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Regulation of the Atheroma-Enriched Protein, SPRR3, in Vascular Smooth Muscle Cells through Cyclic Strain is Dependent on Integrin $\alpha1\beta1$/Collagen Interaction

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Atherosclerotic plaques express high levels of small proline-rich repeat protein (SPRR3), a previously characterized component of the cornified cell envelope of stratified epithelia, where it is believed to play a role in cellular adaptation to biomechanical stress. We investigated the physiological signals and underlying mechanism(s) that regulate atheroma-enriched SPRR3 expression in vascular smooth muscle cells (VSMCs). We showed that SPRR3 is expressed by VSMCs in both human and mouse atheromas. In cultured arterial VSMCs, mechanical cyclic strain, but neither shear stress nor lipid loading induced SPRR3 expression. Furthermore, this upregulation of SPRR3 expression was dependent on VSMC adherence to type I collagen. To link the mechanoregulation of SPRR3 to specific collagen/integrin interactions, we used blocking antibodies against either integrin $\alpha1$ or $\alpha2$ subunits and VSMCs from mice that lack specific collagen receptors. Our results showed a dependence on the $\alpha1\beta1$ integrin for SPRR3 expression induced by cyclic strain. Furthermore, we showed that integrin $\alpha1$ but not $\alpha2$ subunits were expressed on VSMCs within mouse lesions but not in normal arteries. Therefore, we identified the enrichment of the mechanical strain-regulated protein SPRR3 in VSMCs of both human and mouse atherosclerotic lesions whose expression is dependent on the collagen-binding integrin $\alpha1\beta1$ on VSMCs. These data suggest that SPRR3 may play a role in VSMC adaptation to local biomechanical stress within the plaque microenvironment. ***(Am J Pathol 2008, 173:1577–1588; DOI: 10.2353/ajpath.2008.080042)***

Atherosclerosis is known to arise in regions of the vasculature subjected to altered hemodynamic stress.[1] Recent studies have shown that biomechanical stress caused by altered flow not only leads to atherosclerosis, but atherosclerosis itself alters local biomechanics.[2,3] New, more sensitive technological innovations, such as intravascular ultrasound, have revealed that even early atherosclerotic lesions significantly affect vessel compliance.[2,4] Studies have considered changes in VSMCs gene expression in other vascular pathologies,[5,6] however little is known about how locally altered biomechanics affect VSMCs within the context of atheromas.

In a previous study we showed that the protein SPRR3 is highly expressed in advanced atheromas of human arteries.[7] SPRR3 is a member of the family of small proline-rich repeat proteins, consisting of members that all possess glutamine- and lysine-rich head and tail domains and a proline-rich core.[8] The flexible core domain is believed to impart to cells an increased ability to stretch while the head and tail domains are anchored to other proteins.[8,9] Many members of the SPRR family of proteins serve as constituents of the cornified envelope, which is an insoluble protein complex formed under the plasma membrane in the uppermost layers of stratified squamous epithelium.[10–12] The cornified envelope plays a major role in the mechanical and barrier properties of

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these tissues.[13] A recent study identified other SPRR members as stress-inducible, cardioprotective proteins.[14] Both SPRR1a and 2a/b were identified as downstream targets of gp130 signaling that are strongly induced in cardiomyocytes in response to biomechanical stress.[14] Ectopic overexpression of SPRR1a protected cardiomyocytes from ischemic injury both *in vivo* and *in vitro*.[14]

Many proteins have been implicated in mechanosensing in VSMCs, especially integrins.[15–18] Integrins are transmembrane adhesion receptors that primarily bind extracellular matrix (ECM).[19] They function in a non-covalently bound heterodimer composed of an α and a β subunit.[20] Studies have shown that chronic stretch and hypertension increase ECM production, as well as integrin expression on VSMCs.[21–23] Current concepts suggest that mechanical signals are transmitted from integrin-ECM binding sites to the cytoskeleton and hence to other transduction molecules in the cytoplasm and nucleus.[24]

The recognized biomechanical disruptions around atherosclerotic plaques and the putative role of SPRR family members in epidermal and possibly cardiac biomechanics led us to test the hypothesis that SPRR3 gene expression was regulated by mechanical stress in VSMCs. This would represent a novel component of the molecular adaptation of VSMC to biomechanical alterations within atheromas. We investigated the mechanism of transcriptional regulation of SPRR3 by cyclic strain (CS) and implicated signal transduction via integrin $\alpha1\beta1$ and collagen in *SPRR3* gene regulation in VSMCs.

Materials and Methods

Materials

Antibodies: anti-human SPRR3 (clone 4a; Alexis Biochemicals, San Diego, CA), anti- von Willebrand factor (Dako, Glostrup, Denmark), anti-mouse integrin $\alpha1$ subunit (clone Ha 31/8; BD Pharmingen, San Diego, CA), anti-mouse integrin $\alpha2$ subunit (clone Ha1/29; BD Pharmingen), anti-β-actin (clone AC-15; Sigma-Aldrich, St. Louis, MO), anti-smooth muscle myosin heavy chain (clone 1G12; Abcam, Cambridge, MA), and anti-smooth muscle α-actin (clone 1A4, Sigma-Aldrich). We generated and affinity purified a polyclonal Armenian hamster anti-mouse SPRR3 against the peptide spanning amino acids V45-P56 of mSPRR3. By immunoblot, this antibody recognized a 30-kDa band from mouse esophagus lysate as well as COS-7 cells overexpressing SPRR3 (Image Clone ID: 4288753). This band was competed away by preincubation with the immunizing peptide (Supplemental Figure S1A at *http://ajp.amjpathol.org*).

Animals

All animals and procedures were performed in accordance with the Vanderbilt Institutional Animal Care and Use Committee. C57Bl/6 (wild-type, WT), H-2K^b-tsA58[25] (Tag; colony maintained by J.R. at VUMC), and $\alpha1$-integrin[26] and $\alpha2$-integrin[27] subunit null mice were sacrificed between 2 to 3 weeks of age by cervical dislocation and their thoracic aortas were harvested for VSMC isolation. ApoE$^{-/-}$ and syngeneic Bl/6 WT control mice (gift of Dr. A. Hasty,[28] Vanderbilt University) were maintained on regular chow diet and sacrificed at 6 months of age for histological evaluation of proximal aorta lesions.

