THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 35, pp. 21629–21641, August 28, 2015

Expression of V3 Versican by Rat Arterial Smooth Muscle Cells Promotes Differentiated and Anti-inflammatory Phenotypes*[S]

Received for publication, April 9, 2015, and in revised form, June 24, 2015 Published, JBC Papers in Press, July 7, 2015, DOI 10.1074/jbc.M115.657486

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Background: Arterial smooth muscle cells (ASMCs) undergo phenotypic changes during pathophysiological processes.
Results: V3 expression increases contractile SMC markers while decreasing a broad range of proinflammatory chemokines and transcription factors.
Conclusion: V3 expression reprograms rat ASMCs promoting differentiated and anti-inflammatory phenotypes.
Significance: Modifying ECM components via V3 expression could provide a potential therapeutic intervention against vascular and other diseases.

Arterial smooth muscle cells (ASMCs) undergo phenotypic changes during development and pathological processes *in vivo* and during cell culture *in vitro*. Our previous studies demonstrated that retrovirally mediated expression of the versican V3 splice variant (V3) by ASMCs retards cell proliferation and migration *in vitro* and reduces neointimal thickening and macrophage and lipid accumulation in animal models of vascular injury and atherosclerosis. However, the molecular pathways induced by V3 expression that are responsible for these changes are not yet clear. In this study, we employed a microarray approach to examine how expression of V3 induced changes in gene expression and the molecular pathways in rat ASMCs. We found that forced expression of V3 by ASMCs affected expression of 521 genes by more than 1.5-fold. Gene ontology analysis showed that components of the extracellular matrix were the most significantly affected by V3 expression. In addition, genes regulating the formation of the cytoskeleton, which also serve as markers of contractile smooth muscle cells (SMCs), were significantly up-regulated. In contrast, components of the complement system, chemokines, chemokine receptors, and transcription factors crucial for regulating inflammatory processes were among the genes most down-regulated. Consistently, we found that the level of myocardin, a key transcription factor promoting contractile SMC phenotype, was greatly increased, and the pro-inflammatory transcription factors NFκB1 and CCAAT/enhancer-binding protein β were significantly attenuated in V3-expressing SMCs. Overall, these findings demonstrate that V3 expression reprograms ASMCs promoting differentiated and anti-inflammatory phenotypes.

Versican is a large chondroitin sulfate (CS)[3] proteoglycan that is present at low levels in quiescent tissues but accumulates during embryonic development, inflammation, cancer, cardiovascular, and other diseases (1–6). Versican expression is induced by a number of inflammatory stimuli (7, 8) and has been shown to play a key role in leukocyte adhesion (9, 10). Versican exists in at least four major splice variants in humans as follows: V0, V1, V2, and V3. V3, the smallest splice variant, encodes a polypeptide that lacks the glycosaminoglycan binding domain, and thus contains no CS chains. Because versican CS chains can interact with a number of inflammatory mediators, such as MCP1, CD44, PSGL-1, and TLR2 (11–15), and as V3 lacks ADAMTS cleavage sites present in the larger isoforms of versican, V3 has been expected to behave very differently from the larger isoforms. We and others have shown that V3 expression induces significant changes in cell phenotypes. For example, V3 expression significantly decreased proliferation of melanoma cells and cardiomyocytes (16–19) and increased cell-cell association (19). In melanoma cells, this reduction in cell proliferation was partly due to the inhibition of epidermal growth factor (EGF)-dependent signaling pathways (16, 17). In Splotch mice, which have mutations in the *Pax3* gene, versican overexpression was associated with defective neural crest migration characterized by altered expression of V3 (20, 21). In chondrocytes, V3 expression reduced the expression of aggrecan, the principal proteoglycan present in cartilage (22). Simi-

* This work was supported, in whole or in part, by National Institutes of Health Grants EB012558, HL098067, and HL106967 and a Pilot Project under Grant AI101990 (to T. N. W.) and Grants GM103342 and GM103499 (to W. S. A. and J. L. B.). This work was also supported by Medical University of South Carolina through the University Research Resource Facility program to (to W. S. A. and J. L. B. and MUSC Proteogenomics Facility). The authors declare that they have no conflicts of interest with the contents of this article.
† Deceased.
[S] This article contains supplemental Fig. 1 and Table 1.
[1] Supported by the Ann Ramsay-Jenkins and William M. Jenkins Fellowship for Matrix Biology.
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[3] The abbreviations used are: CS, chondroitin sulfate; V3, smallest splice variant of versican; ASMC, arterial smooth muscle cell; ECM, extracellular matrix; FDR, false discovery rate; GO, gene ontology; TRE, transcription factor-response elements; qPCR, quantitative PCR; SMA, smooth muscle α-actin; SRF, serum-response factor; SMC, smooth muscle cell.

larly, we have shown that controlled expression of V3 alters phenotypes of fibroblasts and arterial smooth muscle cells (ASMCs) such that cell growth and migration are reduced while cell adhesion is enhanced (23–25). Moreover, V3 expression in ASMCs induced a significant change in the extracellular matrix (ECM) composition, including a reduction of the larger isoforms of versican, V0/V1, and hyaluronan as well as corresponding hyaluronan synthase expression, although elastic fiber deposition was greatly enhanced (23–25). Furthermore, V3-expressing ASMCs exhibited a reduced capacity to bind monocytes *in vitro* and *in vivo* (26) by altering TGFβ-, EGF-, and NFκB-dependent signaling pathways (27). However, the molecular pathways by which V3 expression induces phenotypic switches in ASMCs, and the key mediator(s) that promote such changes in cell behavior, are not known.

In this study, a microarray analysis was employed to provide an unbiased, top-down approach to examine the changes induced by V3 expression in global gene expression in ASMCs. Our findings indicate that V3 expression reprograms ASMCs to a differentiated, anti-inflammatory phenotype.

Experimental Procedures

Cell Culture—Fischer rat ASMCs were obtained and cultured as described previously (28). Retrovirally transduced cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, glutamine, and penicillin/streptomycin (Invitrogen). Cells were used between 4 and 8 passages after the initial transduction.

Expression of V3 in ASMCs—The versican V3 splice variant was expressed in Fischer rat ASMCs using retroviral vectors, as described previously (23–25). Briefly, the rat V3 sequence was inserted into the BamHI site of the retroviral vector LXSN (courtesy of Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA). The retroviral vector containing the V3 coding sequence (LV3SN), as well as the empty vector control (LXSN), were used to infect Fischer rat ASMCs using PA317 packaging cells as described previously (24, 28).

