genes, *Hapln1, Acan*, and *Col2a1*, and exhibited enrichment of genes associated with GO terms like "cartilage development," "chondrocyte differentiation," and "bone mineralization." SMC-Mod2 may also exhibit an osteochondrogenic phenotype, which was supported by high expression of integrin-binding sialoprotein (*Ibsp*), a major component of bone matrix. SMC-Mod3 likely represents a modulated SMC population devoted to inflammatory cell interactions as it expressed *Lcn2, C3,* and *Lbp* at high levels, all of which are involved in innate immunity and the acute-phase response. SMC-Mod3 marker genes also had an overrepresentation of genes belonging to the GO terms like "inflammatory response" and "defense response." Lastly, SMC-Mod4 exhibited a high expression of transcription and growth factors like *Jun, Egr1,* and *Gdf10* and was enriched for genes belonging to

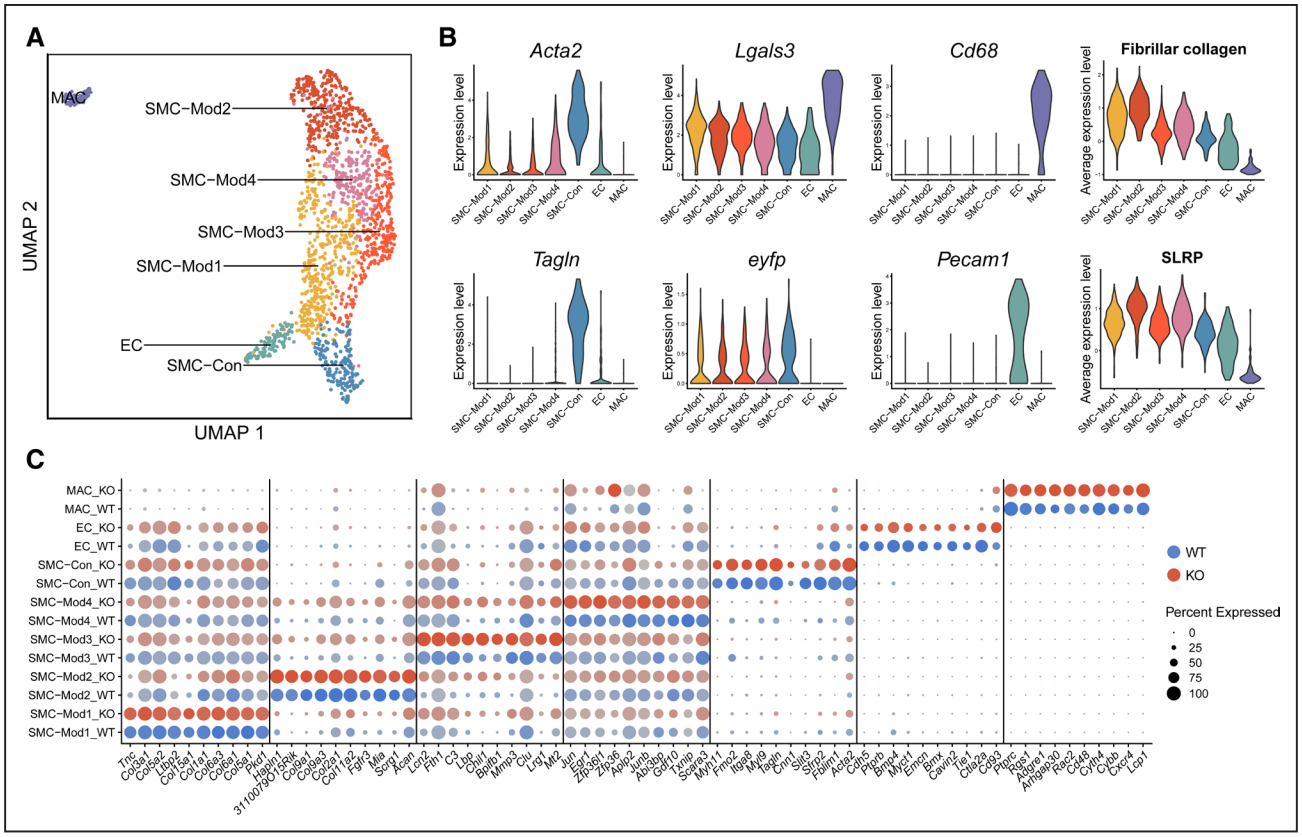


**Figure 3. Single-cell transcriptome profiling of advanced brachiocephalic artery (BCA) lesions from smooth muscle cell (SMC)–Has3 (hyaluronan synthase 3) deficient mice and their respective littermates.**

**A**, Uniform Manifold Approximation and Projection (UMAP) visualization of aggregate SMC-*Has3* wild-type (WT) and SMC-*Has3* knockout (KO) SMC-enriched cell populations of advanced BCA lesions after 15 wk of western diet. **B**, Expression of selected SMC, endothelial, and macrophage markers, including gene signature scores for fibrillar collagens and SLRPs (small leucine-rich proteoglycans) across cell identities for SMC-*Has3* WT and SMC-*Has3* KO mice, visualized using violin plots. Associated statistics can be found in File VII in the Data Supplement. **C**, Dot plot showing the expression of the top 10 conserved marker genes for each cell population broken down by genotype. EC indicates endothelial cell; MAC, macrophage; and SMC-Con, contractile SMC.