Cell Culture

Protocols involving human tissues were reviewed by the Vanderbilt Institutional Review Board. Human (h)VSMCs were isolated from excess aortic tissue from heart explants (in generous collaboration with Dr. Davis Drinkwater and Mr. Paul Chang). VSMCs were isolated from n = four donors. The vessels were cleaned of adventitia, cut longitudinally, and the lumen was scraped to remove the endothelium. Segments from the media were cut into 0.5 cm^2 and cultured in SmGM2 media (Lonza, Basel, Switzerland) until outgrowth of cells. A similar procedure was used to obtain VSMCs from mice. Murine cells were maintained in 15% fetal bovine serum/Dulbecco's Modified Eagle Medium/penicillin/streptomycin with or without 200 pM transforming growth factor (TGF)β.[29] Each independent isolation of VSMCs was assessed by immunofluorescence with anti-smooth muscle α-actin (α-SMA, 1:1000), anti-smooth muscle myosin heavy chain (SM-MHC, 1:250), and anti-von Willebrand factor (1:200). CS experiments were performed with cells that were 95% to 100% α-SMA and SM-MHC positive and von Willebrand factor-negative. VSMCs were maintained at 37°C in 5% CO_2 and used between passages four to nine, except immortal Tag-VSMCs, which were culture expanded at 33°C in the presence of 10 ng/ml interferon-γ and subcultivated for 10 days (five passages) in regular media at 37°C before use.[30] VSMCs were treated with 75 μg/ml of low-density lipoprotein (LDL) or mildly oxidized LDL in medium containing 1% fetal bovine serum (kind gift of W. G. Jerome) for 48 or 72 hours.[31]

Biomechanical Stress Application

VSMCs were exposed to CS by plating cells at 1×10^4 cells/well on Flex I elastomer-bottom dishes or control solid Flex II plates (Flexcell Int, Hillsborough, NC) and allowed to adhere overnight. Plates were commercially prepared with type I collagen or pronectin F coating. Uncoated plates were treated with 100 μg/ml poly-L-lysine for 3 hours at 37°C. In some experiments, VSMCs were preincubated 1 hour on ice with 2 μg antibody/1×10^4 cells. VSMCs were exposed to CS with 15% to 20% elongation for 24 to 72 hours at 1Hz (60 cycles/min) via application of a vacuum (15 to 20 kPa) by a computer-controlled mechanical strain unit (Flexercell 2000, Flexcell Int). All experiments shown were performed a minimum of three times.

VSMCs were exposed to a constant level of shear stress using a cone-plate viscometer. Cone angle and rotational velocity were selected to produce a steady shear stress at either 5 or 10 dynes for 12 and 24 hours (1 dyne = 100mN). Following strain application cells were washed with PBS and harvested in Trizol for RNA extraction, in 8M urea buffer for protein, or fixed for immunofluorescence.

Table 1. Primers Used for Semi-Quantitative RT-PCR

	Primer sequence	Tm	Product size
18S forward	5′-CGCCGCTAGAGGTGAAATTCT-3′	60°C	100 bp
reverse	5′-CGAACCTCCGACTTTCGTTCT-3′		
hMMP-2 forward	5′-ATCGCTCAGATCCGTGGTG-3′	54°C	51 bp
reverse	5′-CCAAATGAACCGGTCCTTGA-3′		
hElastin forward	5′-TTCCCCGCAGTTACCTTTCC-3′	54°C	144 bp
reverse	5′-AACCACCGCACCTGCAGA-3′		
hSPRR3 forward	5′-ATGTCCTTCAACGGTCACTCC-3′	52°C	96 bp
reverse	5′-CTCTTCGGTTGGTGGTCTAC-3′		
mElastin forward	5′-CATCCGTCCATCTTGACTGCCTA-3′	60°C	147 bp
reverse	5′-CAACCAGCCCACACAACCCT-3′		
mSPRR3 forward	5′-CCCTTTGTCCCACCTCCT-3′	59°C	134 bp
reverse	5′-TTGGTGTTTCCTGGTTGTG-3′		

Reverse Transcription and Semi-Quantitative

RNA from VSMCs was isolated with Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and quality was confirmed by an $A_{260/280}$ ratio ≥1.9, as well as clear bands of 28S and 18S rRNA by gel electrophoresis. Following isolation, RNA was treated with RNase-free DNase to eliminate contaminating DNA and processed with the RNeasy RNA cleanup kit (Qiagen, Valencia, CA). cDNA was generated using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) from 1 μg RNA. The cDNA was then used for real-time PCR using SYBR-green iQ PCR supermix and run in an iCycler Real-Time PCR thermal cycler (Bio-Rad). Primer sequences are shown in Table 1. The average cycle threshold from triplicate reactions was used to quantify the relative amount mRNA present based on a standard curve. The differences between samples were determined based on the comparative Ct model.[32] Data were compiled by calculating $\Delta\Delta$Ct relative to the level of 18S RNA present in each sample. A standard curve composed of serial dilutions of a positive control was run with each reaction. A single amplification product was confirmed by the presence of a single band of the correct size by agarose gel electrophoresis, as well as by a single peak in a melt curve analysis of the PCR products. Furthermore, samples were only analyzed when the no-template control reaction was negative. The results are presented as average of fold change from non-stressed samples from multiple, independent experiments; PCR analyses from each experiment performed in triplicate.

Immunofluorescence/Immunohistochemistry

Human tissue was obtained from autopsies (postmortem time ranged from 2 to 12 hours) from males and females whose cause of death was not vascular related. Regions were from both proximal and distal large arteries ($n = 38$) and large veins ($n = 8$). Of these, $n = 12$ of the human aortas contained primarily early atherosclerotic lesions (fatty streaks and intermediate lesions) and $n = 4$ aortas that were histologically normal were analyzed by H&E and SPRR3 immunohistochemistry. Histological analyses were performed by a cardiovascular pathologist (Dr. J. Atkinson). Sections from formalin-fixed, paraffin-embedded human arteries were stained as described.[7] Briefly, the slides were deparaffinized and subjected to antigen retrieval in citrate buffer. The primary antibodies recognized human SPRR3 (1:400) and α-SMA (1:1000). Frozen sections of mouse proximal aortas fixed with cold acetone were stained using antibodies against mouse SPRR3 (3E9.1; 1:250), α1-integrin subunit (1:100), α2-integrin subunit (1:200), and/or α-SMA (1:2000) followed by appropriate fluorescent-conjugated secondary antibodies. Human VSMCs grown on elastomer membranes were fixed for 20 minutes at room temperature with cold acetone, followed by permeabilization with 0.4% Triton in PBS. Subsequently, the membranes were cut out of the dishes. Each membrane was blocked for 1 hour with 10% goat serum and primary antibodies for SPRR3 (1:400), and SM-MHC (1:20) were applied in 3% goat serum overnight at 4°C and incubated with the appropriate secondary antibodies the following day. Slides were viewed under a Zeiss Axioplan microscope (Carl Zeiss MicroImaging, Thornwood, NY), and analyzed with MetaMorph Imaging system (Molecular Devices, Sunnyvale, CA). The staining and colocalization was confirmed by fluorescent confocal microscopy using a Zeiss upright LSM510 confocal microscope and the images were analyzed using LSM Image Browser (Zeiss).