Microarray—Total RNA was first isolated by the Single Step Method by solubilizing the cells cultured for 3 weeks in solution D and 2.0 M sodium acetate (29). The soluble phase from subsequent phenol/chloroform/isoamyl alcohol extraction was precipitated with isopropyl alcohol. Pelleted RNA was further purified using an Agilent total RNA isolation mini kit (Agilent Technologies) according to the manufacturer's specifications. Microarray analysis was performed at the Medical University of South Carolina Proteogenomics Facility (Charleston, SC) using established methods (30–32). Briefly, total RNA quality was evaluated by Agilent 2100 Bioanalyzer, and samples were then converted to biotin-labeled, fragmented cRNA using the 3′ IVT Express Plus kit (Affymetrix). Hybridizations were performed using Rat Genome 230A GeneChips (Affymetrix), and post-hybridization washing, staining, and scanning were done with the Affymetrix Hybridization, Wash and Stain kit, and a 7G scanner according to Affymetrix protocols. Raw data (CEL files) were normalized by Robust Multichip Average (RMA) (33) using Affymetrix Expression Console software. Significant difference in gene expression was defined by absolute fold change of >1.5 and $p < 0.05$ (Student's paired t test). Using these criteria, the false discovery rate (FDR), estimated by iterative permutation of sample groupings, was estimated to be ~1.7%. Gene ontology (GO) analysis and functional annotation clustering were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.7 (NIAID, National Institutes of Health). The total probe list of the microarray was used as the background gene set for the analysis. Microarray data collected for this study have been deposited in NCBI Gene Expression Omnibus in accordance with MIAME conventions (34) under accession number GSE66624.

Transcriptional Regulation Element Promotor Analysis and Interaction Network—Over-represented transcription factor response elements (TRE) were identified within promoter sequences (500 bp upstream of transcription start site(s)) of significantly up- and down-regulated genes using the PAINT version 4.0 (35, 36). For the 261 genes that showed significant down-regulation, 232 corresponding unique upstream sequences were found, with 164 TREs detected based on a core similarity threshold of 1.0. For the 260 genes that showed robust up-regulation, 176 unique upstream sequences were found, with 167 detected TREs. TRE enrichment was determined based on a p value threshold of 0.05 and an FDR threshold of 0.3, relative to the default PAINT reference set of randomly selected *Rattus norvegicus* promoters.

Quantitative Polymerase Chain Reaction (qPCR)—The microarray detected gene expression in ASMCs cultured for 3 weeks at which time elastin deposition was maximal. To confirm whether the differential gene expression was present in V3-expressing ASMCs at earlier time points, qPCR was performed on ASMCs cultured over a time course. DNA-free RNA was obtained from control LXSN- or V3-expressing ASMCs cultured for 1, 4, 7, and 10 days using the total RNA isolation kit from Agilent Technologies, and cDNA was prepared from the isolated RNA by reverse transcription using random primers with a high capacity cDNA archive kit from Applied Biosystems. Real time PCR was carried out using an Applied Biosystems Prism 7500 sequence detection system and a SYBR Green or TaqMan Universal PCR master mix kit from Applied Biosystems as directed by the manufacturer. Primers used with SYBR Green reagents are listed in Table 1. For each group, assays were run on samples isolated from 3 to 4 replicates using the relative standard curve method (Applied Biosystems).

Cytokine ELISA—Conditioned media from control LXSN or V3-expressing ASMCs cultured for 3 days were filtered through 0.22-μm pore syringe filters and spun at 3000 × g for 5 min at 4 °C. Cytokine levels in the conditioned media were quantified by Rat Cytokine/Chemokine 27 Plex Discovery Array (Eve Technologies).

Western Blotting—Cytoplasmic proteins were isolated from control or V3-expressing ASMCs grown for 7 days using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer's protocols. Total protein concentration was determined by BCA assay (Thermo Scientific). 30 μg of total protein from cytoplasmic extracts was run on 8% SDS-PAGE and transferred to a nitrocellulose membrane at 10 V constant voltage overnight at 4 °C. Nitrocellulose membranes were blocked in 2% BSA in Tris-buffered saline

TABLE 1
List of primers

Gene	Forward (5′)	Reverse (3′)
Smooth muscle α-actin (*Acta2*)	TAACGGAGGCGCCGCTGAAC	GGGACAGCACAGCCTGAATAGCC
Calponin (*Cnn1*)	CGGGAGCAACTTCATGGATGGCC	AGATCCCGGCTGGAGCTTGTTGA
Myocardin (*Myocd*)	ACGAGAAGATTGCTCTCCGCCCA	TGCTGCTTTGGAGAGGCTCACCT
Complement component 3 (*C3*)	TGAGCCTGGAGTGGACTACG	TCATCTGAGCCTGACTTGATGAC
Ccl2	CTGTCACGCTTCTGGGCCTGT	GCAGCAGGTGAGTGGGGCAT
Cxcl1	ACTCAAGAATGGTCGCGAGG	ACGCCATCGGTGCAATCTAT
Ccl20	GCACTGAGCAGATCAATTCCTG	TGTGTACGTGAGGCAGCAGT
Cxcl5	CATTGCCCCAAGGTGGAAGTC	GTGCATTCCGCTTTGTTTTCTT
CCAAT/enhancer binding protein (C/EBP), β (*Cebpb*)	CTTCGAACCCGCGGACTG	GACGACGACGTGGACAGG
Nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (*Nfkb1*)	TGTCAACAGATGGCCCATACC	GCACACGTAGCGGAATCGAA
Focal adhesion kinase 1 (*Fak*)	TGAACGGAGCCACGCAGTCC	ACTGCATGTGTCCGCATGCCTT
Focal adhesion related non-kinase (*Frnk*)	GTGCAGCAAGAAGAACGGATG	GGACCTCGGGCTAGGATAAC

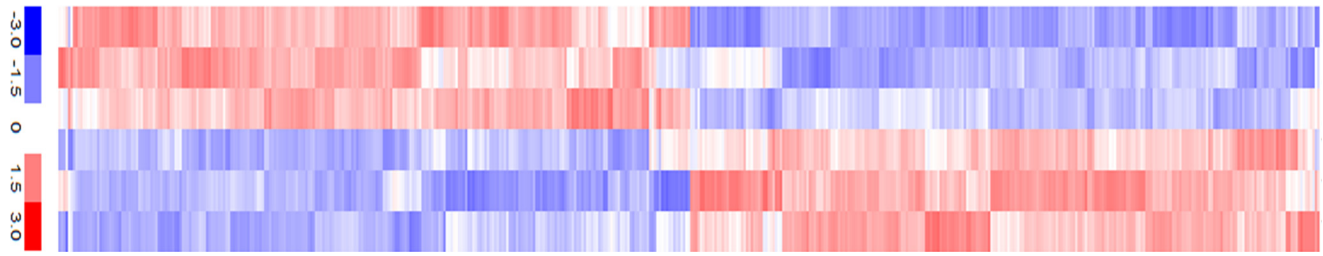

FIGURE 1. **Heat map of 521 genes found to be differentially expressed between control (empty vector LXSN) and V3-transduced rat ASMCs.** Up-regulated genes are shown in *red,* and down-regulated genes are in *blue*. The criteria for inclusion of genes in the differentially expressed gene set were fold change >1.5 and $p < 0.05$ in paired *t* test. FDR was 1.7%.

with 0.1% Tween 20 and incubated with polyclonal antibodies against the C-terminal domain of *Fak* (EMD Millipore; formerly Upstate), which detects both *Fak* and *Frnk* (37), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:80,000). Membranes were developed with a chemiluminescent substrate, West Dura (Pierce Biotech). Densitometry was performed using ImageJ version 1.44 from the National Institutes of Health.