GO terms related to differentiation and response to growth factors/cytokines.

## scRNA-seq Demonstrates SMC-Has3 Deletion Promotes Modulated SMC Phenotypes and Elevates the Acute-Phase Response

Overall, scRNA-seq profiling of advanced BCA lesions from SMC-*Has3* knockout mice showed a greater abundance of modulated SMC and fewer contractile SMC as compared to SMC-*Has3* WT lesions (Figure 4A and 4B). This shift towards the modulated SMC phenotype revealed by scRNA-seq also supports the immunohistochemical analysis that demonstrated a greater abundance of LGALS3$^+$ SMC within the lesion and FC and reduced ACTA2$^+$ SMC (Figure 1). To examine the modulated SMC state as a whole, we grouped the modulated SMC clusters together and performed differential gene expression analysis between SMC-*Has3* knockout and SMC-*Has3* WT mice (Figure 4C, File V in the Data Supplement). Significant and highly upregulated genes in modulated SMC from SMC-*Has3* knockout mice included *Lcn2*, *Saa1*, *Saa2, Saa3*, *C3*, and *Hp* among others. Upregulated genes were examined for overrepresentation of GO terms and showed an enrichment of genes belonging to "acute-phase response," "inflammatory response," "response to bacterium," as well as GO terms involving metal ion homeostasis (Figure 4D). Differential gene expression analysis between SMC-*Has3* knockout and SMC-*Has3* WT cells was also performed within each individual cluster and the significantly upregulated genes were examined with interaction networks to uncover common pathways and functions (Figure XII, File VI in the Data Supplement). SMC-*Has3* knockout cells in the SMC-Mod1 and SMC-Mod2 clusters had elevated expression of defense response genes including (*Chil1*, *Hp*, *Lcn2*, *Lbp*, *C3*, *C1s1*, *Hmgb2*, *Dbi*, *Prdx1*, *Ifitm2,* and *Cebpb).* The inflammatory subpopulation (SMC-Mod3) had the largest amount of significantly regulated genes. Upregulated genes in the SMC-Mod3 group of SMC-*Has3* knockout mice were involved in the acute-phase response (*Lcn2*, *Saa1*, *Saa2*, *Saa3,* and *Lbp)* and collagen biosynthesis and cross-linking (*Col2a1*, *Col9a3*, *Col11a2,* and *Plod2*).