Immunoblotting

VSMC lysates were harvested by scraping in urea buffer (8M Urea, 75 mmol/L Tris pH 8.0, 2% SDS, 5% β-mercaptoethanol, protease inhibitors) and centrifuged at 10,000 × *g* for 10 minutes at 4°C and the debris pellet discarded. Immunoblotting was performed as described elsewhere.[33] Primary antibodies used were anti-mouse SPRR3 (3E9.1, 1:1000) and anti-β-actin (1:5000) with appropriate HRP-conjugated secondary antibodies.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism5. All data are shown as ± SEM. When comparing two samples, a Mann-Whitney *U*-test was used and for experiments with multiple groups, a non-parametric Repeated Measures analysis of variance with Bonferroni

correction was used. *P* values ≤0.05 were considered significant.

Results

SPRR3 Localizes to VSMCs in Atherosclerotic Lesions

SPRR3 was detected even in early atherosclerosis within subendothelial intimal cells (Figure 1, A and C). We detected specific staining in five out of six fatty streaks and all six intermediate lesions examined, but not in normal regions within the same vessel (Figure 1, A–D). Extensive SPRR3 immunoreactive areas were identified in all advanced atheromas (Figure 1, E–F). No specific staining was observed in normal veins (Figure 1J). Immunofluorescence was used to colocalize expression of SPRR3 and α-SMA, a marker for VSMCs, within a human arterial atherosclerotic lesion.[34] Numerous areas of colocalization indicated that a large number of VSMCs within plaques expressed SPRR3 (Figure 1, G–I, arrowheads). There was also some evidence of extracellular deposition of SPRR3, which may be the result of protein secretion or cell death (Figure 1, G–I, arrows). SPRR3 staining was also evident in lesions within the smooth muscle cell-rich fibrous caps of the proximal aortas of Apo $E^{-/-}$ mice (Figure 1, K and L). Human and murine esophagus were stained in parallel as positive control[35] and secondary antibodies alone were used as negative controls (Supplemental Figure S1B at *http://ajp.amjpathol.org).*

SPRR3 Gene Expression Is Regulated by Cyclic Strain in VSMCs but not by Lipids or Shear Stress

Independent preparations of primary hVSMCs were each assessed by immunofluorescence for expression of SM α-actin and SM-MHC (Figure 2A) to confirm the VSMC phenotype in >90% of the cultured cells before use in experiments. Furthermore, we demonstrated codistribution in hVSMCs of SPRR3 with SM-MHC (Figure 2B), supporting the role of SPRR3 in biomechanics.[36,37] To determine whether SPRR3 expression was influenced by biomechanical stress when grown on native type I collagen, we measured SPRR3 mRNA levels by qRT-PCR in response to CS and shear stress. Collagen I was chosen as it is a predominant matrix protein in the arterial wall.[38] Following CS application for 24–72 hours, SPRR3 transcript levels were increased by 2.1 ± 1.1- and 4.95 ± 0.7-fold after 48 and 72 hours, respectively, of continuous strain as compared to unstrained cells (Figure 2C). As a positive control, we assessed concomitant upregulation of the previously studied stress-sensitive transcripts, elastin and MMP-2 (Figure 2, D–E).[21,39] Elastin and MMP-2 transcripts were increased by 5.85 + 1.8- and 2.11 + 0.4-fold, respectively, over unstrained control VSMC after 48 hours of CS. By immunofluorescence, SPRR3 protein levels were shown to increase following 72 hours CS (Figure 2F). As VSMCs are indirectly affected by shear forces,[40–43] we also investigated whether SPRR3 was transcriptionally regulated by shear stress in culture. Human VSMCs were exposed to shear stress at either an arterial level of 10 dynes or a venous level of 5 dynes for 12 or 24 hours (Figure 2G shows data for only 24 hours).[41,42] There were no significant changes in transcript levels of SPRR3 following application of shear stress. Since VSMCs within atheromas are exposed to a lipid-rich environment, we investigated if SPRR3 expression was influenced by lipids. Levels of SPRR3 were significantly reduced after exposure to oxidized LDL (Figure 2 hours), while the addition of either oxidized or unoxidized LDL for 48 hours or 72 hours (only 48 hours data shown, Figure 2) did not increase SPRR3 transcript levels in VSMCs.

Due to the limited availability of primary VSMCs from human aortic explants, it was of interest to determine whether our results could be recapitulated using both mouse primary wild-type VSMCs (WT-VSMC) as well as VSMC lines generated from the transgenic H-$2K^b$-tsA58 mouse (Tag-VSMC). This mouse possesses a heat-labile T-antigen (Tag) expressed behind the mouse major histocompatibility complex H-$2K^b$, which is widely expressed and is further inducible by interferon-γ. Therefore, cells isolated from these animals can be conditionally immortalized by growth at a permissive temperature of 33°C and in the presence of interferon-γ, but can be returned to normal primary culture-like conditions when grown at 37°C.[25] As primary cells must be used within nine passages, conditionally immortal Tag-VSMCs enabled us to circumvent the difficulty of obtaining sufficient numbers for extended analysis. We confirmed that both WT-VSMCs and Tag-VSMCs grown at 37°C expressed α-SMA and SM-MHC (Supplemental Figure S2 at *http://ajp.amjpathol.org*). Both WT and Tag-VSMCs were subjected to CS for 72 hours and both demonstrated statistically significant upregulation of SPRR3 mRNA transcript levels between ~2- to 2.5-fold, respectively (Figure 3, A, C). Interestingly, both types of murine VSMCs showed a less robust CS-associated regulation than human VSMCs under parallel conditions. To determine whether SPRR3 protein levels were regulated by CS, cell homogenates from cyclically strained (72 hours) and non-strained WT-VSMC were analyzed by immunoblot. SPRR3 expression was detected in WT-VSMCs after 72 hours CS, but not in unstrained, control samples. Expression in mouse esophagus was expectedly very high (Figure 3B).[35] Using human and mouse primary VSMCs as well as mouse Tag-VSMCs, we demonstrated consistent upregulation of SPRR3 transcripts and protein by CS.