Statistical Analysis—All data are expressed as the average ± S.E., unless otherwise specified. Differences were identified by two-tailed Student's *t* tests for the comparison of two groups and by one-way analysis of variance followed by Tukey's post hoc tests for the comparison of three or more groups and were regarded significant if $p < 0.05$.

Results

Expression of Versican V3 Significantly Alters Gene Expression Profiles in Rat ASMCs—DNA microarray analysis was performed on RNA isolated from primary rat ASMCs retrovirally transduced with empty vector control (LXSN) and V3-containing vector (LV3SN) and maintained in culture for 3 weeks. The 3-week period was chosen because it is the period of time required for prominent accumulation of elastin. Analysis of microarray data identified 521 genes that were significantly differentially expressed in ASMCs following V3 transduction (supplemental Table 1; Fig. 1). A subset of genes showing the highest magnitude changes is shown in Table 2. Among the genes most up-regulated were components of actin cytoskeleton such as *Acta1*, *Myh10*, and *Myl9*, and ECM components, including *Fbln5*, *Adamts9*, and osteoglycin, a small leucine-rich proteoglycan. Among the most down-regulated genes were complement component 3 and inflammatory cytokine genes such as *Cxcl1*, *Ccl20*, and *Ccl2*.

V3 Expression Affects the Extracellular Microenvironment—To examine the biological processes influenced by V3 expression, genes significantly affected in V3-transduced cells were subjected to functional enrichment analysis based on their GO annotations. As shown in Table 3, the processes most significantly enriched by V3 were linked with extracellular region, extracellular space, and ECM. Table 4 shows expression data for significantly regulated genes categorized as extracellular region (GO:0005576). Review of the genes, their related functions, and their changes in expression revealed a dynamic and complex alteration of the extracellular microenvironment in response to V3. Some ECM components were up-regulated (fibulin 5, elastin, laminin β1, and SPARC-related molecular calcium binding 1 (*Smoc1*)), whereas others were down-regulated (versican, cartilage oligomeric matrix protein, and proline arginine-rich end leucine-rich repeat protein (*Prelp*) and decorin). The levels of V0/V1 were significantly down-regulated by expressing V3 when examined in control and V3 overexpressing ASMCs from four independent transductions. In comparison, the levels of V3 expression achieved by transduction were greater than 10 times the V0/V1 levels naturally expressed in the control ASMCs (supplemental Fig. 1). Some proteases and protease inhibitors were up-regulated (*Adamts9* and carboxypeptidase E), although others were down-regulated (*Timp1*, ectonucleotide pyrophosphatase/phosphodiesterase 2, secretory leukocyte protease inhibitor, and extracellular proteinase inhibitor). Some genes involved in ECM-cell interactions were up-regulated (vinculin) and others were down-regulated (dystroglycan 1). This response of up- and down-regulation of genes within specific functional groups was also seen for several other categories, including cell-cell adhesion, growth factors, and hormones, and the coagulation and complement cascades. However, the chemokine category was nota-

TABLE 2
Genes most up-regulated and down-regulated by V3 expression

Gene title	Gene symbol	Probe set ID	Paired *p* value	-Fold change
Most up-regulated genes				
Actin, α-1, skeletal muscle	*Acta1*	1369928_at	0.015	11.71
Osteoglycin	*Ogn*	1383263_at	0.036	9.00
Osteoglycin	*Ogn*	1376749_at	0.034	8.40
Amine oxidase, copper containing 3 (vascular adhesion protein 1)	*Aoc3*	1372615_at	0.005	8.11
Similar to glypican-6 precursor	*Gpc6*	1372820_at	0.011	7.36
Spermatogenesis-associated, serine-rich 2-like	*Spats2l*	1388791_at	0.033	7.06
A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9	*Adamts9*	1376481_at	0.041	6.32
Transmembrane protein 204	*Tmem204*	1376623_at	0.015	5.94
Fibulin 5 /// thyroid hormone receptor interactor 11	*Fbln5 /// Trip11*	1367866_at	0.018	5.58
Calsequestrin 2 (cardiac muscle)	*Casq2*	1368988_at	0.026	5.58
Guanylate cyclase 1, soluble, β3	*Gucy1b3*	1369097_s_at	0.020	5.24
Olfactomedin-like 2B	*Olfml2b*	1389295_at	0.015	5.10
Myosin, heavy chain 10, non-muscle	*Myh10*	1370158_at	0.027	4.92
Calsequestrin 2 (cardiac muscle)	*Casq2*	1387401_at	0.032	4.59
Most down-regulated genes				
Complement component 3	*C3*	1368000_at	0.001	−114.56
Extracellular proteinase inhibitor	*Wfdc18*	1387715_at	0.001	−110.66
Chemokine (CXC motif) ligand 1	*Cxcl1*	1387316_at	0.011	−39.95
Lipopolysaccharide-binding protein	*Lbp*	1387868_at	0.033	−30.48
Chemokine (C-C motif) ligand 20	*Ccl20*	1369814_at	0.008	−28.05
Retinol-binding protein 1, cellular	*Rbp1*	1367939_at	0.003	−25.11
Chemokine (C-C motif) ligand 2	*Ccl2*	1367973_at	0.010	−24.08
Serpin peptidase inhibitor, clade G, member 1	*Serping1*	1372254_at	0.017	−18.00
Argininosuccinate synthase 1	*Ass1*	1370964_at	0.009	−17.27
Carcinoembryonic antigen-related cell adhesion molecule 1 /// 10	*Ceacam1 /// Ceacam10*	1370371_a_at	0.014	−16.34
Lipocalin 2	*Lcn2*	1387011_at	0.001	−14.42
Superoxide dismutase 2, mitochondrial	*Sod2*	1370173_at	0.001	−11.96
Regulator of G-protein signaling 2	*Rgs2*	1387074_at	0.036	−11.31
Regulator of G-protein signaling 2	*Rgs2*	1368144_at	0.049	−10.06

ble in that all of these genes were down-regulated (lipopolysaccharide-binding protein, angiogenin, *Wnt4*, *Cxcl1, Ccl20, Ccl2, Cxcl6, Cxcl12*, and macrophage migration inhibitory factor). Based on these findings, V3 expression results in extensive remodeling of the extracellular microenvironment and an alteration in the inflammatory status of ASMCs.