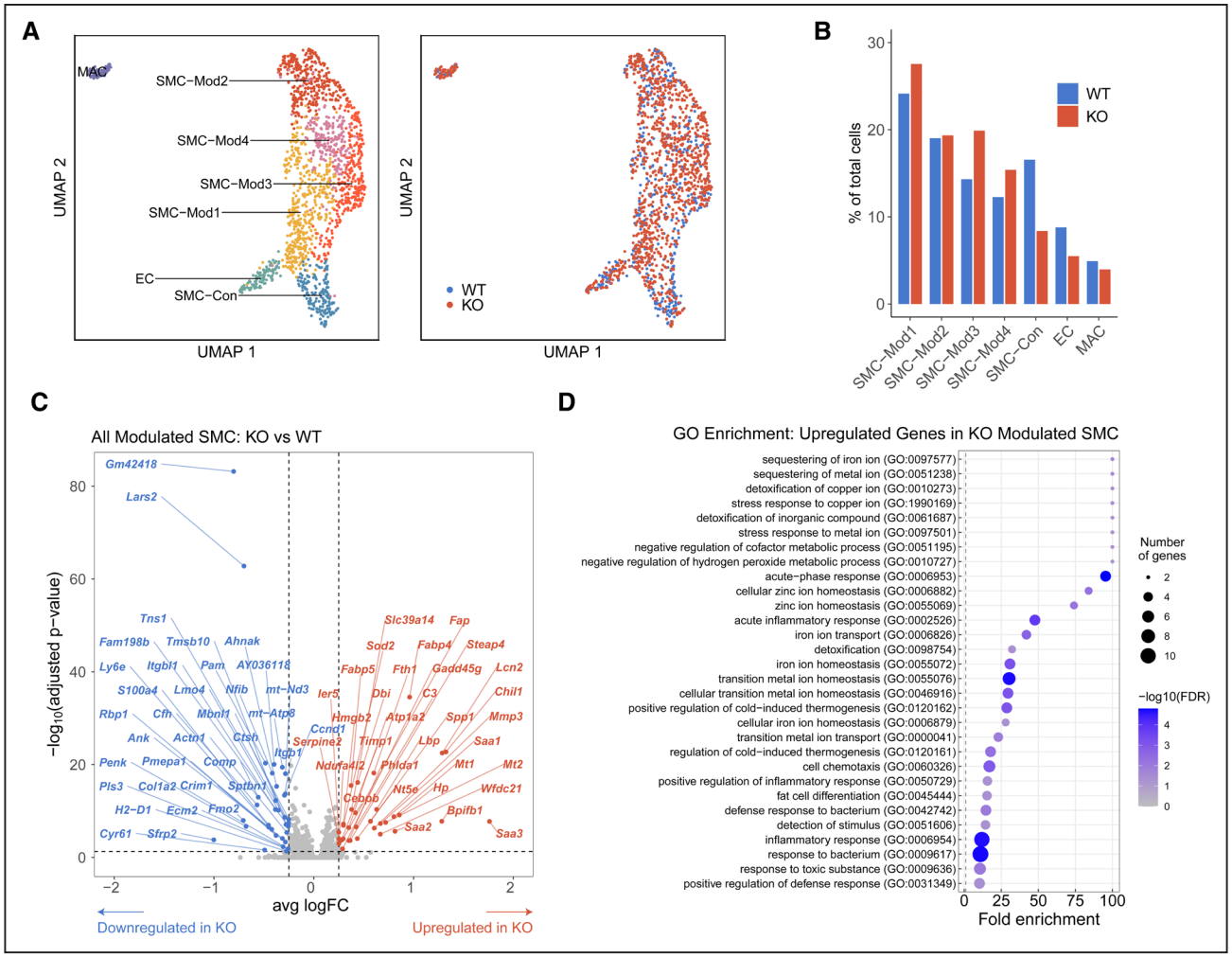


**Figure 4. Single-cell RNA sequencing analysis of advanced brachiocephalic artery (BCA) lesions shows that smooth muscle cell (SMC)–Has3 (hyaluronan synthase 3) knockout (KO) is associated with enhanced SMC phenotypic modulation and acute-phase response gene expression.**

**A**, Uniform Manifold Approximation and Projection (UMAP) visualization of SMC-*Has3* wild-type (WT) and SMC-*Has3* KO (KO) cell distribution. **B**, Proportion of each cell population as a percentage of the total cells in SMC-*Has3* WT and SMC-*Has3* KO data sets. **C**, Differential gene expression analysis of all modulated SMC clusters, SMC-Mod1, SMC-Mod2, SMC-Mod3, and SMC-Mod4, from SMC-*Has3* KO mice compared with SMC-*Has3* WT mice, visualized by volcano plot. Significantly regulated genes are labeled, upregulated in SMC-*Has3* KO (red), downregulated in SMC-*Has3* KO (blue). **D**, Gene Ontology (GO) enrichment analysis (biological process), of upregulated genes in modulated SMC from SMC-*Has3* KO mice. EC indicates endothelial cell; FDR, false discovery rate; logFC, log fold change; MAC, macrophage; and Mod1-4, modulated SMC cluster 1-4.

## Acta2 and ECM Gene Expression Can Be Modulated by In Vitro Has3 Knockdown or Blocking CD44 in Cultured Aortic SMC

Next, in vitro experiments were performed to identify mechanistic pathways that mediate the altered SMC response in the absence of *Has3*. It has been postulated that HAS3 synthesizes a lower molecular weight hyaluronan compared with HAS1 and HAS2[40] and that low molecular weight hyaluronan may elicit inflammatory responses, although these claims have been challenged.[41] Therefore, we sought to better characterize the present hyaluronan species and the specific contribution of HAS3 in murine SMC. Lineage traced murine *e*YFP$^+$ aortic SMC cultures were established and incubated for 48 hours with 10 ng/mL rhPDGF-BB (platelet derived growth factor-BB) and 10 ng/mL mTGF-β1 (transforming growth factor beta 1) to induce an activated, matrix producing state.[4] After incubation, cells were treated with either small interfering RNA (siRNA) targeting *Has3* (si*Has3*) or nontargeting siRNA or CD44 blocking antibody/isotype control or a combined treatment of both. We did not observe statistically significant morphological differences due to the siRNA or antibody treatment (Figure 5A). Administration of *Has3* siRNA resulted in a significant reduction of hyaluronan in the cell culture supernatant and reduced *Has3* expression by 70% without a compensatory increase in *Has1* or *Has2* expression (Figure 5B and 5C). Using agarose gel electrophoresis, we determined the molecular mass distribution of the hyaluronan present by loading equal amounts of secreted hyaluronan isolated from nontargeting siRNA