SPRR3 Mechanoregulation Is Dependent on Binding to Collagen

The mechanisms by which mechanical signals are recognized by VSMCs and translated into molecular responses are not completely understood.[44] Intimal VSMCs within atheromas are exposed to an environment rich in ECM, particularly (type I) collagen.[38] Collagen and other ECM components bind cell surface integrins, which serve

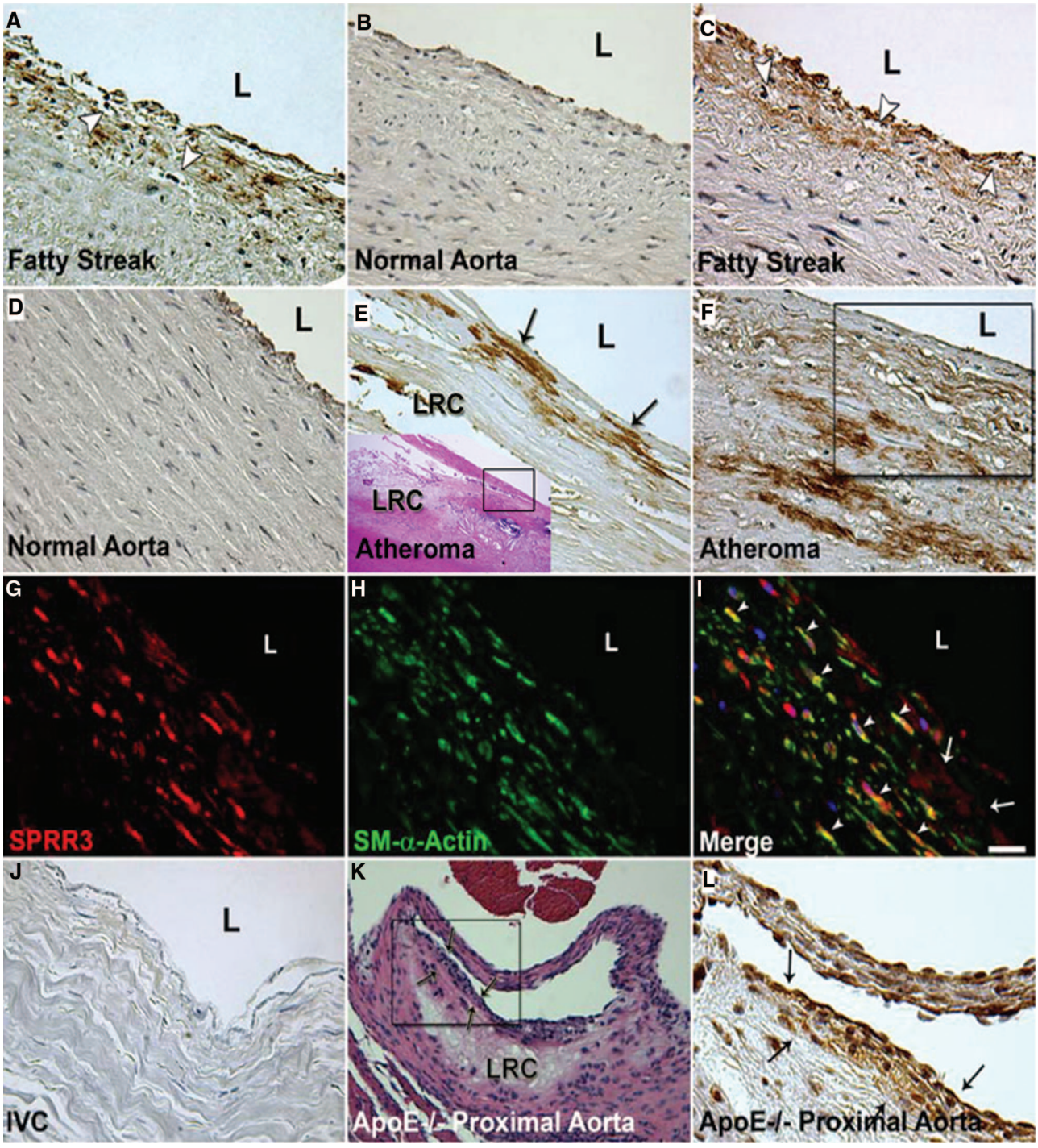

Figure 1. SPRR3 is enriched in VSMCs in human and mouse atheromatous lesions. Representative sections from paraffin-embedded tissues of human arteries obtained from different individuals containing fatty streaks show SPRR3-positive staining (brown) within the affected intima (**A, C**). Corresponding regions with normal histology from the same vessel (**B, D,** respectively) are negative. **Arrowheads** designate foam cells. SPRR3-stained human aortas containing advanced atherosclerotic lesions (**E–F**) show extensive SPRR3 immunopositive staining within the fibrous cap (**E, arrow**) and dispersed through the lesion (**F**). An H&E of the lesion in (**E**) (**inset**) is shown with a box denoting the region magnified in (**E**). The tissue is fractured due to calcification within the atheroma. No SPRR3-staining detected within the vessel wall of histologically normal human inferior vena cava, nor was it found in the medial VSMCs beneath any of the lesions (**J**, data not shown). The boxed area in (**F**) corresponds to the region of the vessel examined by indirect immunofluorescence for costaining of SPRR3 (**G,** red) and α-SMA (**H,** green). **I:** Overlay in which colocalization is denoted with **arrowheads**. Note the presence of extracellular SPRR3, which does not colocalize with 4,6-diamidino-2-phenylindole (**I, arrows**). Representative H&E-stained proximal aortic lesion from ApoE$^{-/-}$ (**K**) and its serial section (**L**) stained for SPRR3. Immunopositive area coincided with the VSMC-rich fibrous cap (indicated by **arrows**; **L** is the magnification of the boxed area in **K**). Vessel lumen (L), Lipid-Rich Core (LRC). Scale bar = 20 μm. Magnification = original ×4 (**inset**, **E**), ×10 objective (**A–F, J–K**), ×40 objective (**G–I, L**).