V3 Expression Significantly Affects Genes Regulating Cell Survival and Inflammation—To more clearly assess the many processes affected by V3 expression, gene annotations were further analyzed to identify significant groups (clusters) of related processes. This analysis again highlighted that the extracellular environment, cytoskeletal system, and inflammatory status were all influenced by V3 (Table 5). Additionally, this analysis highlighted two clusters of functional groups from the prior analyses as follows: apoptosis/cell death and vascular development. Table 6 shows the changes in expression of genes associated with negative regulation of apoptosis. The gene in this category that was most up-regulated in V3-expressing ASMCs was *Ptk2*, which can encode for Fak or its dominant negative form Frnk. This was intriguing because Fak is a key regulator of many important cellular processes, including cell proliferation, adhesion, motility, and survival (38–42). V3 expression up-regulated other signaling molecules and transcription factors involved in the negative regulation of apoptosis, including myocyte enhancer factor 2C (*Mef2c*), a transcription factor promoting cardiac muscle differentiation and vascular development; cadherin 13, a nonclassical glycosylphosphatidylinositol-anchored transmembrane cadherin negatively regulating EGF signaling and a potential tumor suppressor; *Cd59*, a regulator of complement-mediated cell lysis; and Rho kinase (*Rock1*), a key regulator of the actin cytoskeleton promoting contractile force generation. *Tcf4* was increased by 2-fold in V3-expressing ASMCs, whereas *Wnt4* was decreased by 2.2-fold, and recombination signal-binding protein for immunoglobulin κJ region (*Rbpj*), a transcription factor activated by Notch signaling, was decreased 1.74-fold, indicating that the Wnt/β-catenin as well as Notch signaling pathways are also affected by V3 expression. Notably, *Smad3* was decreased by 2.4-fold in V3-expressing ASMCs, indicating that the TGFβ signaling pathway is affected by V3 expression. Many ECM-degrading enzymes, cytokines, and growth factors discussed above that are also involved in cell survival are included within this GO. A number of key regulators of apoptosis were down-regulated by V3 expression, including caspase 3, cell death-inducing DNA fragmentation factor α subunit-like effector A, clusterin, and *Bcl2*. Interestingly, the antioxidant enzymes peroxiredoxin 5 and superoxide dismutase 2 were down-regulated by V3 expression, which may reflect reduced metabolic burden of these cells after 3 weeks of culture because V3 expression significantly reduced proliferation. Proinflammatory transcription factors such as *Nfkb1* and CCAAT/enhancer-binding protein β (*Cebpb*) were also decreased by 1.9- and 2.6-fold, respectively, in V3-expressing ASMCs.

The expression patterns of several genes involved in apoptosis were interrogated by qPCR at days 1, 4, 7, and 10 after V3 transduction. Expression levels of *Cebpb* and *Nfkb1* in V3-expressing ASMCs were lower than those of control ASMCs at day 1, but the difference was not statistically significant (Fig. 2, $p = 0.22$ and 0.21, respectively). However, these levels increased in control ASMCs but were significantly reduced in V3-expressing ASMCs at days 4, 7, and 10. Expression levels of caspase 3 and *Rela* were also significantly reduced in V3-ex-

TABLE 3
DAVID functional annotation chart

Term	Count	%	*p* value	Benjamini
Extracellular region part	51	11.62	1.16E-07	2.05E-05
Extracellular region	74	16.86	6.68E-08	2.35E-05
Extracellular space	37	8.43	1.52E-05	0.002
Extracellular matrix	21	4.78	1.11E-04	0.010
Negative regulation of cell death	30	6.83	2.88E-05	0.015
Negative regulation of programmed cell death	30	6.83	2.70E-05	0.018
Response to hypoxia	22	5.01	2.69E-05	0.024
Response to oxygen levels	22	5.01	6.27E-05	0.027
Negative regulation of apoptosis	30	6.83	2.36E-05	0.031
LIM	9	2.05	2.55E-04	0.039
Response to wounding	34	7.74	1.22E-04	0.045
LIM domain	9	2.05	1.58E-04	0.053
Regulation of endothelial cell proliferation	9	2.05	2.18E-05	0.056
Soluble fraction	25	5.69	8.31E-04	0.057
Proteinaceous extracellular matrix	17	3.87	1.31E-03	0.064
Cell fraction	56	12.76	1.18E-03	0.067
Regulation of programmed cell death	44	10.02	2.70E-04	0.069
Regulation of apoptosis	44	10.02	2.21E-04	0.071
Regulation of cell death	44	10.02	3.06E-04	0.071
Negative regulation of cell differentiation	19	4.33	2.66E-04	0.076
Zinc finger, LIM-type	9	2.05	1.44E-04	0.102
Positive regulation of endothelial cell proliferation	6	1.37	7.09E-04	0.126
Anti-apoptosis	16	3.64	7.00E-04	0.134
Secreted	46	10.48	8.43E-04	0.135
Regulation of smooth muscle cell proliferation	10	2.28	6.92E-04	0.142
Regulation of cell proliferation	42	9.57	8.97E-04	0.147
Heart development	19	4.33	9.75E-04	0.150
Regulation of cell migration	16	3.64	1.21E-03	0.164
Peptide metabolic process	9	2.05	1.21E-03	0.172
Regulation of locomotion	17	3.87	1.69E-03	0.177
Inflammatory response	19	4.33	1.62E-03	0.178
Actin filament organization	9	2.05	1.81E-03	0.182
Response to endogenous stimulus	39	8.88	1.61E-03	0.185
Regulation of cell motion	17	3.87	1.47E-03	0.186
Fat cell differentiation	9	2.05	1.59E-03	0.191
Negative regulation of cell proliferation	21	4.78	2.19E-03	0.208
Antigen processing and presentation	8	1.82	2.43E-03	0.213
Muscle cell differentiation	13	2.96	2.35E-03	0.214
Egf-like domain	13	2.96	2.83E-03	0.217
Cleavage on pair of basic residues	15	3.42	4.43E-03	0.225
Inflammatory response	7	1.59	2.29E-03	0.231
Positive regulation of epithelial-to-mesenchymal transition	4	0.91	2.77E-03	0.231
Response to lipopolysaccharide	13	2.96	2.99E-03	0.240
Actin filament-based process	16	3.64	3.18E-03	0.246
Actomyosin	6	1.37	6.44E-03	0.247
Positive regulation of transport	19	4.33	3.81E-03	0.252
Blood vessel morphogenesis	16	3.64	3.39E-03	0.253
Brown fat cell differentiation	6	1.37	3.76E-03	0.255
Iron transport	4	0.91	4.31E-03	0.258
Response to bacterium	16	3.64	3.61E-03	0.260
Positive regulation of response to external stimulus	9	2.05	3.72E-03	0.260
Vascular smooth muscle contraction	13	2.96	4.25E-03	0.261
Regulation of epithelial to mesenchymal transition	4	0.91	4.28E-03	0.265
Positive regulation of cell morphogenesis involved in differentiation	4	0.91	4.28E-03	0.265
Blood vessel development	18	4.10	4.40E-03	0.266
Protein homo-oligomerization	11	2.51	4.25E-03	0.270
Cytoskeleton organization	22	5.01	4.61E-03	0.270
Response to molecule of bacterial origin	13	2.96	5.04E-03	0.285
Response to organic substance	53	12.07	5.96E-03	0.287
Vasculature development	18	4.10	5.73E-03	0.288
Actin cytoskeleton organization	15	3.42	5.91E-03	0.290
Response to hormone stimulus	34	7.74	5.28E-03	0.291
Positive regulation of smooth muscle cell proliferation	7	1.59	5.67E-03	0.291
Defense response	24	5.47	5.44E-03	0.292
Immune response	23	5.24	5.61E-03	0.294

pressing ASMCs, although *Rela* was statistically significant only at day 4 ($p = 0.004$, Fig. 2). These findings indicate that V3 expression prevents the increase of pro-apoptotic and pro-inflammatory transcription factors during cell culture.