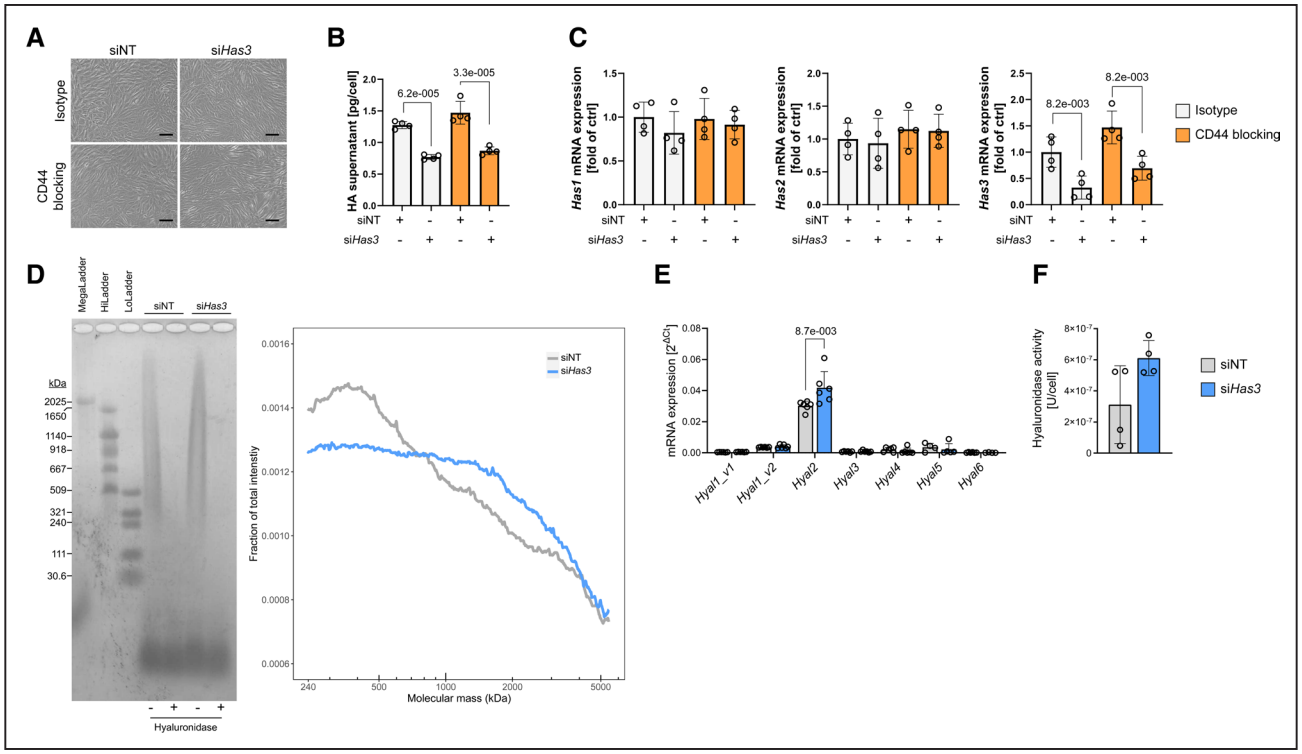


**Figure 5. Hyaluronan (HA) synthesis, molecular mass distribution, and hyaluronidase activity in murine smooth muscle cells (SMC) after Has3 (HA synthase 3) knockdown.**

Murine SMC cultures were stimulated with PDGF-BB and TGF-β1 followed by treatment with small interfering RNA against *Has3* and a CD44 (cluster of differentiation-44) blocking antibody. **A**, No observable morphological differences. **B** and **C**, Quantification of secreted HA and *Has* gene expression. **D**, Molecular mass distribution of secreted HA. HA content was previously quantified and equal amounts were loaded in each lane. **E**, Hyaluronidase gene expression. **F**, Hyaluronidase activity of cell culture supernatant. Statistical analysis was performed with aligned rank transform ANOVA, post hoc pairwise multiple comparisons were adjusted with Sidak correction (**B** and **C**) or Mann-Whitney test (**E** and **F**). Error bars represent mean±SD, n=4/4 (**B** and **C**), n=6/6 (**E**), n=4/4 (**F**) technical independent samples. Scale bars=100 µm. PDGF-BB indicates platelet derived growth factor-BB; si*Has3*, siRNA targeting *Has3*; siNT, nontargeting siRNA; and TGF-β1, transforming growth factor beta 1.

and si*Has3* treated cells. This demonstrated that the hyaluronan in the present system is of intermediate to high molecular weight (300 kDa–2+ MDa) and the *Has3* silenced SMC have a modest shift towards higher molecular weight hyaluronan (Figure 5D). Hyaluronidase (*Hyal*) gene expression and hyaluronidase activity were also profiled. *Hyal2* expression was significantly upregulated and hyaluronidase activity demonstrated a subtle but not statistically significant increase (Figure 5E and 5F). This is likely inconsequential, as there was rather a small increase in molecular mass distribution (Figure 5D). Additionally, CD44 splice variant expression was analyzed, SMC largely expressed the standard variant (cluster of differentiation-44 [CD44s]; Figure XIII in the Data Supplement).

Consistent with our in vivo observations in this model, *Has3* targeted siRNA treatment as well as CD44 blocking significantly reduced *Acta2* expression (Figure 6A). Moreover, si*Has3* treatment and CD44 blocking increased fibrillar collagen *Col1a1*, as well as the ECM organizing collagen *Col15a1.* In addition, *Col3a1* and *Fn1*, both already known to be induced in SMC transition states,[7,8,12] were increased in si*Has3* and CD44 blocked samples (Figure 6B). Combined treatment of si*Has3* and CD44 blocking did not show additive effects. These in vitro investigations support the in vivo findings outlined in this study and suggest that increased SMC phenotypic switching after SMC-*Has3* knockout may be driven by decreased hyaluronan/CD44 signaling, as blocking CD44 reproduced the *Has3* knockdown phenotype.