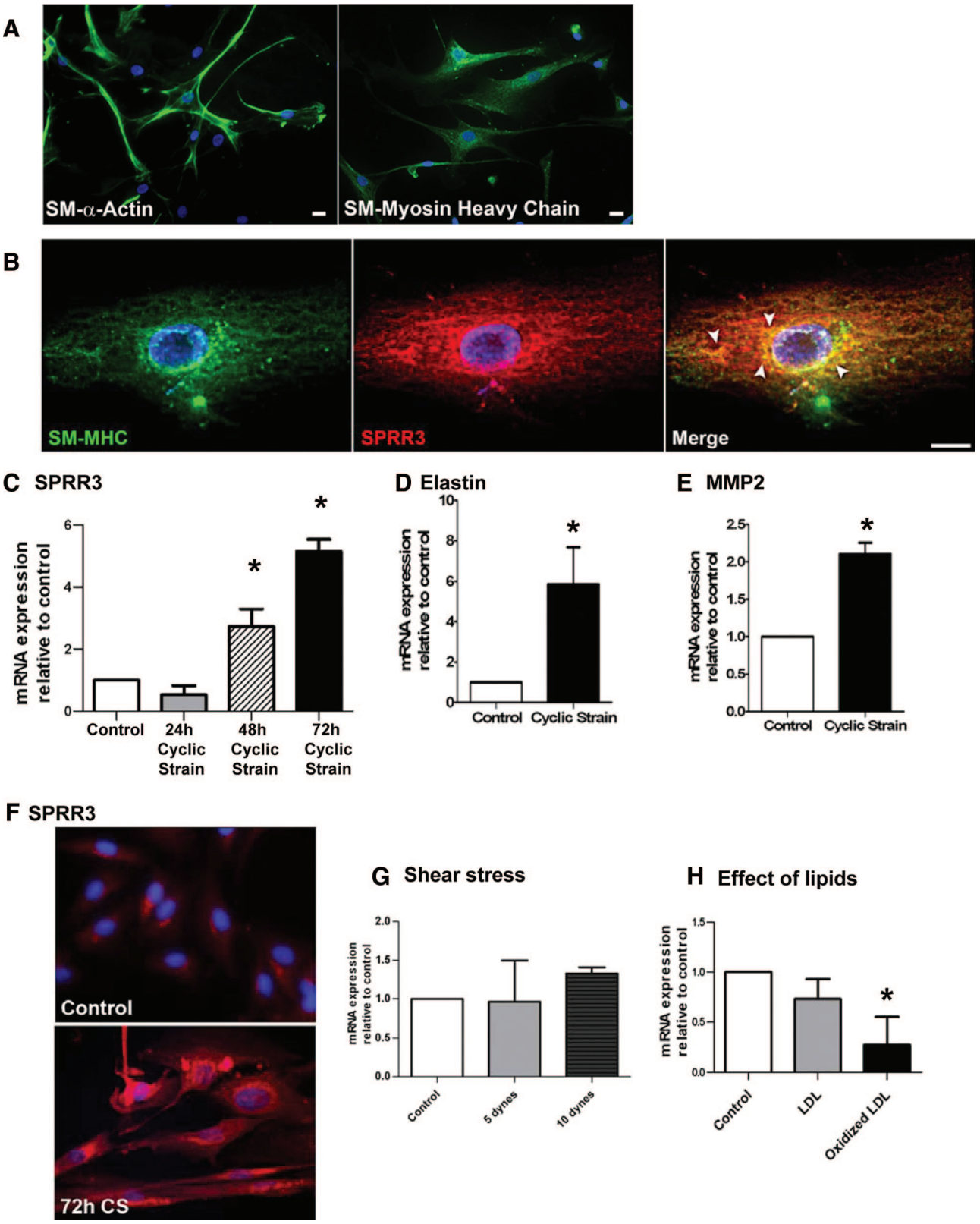

Figure 2. SPRR3 is regulated by CS in human VSMCs. **A:** Primary hVSMC cultures expressed both α-SMA and SM-MHC. **B:** SM-MHC (green) and SPRR3 (red) codistributed (yellow, **arrowheads**). mRNA levels of SPRR3 (**C**), elastin (**D**, 48 hours), MMP2 (**E**, 48 hours) after exposure to CS relative to unstrained controls (n = 4 independent CS experiments). Immunofluorescence of representative strained and unstrained cells from the same experiment demonstrated clearly increased cellular SPRR3 protein expression following 72 hours CS (**F**). Shear stress (24 hours) had no effect on SPRR3 transcript levels at either 5 or 10 dynes (**G**) (n ≥3 independent primary VSMC preps and CS experiments). Culturing hVSMCs with lipids (**H**, only 48 hours data shown) did not induce SPRR3 transcripts over control (n = 4 independent experiments in triplicate). Scale bar = 20 μm. Magnification = original ×20 (**A**), ×20 (**B**). *$P \leq 0.05$.

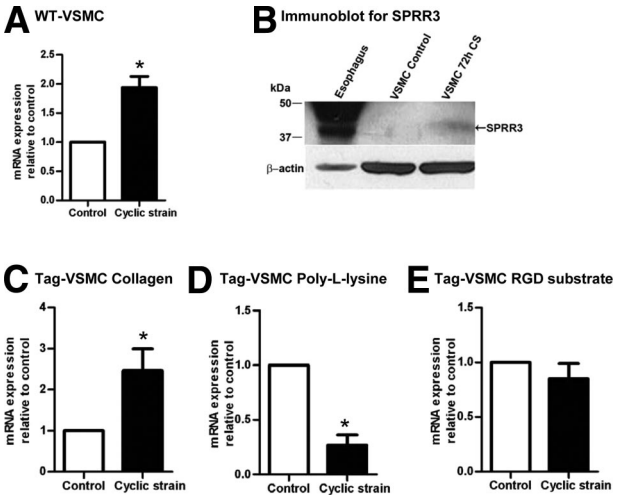

Figure 3. SPRR3 upregulation in murine VSMCs requires collagen. SPRR3 transcripts (**A**) and protein (**B**) up-regulated by 72 hours CS in WT-VSMCs ($n \geq 3$ independently isolated VSMCs; $n = 4$ independent CS experiments). Similar fold increase in SPRR3 transcripts observed in Tag-VSMCs cultured on type I collagen ($n = 5$) (**C**), but not on poly-L-lysine ($n = 3$, **D**), or pronectin F ($n = 3$, **E**, RGD substrate). $^*P \leq 0.05$.

to connect the ECM to the actin cytoskeleton of cells.[45] Moreover, changes in traction force initiate intracellular signal transduction cascades via "outside in" integrin signaling.[45] We investigated whether regulation of SPRR3 by mechanical forces was modulated by specific extracellular matrices.

To determine the specificity of SPRR3 regulation for type I collagen, we exposed VSMCs to CS on various substrates. Tag-VSMCs were plated onto poly-L-lysine (which allows non-integrin mediated adhesion) and pronectin F (which contains multiple RGD repeat peptides from human fibronectin) and subjected to CS conditions.[46,47] Growth and gross morphology of the VSMCs were not markedly altered on these substrates. SPRR3 transcript levels were not increased in VSMCs after 72 hours of CS when cultured on either poly-L-lysine or on pronectin F (Figure 3, D–E). In fact, the poly-L-lysine substrate resulted in a significant relative decrease (>2-fold) in SPRR3 transcript level with 72 hours of CS. These data indicated that SPRR3 regulation required VSMC contact with (type I) collagen.