Table 7 shows the expression changes of genes regulating response to wounding, many of which overlap with the genes listed in Table 5, including components of the complement cascades, growth factors, and cytokines. Notably, retinoid X receptor α was increased by 3.36-fold, which suggests that the elastogenic effect of V3 may be partially dependent on retinoic acid receptor-dependent signaling pathways that have also been shown to promote elastogenesis, especially in the lungs (43, 44). *Notch3* was increased by 2.48-fold, along with the decrease of *Rbpj* shown in Table 6, indicating that V3 affects Notch signaling pathways. Moreover, components of the cytoskeletal network, such as myosin heavy chain 10, tropomyosin α, and microtubule-associated protein 1B were also increased by 4.92-, 2.69-, and 2.06-fold in V3-expressing ASMCs.

TABLE 4
Genes in extracellular region (GO:0005576) affected by V3 expression

Categories	ID	Gene name	Fold change
ECM	1367866_at	Fibulin 5	5.58
	1388111_at	Elastin	3.36
	1388545_at	SPARC-related modular calcium-binding 1	1.92
	1373210_at	Laminin, β1	1.87
	1387137_at	Cartilage oligomeric matrix protein	−2.27
	1388142_at	Versican	−2.50
	1372647_at	Proline arginine-rich end leucine-rich repeat protein	−3.29
	1370956_at	Decorin	−3.63
	1387886_at	Proline arginine-rich end leucine-rich repeat protein	−4.11
Proteases/protease inhibitors	1376481_at	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 9	6.32
	1367835_at	Proprotein convertase subtilisin/kexin type 1 inhibitor	3.46
	1386921_at	Carboxypeptidase E	2.45
	1392773_at	Proprotein convertase subtilisin/kexin type 5	2.06
	1367998_at	Similar to secretory leukocyte protease inhibitor	−1.96
	1367712_at	TIMP metallopeptidase inhibitor 1	−2.38
	1387715_at	Extracellular proteinase inhibitor	−110.66
ECM-cell interactions	1372905_at	Vinculin	1.79
	1375538_at	Vinculin	1.69
	1371430_at	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	−1.53
	1389105_at	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	−1.83
Cell-cell interactions	1373102_at	Cadherin 13	2.48
	1387202_at	Intercellular adhesion molecule 1	−3.78
Growth factors/hormones	1368677_at	Brain-derived neurotrophic factor	2.17
	1367859_at	Transforming growth factor, β3	2.16
	1367949_at	Proenkephalin 1	2.22
	1370082_at	Transforming growth factor, β1	−1.65
	1373807_at	Vascular endothelial growth factor A	−2.16
	1387450_at	Transforming growth factor α	−2.23
	1370081_a_at	Vascular endothelial growth factor A	−2.66
	1387219_at	Adrenomedullin	−2.66
	1377163_at	Inhibin β-B	−3.16
	1372750_at	Follistatin	−5.66
	1387843_at	Follistatin	−5.78
Coagulation and complement cascade components	1367929_at	CD59 molecule, complement regulatory protein	2.48
	1383241_at	Complement component 1, r subcomponent	−1.78
	1370892_at	Complement component 4B (Childo blood group)	−1.84
	1387893_at	Similar to complement component 1, s subcomponent; complement component 1, s subcomponent	−2.31
	1369182_at	Coagulation factor III (thromboplastin, tissue factor)	−2.91
	1372254_at	Serine (or cysteine) peptidase inhibitor, clade G, member 1	−18.00
	1368000_at	Complement component 3	−114.56
Chemokines	1367609_at	Macrophage migration inhibitory factor	−1.93
	1387655_at	Chemokine (CXC motif) ligand 12 (stromal cell-derived factor 1)	−3.32
	1388583_at	Chemokine (CXC motif) ligand 12 (stromal cell-derived factor 1)	−4.14
	1387648_at	Chemokine (CXC motif) ligand 6 (granulocyte chemotactic protein 2)	−6.19
	1367973_at	Chemokine (C-C motif) ligand 2	−24.08
	1369814_at	Chemokine (C-C motif) ligand 20	−28.05
	1387316_at	Chemokine (CXC motif) ligand 1 (melanoma growth stimulating activity, α)	−39.95
Others	1390075_at	Olfactomedin-like 2B	3.48
	1376575_at	Twisted gastrulation homolog 1 (*Drosophila*)	2.95
	1376457_at	Cysteine-rich secretory protein LCCL domain containing 2	2.81
	1370907_at	ST6 β-galactosamide α-2,6-sialyltranferase 1	2.71
	1367687_a_at	Peptidylglycine α-amidating monooxygenase	2.22
	1387772_at	Calmodulin pseudogene 2; calmodulin 3; calmodulin 2; calmodulin 1	1.88
	1369936_at	Calmodulin pseudogene 2; calmodulin 3; calmodulin 2; calmodulin 1	1.71
	1387127_at	Attractin	1.66
	1370307_at	Agrin	1.65
	1374703_at	Chromobox homolog 6; neuronal pentraxin receptor	1.60
	1369186_at	Caspase 1	−1.58
	1393427_s_at	EGF-like domain, multiple 7	−1.62
	1387027_a_at	Lectin, galactoside-binding, soluble, 9	−1.69
	1388924_at	Angiopoietin-like 4	−1.83
	1373850_at	Sphingomyelin phosphodiesterase	−1.85
	1389911_at	Meteorin, glial cell differentiation regulator-like	−1.89
	1369906_s_at	Multiple coagulation factor deficiency 2	−2.07
	1376435_at	Lysyl oxidase-like 4	−2.16
	1368641_at	Wingless-type MMTV integration site family, member 4	−2.16
	1390326_at	Angiogenin, ribonuclease A family, member 1	−2.17
	1372301_at	AE-binding protein 1	−2.28
	1368441_at	Mesothelin	−2.31
	1368943_at	Ribonuclease, RNase A family 4	−2.31
	1371462_at	Insulin-like growth factor binding protein 4	−2.53
	1370862_at	Apolipoprotein E	−2.57
	1386879_at	Lectin, galactoside-binding, soluble, 3	−2.93
	1367784_a_at	Clusterin	−2.95
	1370012_at	Prostaglandin I2 (prostacyclin) synthase	−3.29
	1368128_at	Phospholipase A2, group IIA (platelets, synovial fluid)	−3.53
	1368536_at	Ectonucleotide pyrophosphatase/phosphodiesterase 2	−3.78
	1374726_at	Fibronectin type III domain containing 1	−3.97
	1387835_at	Interleukin 1 receptor antagonist	−7.06
	1387011_at	Lipocalin 2	−14.42
	1387868_at	Lipopolysaccharide-binding protein	−30.48