## CD44 Blocking Induces the Acute-Phase Response in Murine SMC

To further evaluate the role of CD44, we profiled the acute-phase response in murine aortic SMC after CD44 blocking, as this was a highly upregulated pathway identified by scRNA-seq. Indeed, CD44 blocking strongly upregulated the expression of acute-phase response genes; *Lcn2 (Lipocalin-2), Saa3*, and *Hp* (Figure 7A), as well as secretion of LCN2 into the supernatant (Figure 7 B). CD44 blocking also led to the statistically significant increase of *Col1a1* and *Dcn* expression (Figure 6C). Inhibiting NF-κB (nuclear factor κB) nuclear translocation by the addition of specific inhibitor, JSH-23, diminished the upregulation of *Lcn2*

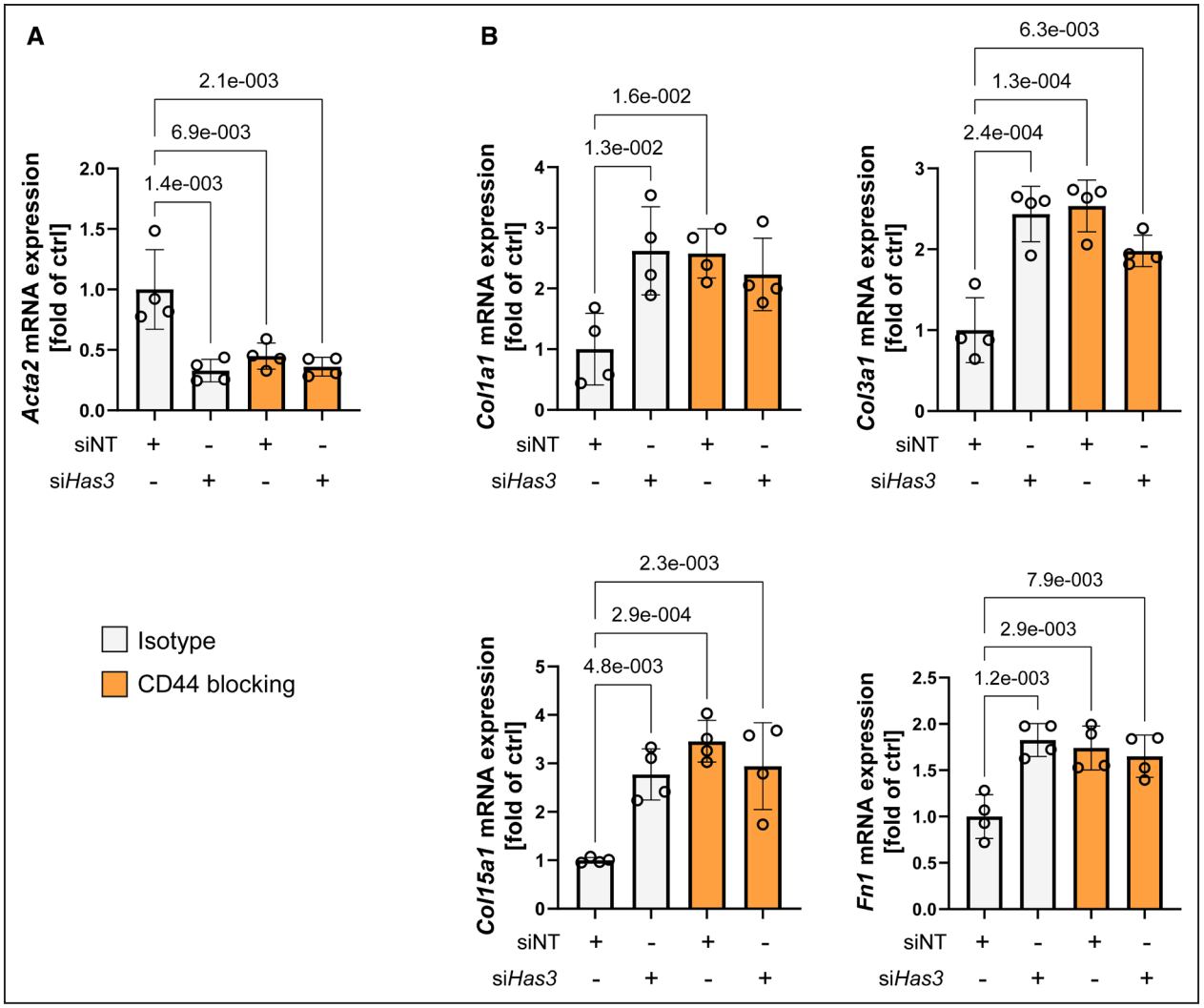


**Figure 6. Has3 (hyaluronan synthase 3) knockdown or CD44 (cluster of differentiation-44) blocking enhances a modulated phenotype in murine smooth muscle cell (SMC) in vitro.**

Murine SMC cultures were stimulated with PDGF-BB and TGF-β1 followed by treatment with small interfering RNA against *Has3* and a CD44 blocking antibody. **A**, *Has3* silencing or CD44 blocking reduces the expression of the contractile marker gene *Acta2*. **B**, *Has3* silencing or CD44 blocking induces extracellular matrix gene expression (*Col1a1, Col3a1, Col15a*, and *Fn1*). No additive effects were observed with simultaneous *Has3* silencing and CD44 blocking. Statistical analysis was performed with 2-way ANOVA, post hoc pairwise multiple comparisons were adjusted with Sidak correction. Error bars represent mean±SD, n=4/4, technical independent samples.

(Figure 7D), suggesting at least a partial involvement of the NF-κB pathway. It has been previously shown that serum amyloid A and LCN2 can drive phenotypic switching.[42,43] To confirm their potential to drive SMC modulation, we stimulated our murine SMC cultures with recombinant LCN2 and Apo-SAA (serum amyloid A apolipoprotein) and found that their addition significantly upregulated *Col1a1* and *Dcn* and downregulated *Acta2* expression respectively (Figure 7E).