Integrin α1β1 Is Necessary for Mechanosensitive Up-Regulation of SPRR3

Based on the above data, we chose to investigate the role of the major collagen binding integrins, $\alpha1\beta1$ and $\alpha2\beta1$,[20] in SPRR3 regulation using function blocking antibodies to each of the integrin $\alpha1$ or $\alpha2$ subunits. Tag-VSMCs were preincubated with function blocking monoclonal antibodies against $\alpha1$ or $\alpha2$ integrin subunits before plating on type I collagen and application of 72 hours of CS. Incubation with $\alpha1$ integrin subunit blocking antibodies abrogated the CS-mediated increase in SPRR3 expression (Figure 4A). By contrast, preincubation of VSMCs with $\alpha2$ integrin subunit blocking antibodies induced a modest (1.5 ± .4-fold), but statistically insignificant up-

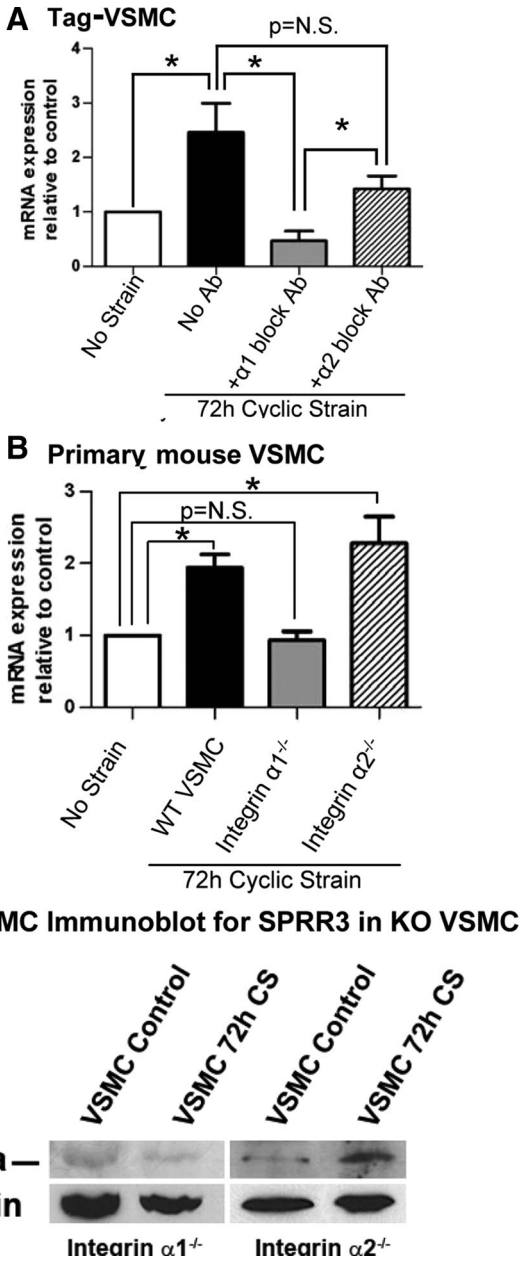

Figure 4. Transcriptional regulation by CS requires integrin $\alpha1$, but not $\alpha2$. **A:** Relative fold change in SPRR3 mRNA transcripts in Tag-VSMCs exposed to CS following pretreatment with function blocking antibodies ($n = 4$). **B:** SPRR3 transcript levels were measured in primary VSMCs from WT or mice lacking designated integrin subunits after 72 hours CS ($n = 4$ CS experiments from single isolation of VSMCs). **C:** SPRR3 protein levels were increased in primary VSMCs from integrin $\alpha2\beta1^{-/-}$ mice following 72 hours CS, but not in integrin $\alpha2\beta1^{-/-}$ mice, as determined by immunoblot. $^*P \leq 0.05$, NS: not significant.

regulation of SPRR3 transcripts. There was a statistically significant difference in fold-change of SPRR3 transcripts between cells exposed to antibodies against $\alpha1$ versus $\alpha2$ (Figure 4A). To further strengthen these findings we used genetic models of integrin $\alpha1$ or $\alpha2$ subunit deletion. Primary VSMCs were isolated from aortas of mice lacking integrin $\alpha1\beta1$[26] or $\alpha2\beta1$.[27] VSMC cells derived from both integrin $\alpha1\beta1$ and $\alpha2\beta1$ knockout mice were confirmed to express α-SMA and SM-MHC (Supplemental Figure S2 at