TABLE 5
DAVID functional annotation clustering

Term	Count	*p* value	Benjamini
Cell death/apoptosis: enrichment score 2.903			
Negative regulation of apoptosis	30	2.36E-05	0.031
Negative regulation of programmed cell death	30	2.70E-05	0.018
Negative regulation of cell death	30	2.88E-05	0.015
Regulation of apoptosis	44	2.21E-04	0.071
Regulation of programmed cell death	44	2.70E-04	0.069
Regulation of cell death	44	3.06E-04	0.071
Anti-apoptosis	16	7.00E-04	0.134
Extracellular region: enrichment score 2.888			
Extracellular region	74	6.68E-08	2.35E-05
Extracellular region part	51	1.16E-07	2.05E-05
Extracellular space	37	1.52E-05	0.002
Secreted	46	8.43E-04	0.135
Extracellular matrix: enrichment score 2.821			
Extracellular matrix	21	1.11E-04	0.010
Proteinaceous extracellular matrix	17	0.001	0.064
Inflammatory response: enrichment score 2.596			
Response to wounding	34	1.22E-04	0.045
Inflammatory response	19	0.002	0.178
Defense response	24	0.005	0.292
Immune response	23	0.006	0.294
Actin cytoskeleton: enrichment score 2.451			
Actin filament organization	9	0.002	0.182
Actin filament-based process	16	0.003	0.246
Cytoskeleton organization	22	0.005	0.270
Actin cytoskeleton organization	15	0.006	0.290
Vascular development: enrichment score 2.129			
Blood vessel morphogenesis	16	0.003	0.253
Blood vessel development	18	0.004	0.266
Vasculature development	18	0.006	0.288

TABLE 6
Genes involved in negative regulation of apoptosis (GO:0043066) affected by V3 expression

ID	Gene name	-Fold change
1387875_at	*Ptk2* protein-tyrosine kinase 2 (Fak1, Frnk)	3.10
1372332_at	Myocyte enhancer factor 2C	3.10
1373102_at	Cadherin 13	2.87
1367929_at	*Cd59* molecule, complement regulatory protein	2.48
1368677_at	Brain-derived neurotrophic factor	2.17
1367859_at	Transforming growth factor, β3	2.16
1377156_at	Similar to transcription factor 7-like 2, T-cell specific, HMG-box (*Tcf-4*)	1.95
1372728_at	Sortilin 1	1.77
1368932_at	Rho-associated coiled-coil containing protein kinase 1 (*Rock1*)	1.59
1374227_at	Tetratricopeptide repeat domain 27; baculoviral IAP repeat-containing 6	1.57
1370376_a_at	Cold shock domain protein A	−1.52
1387690_at	Caspase 3, apoptosis related cysteine protease	−1.59
1379302_at	Recombination signal binding protein for immunoglobulin κJ region	−1.74
1388924_at	Angiopoietin-like 4	−1.83
1370968_at	Nuclear factor of κ light polypeptide gene enhancer in B-cells 1	−1.91
1367609_at	Macrophage migration inhibitory factor (*Mif*)	−1.93
1389179_at	Cell death-inducing DNA fragmentation factor, α subunit-like effector A	−1.95
1373807_at	Vascular endothelial growth factor A	−2.16
1387450_at	Transforming growth factor α	−2.23
1389127_at	SMAD family member 3	−2.36
1367712_at	TIMP metallopeptidase inhibitor 1	−2.38
1370862_at	Apolipoprotein E	−2.57
1387087_at	CCAAT/enhancer binding protein (C/EBP), β	−2.60
1370081_a_at	Vascular endothelial growth factor A	−2.66
1387805_at	*Bcl2*/adenovirus E1B 19-kDa interacting protein 3	−2.71
1367784_a_at	Clusterin	−2.95
1367677_at	Peroxiredoxin 5	−3.03
1389538_at	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α	−3.23
1387835_at	Interleukin 1 receptor antagonist	−7.06
1370172_at	Superoxide dismutase 2, mitochondrial	−7.73
1370173_at	Superoxide dismutase 2, mitochondrial	−11.96
1367973_at	Chemokine (C-C motif) ligand 2	−24.08

To confirm that V3-expressing ASMCs had a marked reduction in the inflammatory mediators shown in Table 7, we examined transcript levels of *C3, Ccl2, Cxcl1, Ccl20,* and *Cxcl5* from control LXSN or V3-expressing ASMCs cultured for 10 days. As shown in Fig. 3, V3-expressing ASMCs had reduced transcript levels of these inflammatory mediators. Among these, we also examined Ccl2 and Cxcl1 protein levels, verifying that both were significantly lower in conditioned media from V3-expressing ASMCs. These data demonstrate that V3 expression negatively impacts expression of

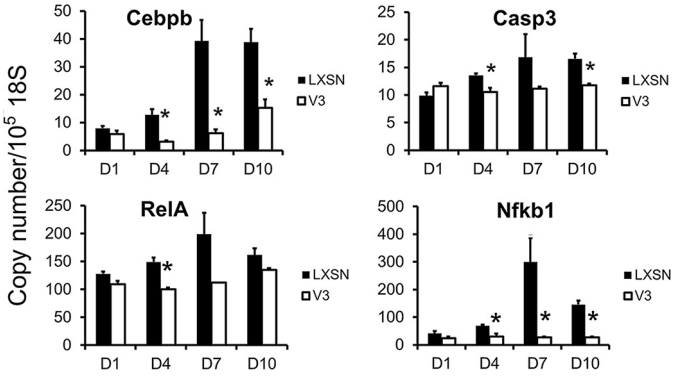

FIGURE 2. **Some genes involved in regulation of apoptosis were validated by qPCR at days 1, 4, 7, and 10 in culture.** *Casp3* was down-regulated at days 4 and 10, and *RelA* was down-regulated at day 4. *Cebpb* and *Nfkb1* showed a robust down-regulation between days 4 and 10. *, $p < 0.05$ in unpaired *t* test.

TABLE 7

Genes involved in response to wounding (GO:0009611) affected by V3 expression

ID	Gene name	-Fold change
1370158_at	Myosin, heavy chain 10, nonmuscle	4.92
1374077_at	Retinoid X receptor α	3.36
1379936_at	Tropomyosin 1, α	2.69
1369329_at	Notch homolog 3 (*Drosophila*)	2.48
1367859_at	Transforming growth factor, β3	2.16
1373363_at	Microtubule-associated protein 1B	2.06
1376082_at	Ecotropic viral integration site 1	2.00
1387127_at	Attractin	1.66
1387690_at	Caspase 3, apoptosis related cysteine protease	−1.59
1370219_at	Cytochrome *b*-245, α polypeptide	−1.59
1370082_at	Transforming growth factor, β1	−1.65
1387893_at	Complement component 1, s subcomponent	−1.78
1370892_at	Complement component 4B (Childo blood group)	−1.84
1367609_at	Macrophage migration inhibitory factor	−1.93
1370281_at	Fatty acid-binding protein 5, epidermal	−2.27
1367712_at	TIMP metallopeptidase inhibitor 1	−2.38
1388142_at	Versican	−2.50
1371462_at	Insulin-like growth factor binding protein 4	−2.53
1370862_at	Apolipoprotein E	−2.57
1387219_at	Adrenomedullin	−2.66
1369182_at	Coagulation factor III (thromboplastin, tissue factor)	−2.91
1367784_a_at	Clusterin	−2.95
1370956_at	Decorin	−3.63
1387648_at	Chemokine (C*X*C motif) ligand 6 (granulocyte chemotactic protein 2)	−6.19
1373654_at	Annexin A8	−6.41
1387835_at	Interleukin 1 receptor antagonist	−7.06
1370172_at	Superoxide dismutase 2, mitochondrial	−7.73
1370173_at	Superoxide dismutase 2, mitochondrial	−11.96
1370964_at	Argininosuccinate synthetase 1	−17.27
1372254_at	Serine (or cysteine) peptidase inhibitor, clade G, member 1	−18.00
1367973_at	Chemokine (C-C motif) ligand 2	−24.08
1369814_at	Chemokine (C-C motif) ligand 20	−28.05
1387868_at	Lipopolysaccharide binding protein	−30.48
1387316_at	Chemokine (C*X*C motif) ligand 1	−39.95
1368000_at	Complement component 3	−114.56

a number of key complement components and inflammatory chemokines.