## DISCUSSION

In recent years, the hyaluronan matrix has been shown to fulfill diverse functions in the context of cardiovascular diseases. In addition to facilitating neointimal matrix expansion and SMC migration,[20,21] an immunomodulatory function and an important role in endothelial (dys)function has been shown.[22,24] It is also known that T-cell responses are modulated by hyaluronan based on its function in the T-cell immune synapse to reduce Th1 cell polarization.[24] In addition, in the context of recovery after myocardial infarction, it has been shown that hyaluronan increases macrophage survival and myofibroblast responses in mice.[44]

The aforementioned studies highlight the divergent, and in part, opposing roles of hyaluronan in different functional organ compartments and in different cell types. Therefore, in this study, we aimed to unravel the role of HAS3 expression by SMC in atherosclerosis development. Ubiquitous, constitutive deletion of *Has3* in *Apoe*$^{-/-}$ mice reduced Th1 cell polarization, resulting in reduced macrophage-driven inflammation, reduced lesion size,

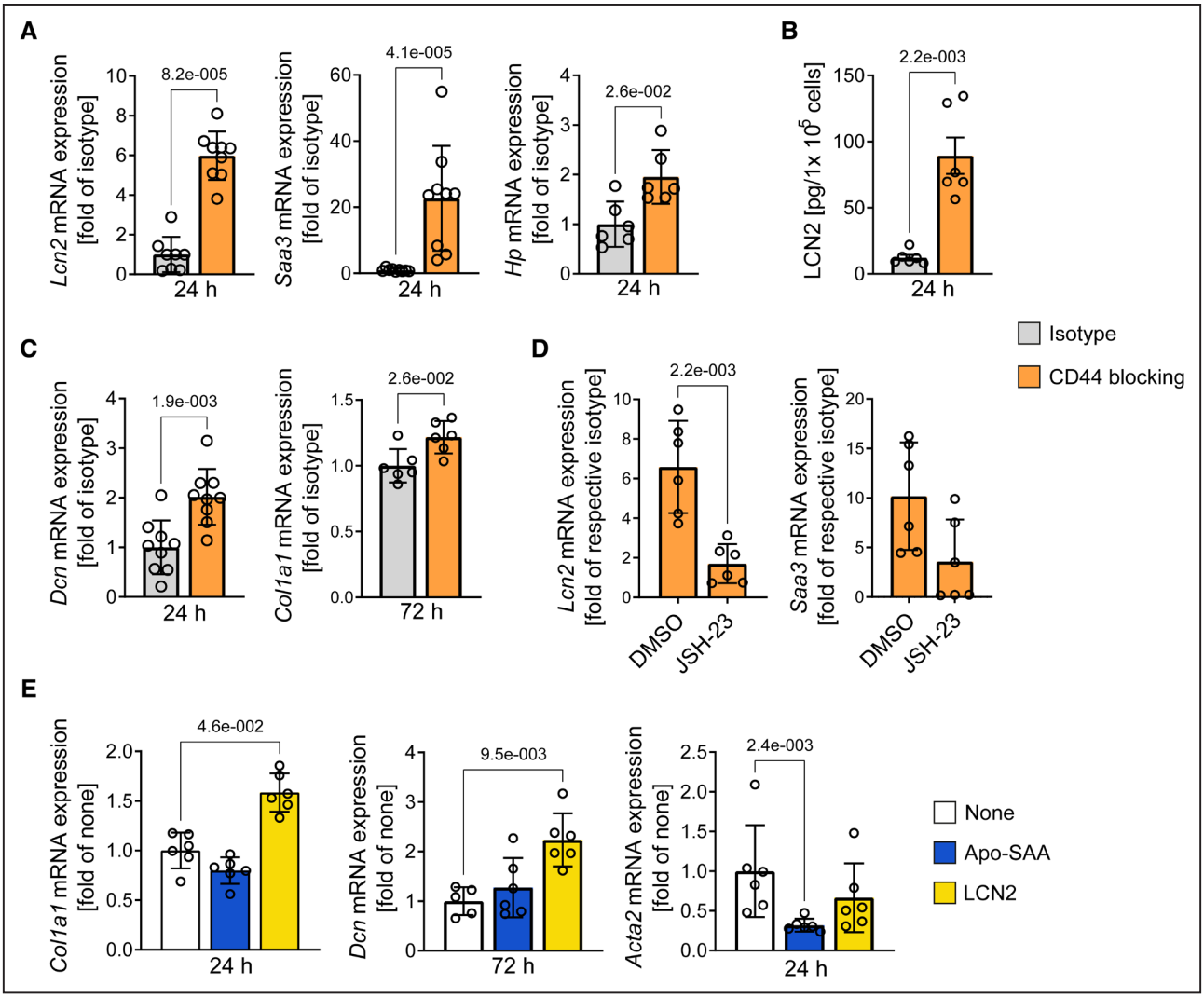


**Figure 7. CD44 (cluster of differentiation-44) blocking in murine smooth muscle cells (SMC) induces acute-phase protein gene expression which drives phenotypic modulation.**