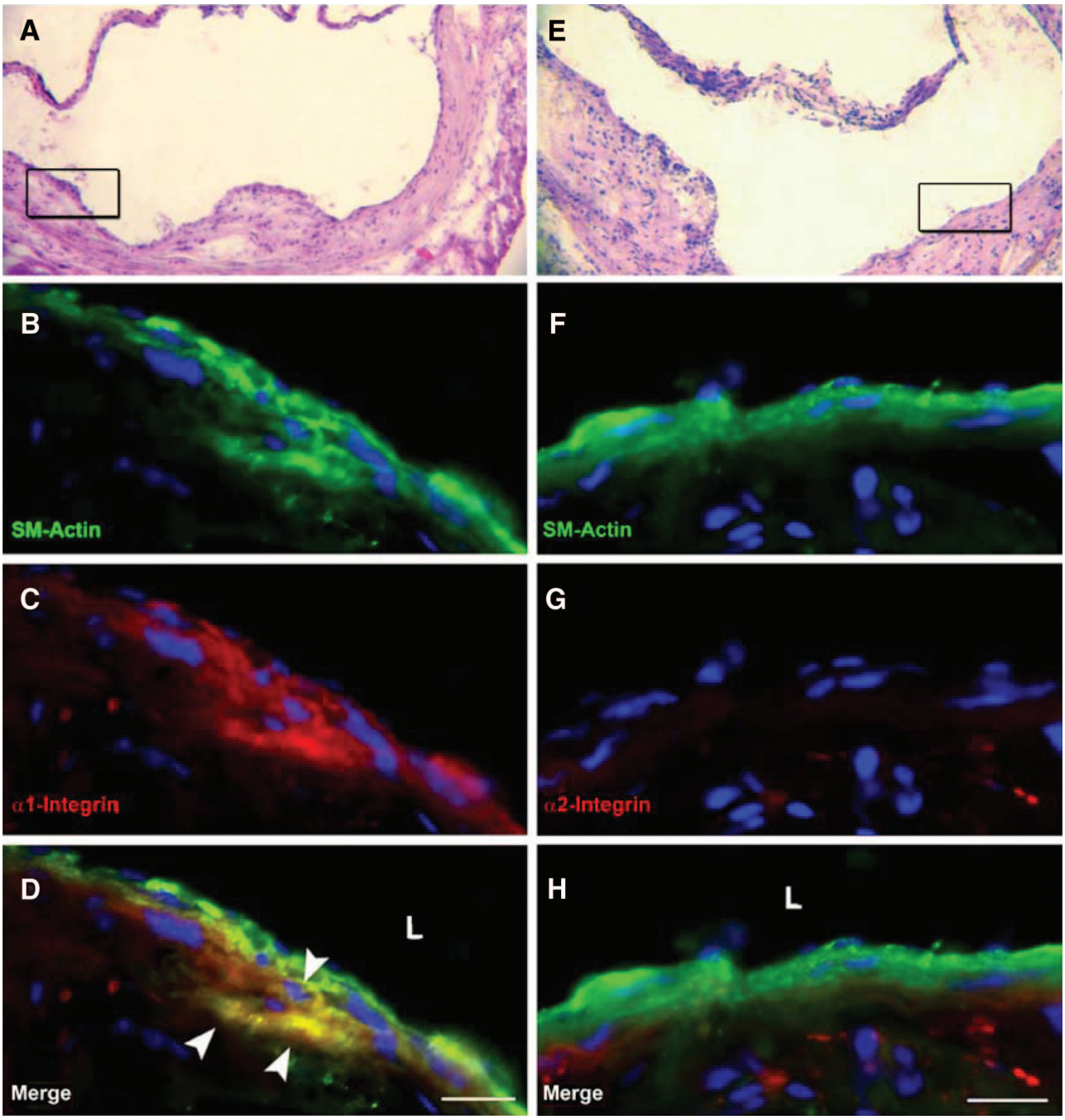

Figure 5. Integrin $\alpha 1$, but not $\alpha 2$, is expressed in VSMCs in atheromas. Indirect immunofluorescence of representative proximal aorta lesion from ApoE$^{-/-}$ mice (H&E, **A,E**) showed coexpression of α-SMA (**B, F**, green) and integrin $\alpha 1$ (**C**, red). **D:** Merge, colocalization (yellow) marked by **arrowheads**. Integrin $\alpha 2$ expression was not detected (**G,H**). Scale bar = 20 μm. Magnification = original ×40. L indicates vessel lumen.

http://ajp.amjpathol.org). KO-VSMC primary cells were plated on type I collagen, subjected to 72 hours CS, and assessed for changes in SPRR3 levels as compared to control cells without strain. As expected, the VSMCs from mice lacking integrin $\alpha 1\beta 1$ failed to up-regulate SPRR3 whereas those from integrin $\alpha 2\beta 1^{-/-}$ animals showed a 2.3 ± 0.6-fold ($P < 0.05$) increase in SPRR3 transcript and a corresponding increase in protein similar to WT-VSMCs (Figure 4, B and C). These data help explain why SPRR3 expression is limited to atherosclerotic regions of the vasculature; SPRR3 regulation will not occur in the absence of integrin $\alpha 1\beta 1$. It is of note that WT-VSMCs and Tag-VSMCs demonstrated enhanced SPRR3 regulation when the cells had been cultured for 48 hours before stress in 200 pM TGF-β (Supplemental Figure S3B at *http://ajp.amjpathol.org*). This observation was likely due to the upregulation of the integrin $\alpha 1$ subunit when VSMCs were cultured in 200 pM TGF-β (Supplemental Figure S3C at *http://ajp.amjpathol.org*); Furthermore, it is known that VSMCs in prolonged culture down-regulate integrin $\alpha 1\beta 1$.[48] Consistent with this, we did not observe SPRR3 regulation with cyclic strain using cells in pro-

longed culture (over nine passages) (Supplemental Figure S3A at *http://ajp.amjpathol.org*). It should be noted, however, that there are numerous other cytokines and signaling factors that are known to affect integrin expression and these, as well as or in addition to TGF-β, may be influencing $\alpha1\beta1$ integrin expression in atheromas.[49]

To confirm the relevance of integrin $\alpha1\beta1$ in this system, we assessed whether $\alpha1\beta1$ integrins were expressed in VSMCs of atheromas. Proximal aortas from ApoE$^{-/-}$ mice containing atherosclerotic lesions were costained with antibodies against $\alpha1$ or $\alpha2$ integrin subunits and anti-α-SMA antibodies. Expression of integrin $\alpha1$ subunit was colocalized with the VSMC marker (Figure 5 A–D), whereas no discernible integrin $\alpha2$ staining was observed in this mouse atherosclerosis model (Figure 5, E–H).

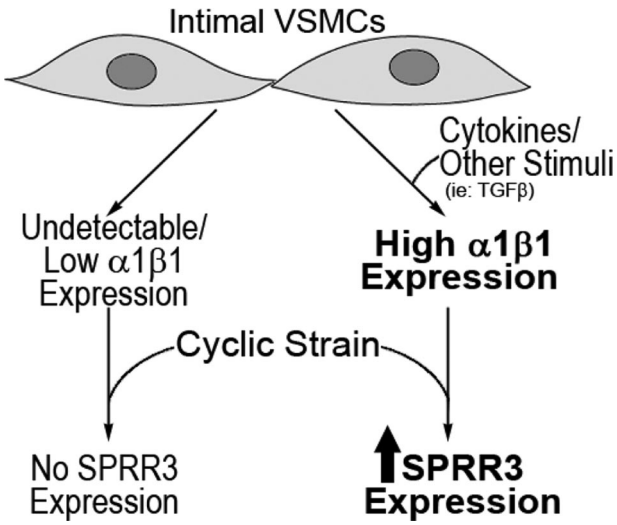

Figure 6. Model of SPRR3 Regulation in VSMCs.

Discussion

This study was designed to investigate the physiological basis for atheroma-enriched expression of the SPRR3 protein demonstrated in both human and murine vasculature. Our results indicated that prolonged (>48 hours) CS of 20% resting length produced upregulation of SPRR3 RNA and protein in VSMCs. By contrast, exposure of VSMCs to varying amounts of shear stress or lipids failed to increase SPRR3 transcript levels (Figure 2). To our knowledge this is the first atheroma-specific protein whose expression is biomechanically regulated. It is of some interest that SPRR3 codistributed with SM-myosin heavy chain in hVSMCs (Figure 2B). SM-MHC has previously been shown to be both biomechanically regulated as well as biomechanically active and hence this finding supports a role for SPRR3 in biomechanics.[36,37] Because integrins are hypothesized to serve as prime cellular mechanosensors, as they link the interior and exterior of the cell,[22,50] we sought to elucidate the effect of ECM and integrins in mechanoregulation of SPRR3 transcription. By exposing VSMCs to CS on various substrates, we demonstrated that SPRR3 regulation by CS required type I collagen, whereas cells grown on poly-L-lysine or pronectin F failed to regulate SPRR3 with CS. Since type I collagen constitutes >70% of all collagen in atheromatous plaques, it represents the primary ECM circumscribing VSMCs within lesions.[38] Given that the CS effect required a collagenous substrate, we evaluated the role of the major collagen binding receptors, $\alpha1\beta1$ and $\alpha2\beta1$, in SPRR3 gene regulation using both an immunoblocking approach and a genetic deletion model. Taken together, the data suggest that the $\alpha1\beta1$ collagen-binding integrin is required for mechanoregulation of SPRR3. Consistent with published studies, we confirmed integrin $\alpha1$ subunit expression in VSMCs within murine lesions.[51] Expression of the integrin $\alpha2$ subunit was not evident in murine lesions as is consistent with previous findings.[48,52]