V3 Expression Promotes Mature, Differentiated ASMC Phenotypes—V3 expression increased the expression of *Mef2c*, which promotes muscle differentiation and influences the actin cytoskeletal network. We therefore sought to examine whether V3 expression in ASMCs promoted a differentiated SMC phenotype. As shown in Fig. 4, V3 expression significantly up-regulated levels of smooth muscle α-actin (SMA) and calponin, which are components of the cytoskeletal network as well as markers of contractile SMCs. V3 expression also up-regulated myocardin, which is a key transcription factor promoting contractile phenotypes of SMCs (45). Notably, V3-expressing ASMCs exhibited increases in these SMC markers at day 1, which persisted up to day 10. These findings indicate that V3 expression increases differentiated and mature SMC markers.

TRE for SRF, Stat1, and NFκB Are Enriched in Promoter Regions of Genes Affected by V3 Expression—To elucidate transcriptional mechanisms by which V3 influenced differential gene expression in ASMCs, we conducted an analysis of TREs enriched in promoters of the 521 genes significantly affected by V3 expression. Among the genes up-regulated by V3 expression (fold change >1.5, $p < 0.05$), the TRE for binding to the serum-response factor (SRF), a key transcription factor promoting contractile SMC phenotypes, was significantly over-represented (Table 8, FDR <0.3). For the genes down-regulated by V3 expression (fold change < -1.5, $p < 0.05$), TREs significantly over-represented included those for binding to Stat1 and NFκB (Table 9, FDR <0.3), indicating that V3 expression blocks these pro-inflammatory transcription factors.

Frnk, but Not Fak, Is Significantly Up-regulated in V3-expressing ASMCs—One of the genes significantly up-regulated by V3 expression was *Ptk2*, which can encode either Fak or its endogenous inhibitor Frnk (Table 6). Fak is one of the key signaling molecules in the focal adhesion complex that is activated via integrin or growth factors, and it further activates downstream signaling pathways mediated by PI3K, MAPK, and/or NFκB, promoting cell proliferation and migration (38–42). Therefore, we further explored whether V3 expression affects Fak or its dominant negative isoform Frnk. As shown in Fig. 5, V3-expressing ASMCs had significantly greater transcript levels of Frnk, but not Fak, at day 1 of culture, which persisted up to day 10. V3-expressing ASMCs had greater amounts of Frnk at protein levels, when examined by Western blotting (Fig. 5*B*). These findings suggest that V3-expressing ASMCs have altered focal adhesion signaling pathways that can further impact other cell phenotypes.

Discussion

In this study, we demonstrated that V3 expression reprograms ASMCs to enhance expression of differentiated, mature SMC markers while repressing components of the complement cascades, inflammatory cytokines, and cell adhesion molecules, promoting an anti-inflammatory phenotype.

Expression of the V3 transcript *in vivo* has been detected in mouse, rat, and human fetal and adult tissues and cells (46–50), and most recently, secretion of V3 protein by primary human skeletal muscle cells was detected by proteomic analysis (51). Previous studies have shown that V3 expression induces increases in cell-cell and cell-ECM association (19, 23–25). This is accompanied by a remodeling of the ECM such that accumulation of aggrecan, hyaluronan, and the larger isoforms of versican, V0/V1, was reduced (22–25), whereas elastic fiber deposition was greatly enhanced (23–25), generating a microenvironment resistant to monocyte adhesion both *in vitro* and *in vivo* (26, 27). Our present findings show that V3 expression induces significant remodeling of the extracellular microenvironment, indicated by the changes observed in the ECM molecules, growth factors, cytokines, components of the complement cascades, proteases, and protease inhibitors, as

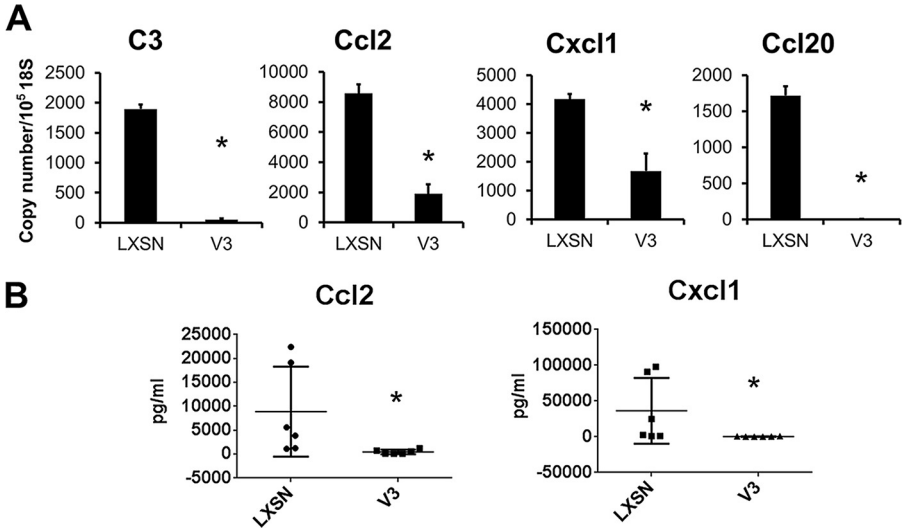

FIGURE 3. *A,* changes in *C3*, *Ccl2*, *Cxcl1*, *Ccl20*, and *Cxcl5* transcript levels were validated by qPCR using control LXSN or V3-expressing ASMCs cultured for 10 days. $n = 3$, *, $p < 0.05$ in unpaired *t* test. *B*, changes in *Ccl2* and *Cxcl1* at protein levels were examined from conditioned media after a 3-day culture of LXSN or V3 ASMCs by a multiplex cytokine array. $n = 6$, *, $p < 0.05$ in unpaired *t* test.