Murine SMC cultures were treated with a CD44 blocking antibody or recombinant acute-phase response proteins, without previous growth factor stimulation. **A**, mRNA expression of acute-phase response genes (*Lcn2,* n=8/9*; Saa3*, n=8/9; and *Hp,* n=6/6), is upregulated in response to CD44 blocking compared to isotype control. **B**, LCN2 protein quantification via ELISA of the cell culture supernatant (n=6/6). **C**, Fibrotic gene expression (*Col1a1,* n=9/9 and *Dcn,* n=6/6) is upregulated in response to CD44 blocking. **D**, Acute-phase response gene expression stimulated by CD44 blocking is blunted by the NF-κB translocation inhibitor JSH-23 (10 μmol/L; n=6/6). **E**, Stimulation of murine SMC with recombinant acute-phase proteins induces *Col1a1* and *Dcn* expression (LCN2, 1 μg/mL) and downregulates *Acta2* (Apo-SAA, 6.5 μg/mL; n=6/6). Statistical analysis was performed with Mann-Whitney tests (**A**–**D**) or Kruskal-Wallis (**E**), post hoc pairwise multiple comparisons were adjusted with Dunn's correction. Error bars represent mean±SD. Apo-SAA indicates serum amyloid A apolipoprotein; DMSO, dimethyl sulfoxide; and LCN2, lipocalin-2.

and atheroprogression.[24] Here, loss of SMC-*Has3* did not affect lesion size or macrophage accumulation. But notably, *Has3* deficiency in SMC had a profound effect on phenotypic switching of SMC and composition of lesion ECM. Specifically, SMC-*Has3* knockout reduced the percentage of ACTA2$^+$ SMC within the lesion by 50% with a concomitant increase in LGALS3$^+$ SMC within the lesion and FC. These results indicate an important role of SMC-*Has3* with respect to overall lesion composition and indices of plaque stability. Therefore, it appears that HAS3 has two detrimental roles in atherosclerosis: (1) promoting Th1 (T-helper cell 1) cell response and macrophage accumulation and (2) inhibiting the transition of SMC to an ECM-synthesizing and plaque stabilizing phenotype.

Recently, Alencar et al,[7] Pan et al,[12] and Wirka et al[8] demonstrated that *Lgals3* is not a marker of a terminally differentiated SMC-derived macrophage-like cell state as previously thought[9,11,45] but rather represents the transition of contractile cells into an ECM remodeling pioneer cell phenotype.[7,8,12,13] Moreover, they showed that the majority of SMC-derived cells in advanced BCA lesions express and likely go through a *Lgals3*$^+$ transition state from which, depending on environmental cues, SMC proceed to phenotypically modulated SMC that can either be beneficial or detrimental for lesion pathogenesis. Of