Since VSMCs are exposed to mechanical forces such as CS in healthy arteries as well as in atheromas, our findings do not fully explain why SPRR3 expression was restricted to plaques. One possibility for this phenomenon relates to the microenvironment of the plaque itself. VSMCs as well as macrophage foam cells are known to take up lipid in atheromas, so we investigated if lipid loading could up-regulated SPRR3 transcript.[53] LDL and ox-LDL failed to enhance SPRR3 expression, although ox-LDL loading resulted in decreased SPRR3 transcription. While it is unclear why this may have occurred, it is known that lipid-loading VSMCs induces a variety of signaling pathways, as well as inducing an even more synthetic cellular phenotype.[54–56] Therefore, cells with an altered phenotype, in the absence of biomechanical stress, may down-regulate SPRR3. However, this raises the issue of VSMC phenotype (eg, contractile/differentiated versus synthetic) as a variable in this system. VSMCs in the context of the atherosclerotic plaque are widely considered to reside in the synthetic state.[56,57] Because this study investigates the regulation of SPRR3 in the context of the atherosclerotic plaque, it is optimal to use cells *in vitro* under culture conditions that promote a synthetic phenotype. SM-MHC is a marker of a differentiated/contractile VSMC. As seen in Figure 2 and in Supplemental Figure 2, the VSMCs used in this study express low levels of this protein, suggesting that they are in a relatively synthetic state. Another potential explanation may be that VSMCs within atheromas up-regulate $\alpha1\beta1$ expression. We have observed *in vitro* down-regulation of the integrin $\alpha1$ subunit in some VSMCs after prolonged culture; these cells fail to demonstrate SPRR3 regulation (Supplemental Figure S3A at *http://ajp.amjpathol.org*).[48] It is known that lesions locally express high levels of TGFβ, which is known to up-regulate collagen and surface integrin expression.[58–60] We confirmed this finding by demonstrating that TGFβ increased expression of $\alpha1\beta1$ in cultured VSMCs. Accordingly, VSMCs up-regulated SPRR3 in response to CS more robustly when cultured in the presence of 200 pM TGFβ (Supplemental Figure S3B,C at *http://ajp.amjpathol.org*). We also demonstrated highly localized expression of $\alpha1\beta1$ on VSMCs constituting murine proximal aorta lesions. Hence, one hypothesis is that the atheroma microenvironment (inflammation, altered local compliance, etc), perhaps through increased TGFβ, al-

tered VSMC integrin expression and subsequent ECM/integrin signaling. However, other cytokines also present in this microenvironment may also be responsible for the integrin regulation.[49] Moreover, to further examine if biomechanical stress alone could up-regulate SPRR3, we examined aortas isolated from mice subjected to continuous infusion of angiotensin II (Ang II) for 2 months, which induces systemic hypertension associated with increased medial thickness as compared to saline controls (samples obtained from Dr. Nancy J. Brown, Vanderbilt University).[61] We failed to detect either $\alpha 1\beta 1$ or SPRR3 expression in arterial wall VSMCs in treated or control aortas (Supplemental Figure S4 at *http://ajp.amjpathol.org*). Together, the data suggest that biomechanical stimulation alone *in vivo* or *in vitro* was not sufficient for upregulation of SPRR3 transcript and protein expression in the absence of VSMC $\alpha 1\beta 1$ integrin expression. A model of this proposed mechanism is shown in Figure 6. Nevertheless, the atherosclerotic plaque is a complex tissue with multiple variables that may affect the transcription of SPRR3. While many excellent studies have highlighted integrin/matrix interactions in regulating the physiology and gene expression in VSMCs, to our knowledge our study is among the first to delineate a direct relationship between transcription of a mechanosensitive target gene and specific integrin/ECM signaling.

The function of SPRR3 in VSMCs within atheromas remains unclear. The architecture of SPRR3, which is transglutaminated via its N- and C-terminal domains to other structural proteins, including other SPRRs, is believed to play a central role in the barrier and stress function of the cornified envelope.[8] The central core of SPRR3 is considered to have virtually no secondary structure, a vital characteristic in its function as a flexible cross-bridge between its binding partners.[62,63] SPRR3 is a substrate for transglutaminase types I and III, which are expressed in murine vessels.[7,62,63] Interestingly, other studies have linked increased transglutaminase activity to VSMCs and sites of atherosclerosis,[64,65] although no reports are available on the relationship between SPRR3 (or any substrates) and transglutaminase gene family in VSMCs. It is tempting to speculate that SPRR3 may serve as a crosslinking substrate for transglutaminase to stabilize the cytoarchitecture of VSMCs, although the relationship between SPRR3 and transglutaminases in the vasculature remains unexamined. Our data showing that SPRR3 is exclusively enriched in VSMCs within atheromas in response to mechanical stress implies that it may play a role in altered biomechanical compliance of the smooth muscle cell within an atheromatous lesion. Efforts in our lab are currently underway to better understand the role of SPRR3 within VSMCs using gene deletion models and identifying its crosslinked partners.

It has been reported that early atherosclerotic lesions (undetectable by angiography but evident by intravascular ultrasound and postmortem histopathology) result in significantly reduced aortic compliance in a hyperlipidemic rabbit model.[2] These data have been confirmed by other studies by multiple groups.[66–68] One mechanism by which even an early atheroma may alter local biomechanics is from the direct effect of cholesterol on VSMC membrane fluidity. Alternatively, alterations of the physical properties of the ECM can be transduced to VSMCs.[69–71] While much data support that atherosclerosis itself alters vessel compliance and, thereby, biomechanical properties, the molecular changes within VSMCs in response to altered local biomechanics are poorly understood. This paper elucidates the mechanism by which SPRR3 is regulated by mechanotransduction in VSMCs within the context of the microenvironment of the atherosclerotic plaque.

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