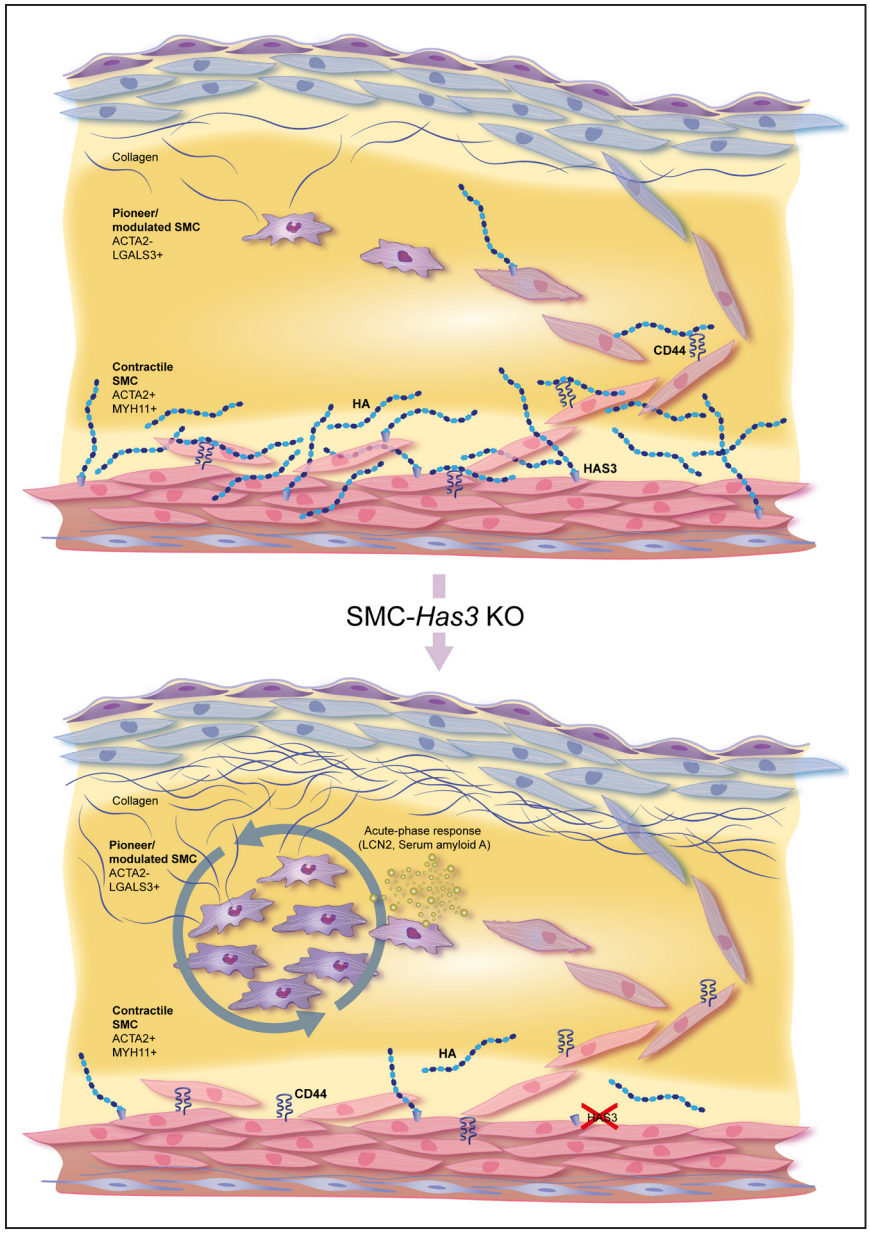


**Figure 8. Schematic overview.**
Hyaluronan (HA) is known to have a crucial influence on volume expansion in atherosclerotic lesion development as well as smooth muscle cells (SMC) proliferation and migration. HAS3 (HA synthase 3) is a strong contributor to HA deposition in early lesion development. Immunostaining of SMC-*Has3* knockout (KO) mice revealed an increase in LGALS3$^+$ (galectin-3) pioneering/modulated SMC which contribute to collagen deposition and maturation within the lesion and the fibrous cap. Single-cell RNA sequencing of brachiocephalic artery (BCA) lesions and in vitro analysis of murine SMC revealed this phenotype is driven by an excessive acute-phase response by SMC, which may drive phenotypic modulation. Presumably, under normal conditions, HA/CD44 (cluster of differentiation-44) interaction serves to negatively regulate the acute-phase response of SMC and, therefore, controls phenotypic switching during the progression of atherosclerosis. ACTA2 indicates alpha-smooth muscle actin; and LCN2, lipocalin-2.

major significance, results of the present study show that the loss of SMC-specific *Has3*, while resulting in an increased fraction of LGALS3$^+$ SMC and decreased ACTA2$^+$ SMC, surprisingly appears to promote beneficial changes within lesions including significantly more decorin and collagen deposition as well as collagen maturation in the FC and lesion core.

Our scRNA-seq analysis of isolated plaque lesions, which identified 5 distinct SMC populations, enabled further insight into the phenotypic status of SMC in SMC-*Has3* knockout mice and possible underlying mechanisms. Consistent with Alencar et al,[7] Pan et al,[12] and Wirka et al,[8] the modulated SMC populations were positive for markers associated with SMC transition, as *Lgals3, Lum, Fn1, or Tnfrsf11b*.[7,8,12] These observations agree with previous results, which demonstrated that 80% of lesion SMC lose their traditional, contractile marker proteins under pathological conditions, such as atherosclerosis.[9]

In general, inflammation develops due to its essential and beneficial roles in tissue damage repair and pathogen resistance. However, in atherosclerosis, inflammatory responses, and, especially, macrophage-driven plaque inflammation, drive atherogenesis and progression. But notably, certain pathways associated with inflammation have recently been shown to play a beneficial role in atherosclerosis. This is shown in a study on the role of IL (interleukin) 1- and IL1R1 (interleukin 1 receptor type 1)-signal transduction in SMC phenotype and collagen deposition in atherosclerosis.[46] Although the IL1R1 signaling pathway in SMC has been shown to exacerbate the development of atherosclerosis,

paradoxically, it also plays a critical role in promoting the formation and maintenance of a protective FC. Here, we found that in addition to collagen, inflammatory genes involved in the acute-phase response; *Saa1, 2,* and *3*, and *Lcn2,* among others were upregulated in *Has3*-deficient SMC-modulated clusters as well as in vitro after CD44 blocking. Acute-phase proteins Serum amyloid A and LCN2 have been shown to promote SMC phenotypic switching[42] and collagen deposition in experimental atherosclerosis.[43] To confirm their potential to drive SMC modulation, we stimulated our murine SMC cultures with recombinant Apo-SAA and LCN2. This treatment indeed stimulated *Col1a1* and *Dcn* and downregulated *Acta2.* Decorin is interesting because it is known to promote collagen fibrillogenesis and collagen matrix stability which has been shown originally in the skin by knock out[39] but also in the vasculature by overexpression.[47] Our results also indicate that CD44, one of the main hyaluronan receptors, plays a role in preventing beneficial increases in ECM deposition by suppressing the SMC expression of acute-phase proteins.

As schematically summarized in Figure 8, LGALS3$^+$ modulated SMC increase after loss of SMC-*Has3*, and likely modulate an acute-phase response, which in an autocrine manner stimulates further collagen deposition, organization, and maturation in the plaque and the FC. This effect on phenotypic switching of SMC is presumably dependent on reduced hyaluronan/CD44 signaling as suggested by experiments in vivo and in vitro. Therefore, the present results suggest strategies to inhibit SMC-*Has3*/CD44 as a possible means to increase atherosclerotic lesion stability.

## ARTICLE INFORMATION

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### Disclosures

None.

### Supplemental Materials

Expanded Materials and Methods
Data Supplement Figures I–XIII
Data Supplement Files I–VII
Data Supplement Tables I and II

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