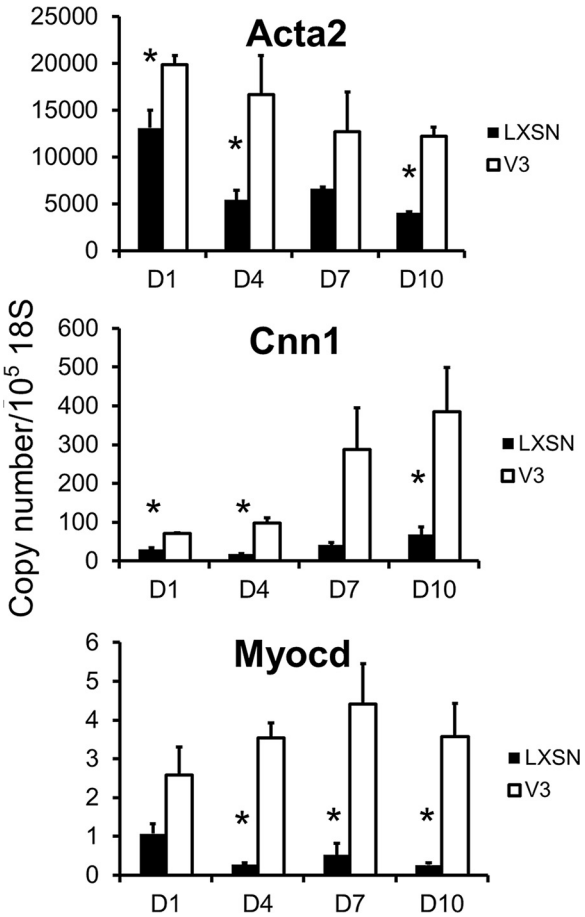

FIGURE 4. **Changes in markers of contractile SMCs such as smooth muscle α-actin (*Acta2*) and calponin (*Cnn1*) were validated by qPCR at days 1, 4, 7, and 10.** V3 expression induced a significant increase in myocardin (*Myocd*) as shown by qPCR. $n = 3$, *, $p < 0.05$ in unpaired *t* test.

TABLE 8
TRE over-represented in up-regulated genes

TRE\external group	FDR
ZF5/V$ZF5_B	0
Kid3/V$KID3_01	0
AP-2α/V$AP2ALPHA_01	3.34E-04
KROX/V$KROX_Q6	3.34E-04
ETF/V$ETF_Q6	3.34E-04
AhR, Arnt, HIF-1/V$AHRHIF_Q6	0.001
MAZ/V$MAZ_Q6	0.005
CKROX/V$CKROX_Q2	0.008
WT1/V$WT1_Q6	0.012
Sp1/V$SP1_Q2_01	0.018
LRF/V$LRF_Q2	0.018
AP-2/V$AP2_Q6	0.018
SRF/V$SRF_C	0.156
Arnt/V$ARNT_01	0.170
SRF/V$SRF_Q4	0.225
SRF/V$SRF_Q6	0.272

well as molecules involved in cell-cell adhesions. Components of elastic fiber, fibulin 5, and elastin were up-regulated by V3 expression, as demonstrated previously in our studies (27, 52). Versican expression was down-regulated, confirming our previous findings that V3 negatively regulates the larger isoforms of versican (23). Additionally, basement membrane components such as laminin β1 and *Smoc1* were up-regulated by V3 expression, whereas collagen-binding proteins such as decorin, *Prelp*, and cartilage oligomeric matrix proteins were down-regulated. Among the many growth factors and cytokines affected by V3 expression, TGFβ superfamily members, including TGFβ1, follistatin, inhibin β-B, and TGFβ3, were significantly modulated. This is consistent with our previous findings that TGFβ signaling pathways were affected by V3 expression (27).

We also found that a number of molecules mediating Wnt/β-catenin and Notch signaling pathways were significantly affected by V3 expression, such as *Wnt4, Tcf4, Notch3*, and *Rbpj*. Given that these signaling pathways regulate tumorigenesis and that V3 expression in melanoma cells has been shown to significantly reduce tumorigenesis (16–18), V3 may negatively impact tumorigenesis by affecting Wnt/β-catenin and Notch signaling pathways. Recently, we have also found that negatively regulating versican in leiomyosarcoma cells significantly blocked tumorigenic potential when subcutaneously implanted into nude mice (53), which suggests V3 as a negative regulator of larger isoforms of versican may provide a novel anti-tumorigenic approach.

TABLE 9
TRE over-represented in down-regulated genes

TRE\external group	FDR
Sp1/V$SP1_Q2_01	0
KROX/V$KROX_Q6	0
Kid3/V$KID3_01	0
myogenin/NF-1/V$MYOGNF1_01	8.2E-04
STAT1/V$STAT1_01	0.002
AP-2/V$AP2_Q6_01	0.002
E2F/V$E2F_03	0.004
AP-2/V$AP2_Q6	0.004
ZF5/V$ZF5_B	0.006
CKROX/V$CKROX_Q2	0.007
GABP/V$GABP_B	0.010
ETF/V$ETF_Q6	0.010
WT1/V$WT1_Q6	0.014
c-Myc:Max/V$MYCMAX_03	0.015
CP2/LBP-1c/LSF/V$CP2_02	0.015
NF-κB/V$NFKB_Q6_01	0.018
GLI/V$GLI_Q2	0.024
MAZ/V$MAZ_Q6	0.028
AP-2α/V$AP2ALPHA_01	0.036
E2F/V$E2F_Q6_01	0.037
MyoD/V$MYOD_Q6_01	0.049
VDR/V$VDR_Q3	0.050
LRF/V$LRF_Q2	0.056
HIC1/V$HIC1_02	0.058
v-Myb/V$VMYB_02	0.076
NF-Y/V$NFY_01	0.095
AhR, Arnt, HIF-1/V$AHRHIF_Q6	0.103
NF-1/V$NF1_Q6_01	0.104
Pax-3/V$PAX3_B	0.111
USF/V$USF_Q6_01	0.118
Ets/V$ETS_Q6	0.118
Muscle initiator sequences-19/V$MINI19_B	0.130
Ebox/V$EBOX_Q6_01	0.133
LXR/V$LXR_Q3	0.157
SZF1–1/V$SZF11_01	0.157
IRF/V$IRF_Q6	0.162
PPAR, HNF-4, COUP, RAR/V$DR1_Q3	0.238
SREBP/V$SREBP_Q6	0.238
Egr-1/V$EGR1_01	0.246
HMG IY/V$HMGIY_Q6	0.257
HNF4/V$HNF4_Q6_01	0.270
PPARα:RXRalpha/V$PPARA_02	0.278
CREB/V$CREB_02	0.278
Arnt/V$ARNT_01	0.285

Vascular SMCs undergo phenotypic changes during development and pathological processes *in vivo* and during cell culture *in vitro* (45, 54–57). For example, medial SMCs exhibit a mature and differentiated phenotype in which the cells express greater amounts of proteins necessary for the contractile function of the cells, including smooth muscle myosin heavy chain and SMA, compared with intimal SMCs that exhibit a "synthetic" phenotype with greater proliferative and migratory capacity and express more ECM proteins such as type I collagen (45, 54, 55). Our study clearly demonstrates that V3 expression increases a number of contractile SMC markers such as SMA, calponin 1, and myocardin, a key transcription factor promoting muscle differentiation, which occurred as early as 24 h after culture. Previously, myocardin had been shown to repress versican through induction of miR143 (58, 59), consistent with the decrease in total versican transcript induced by V3 expression, suggesting that V3 may promote SMC differentiation by decreasing the amount of larger isoforms of versican. Consistently, genes up-regulated by V3 expression were enriched with TRE for SRF, a transcription factor promoting mature and differentiated SMC phenotypes. Changes in the TGFβ superfamily induced by V3 expression, as discussed above, may be responsible for the increases in the markers of differentiated mature SMC phenotypes (60, 61). Our findings that V3 expression induces increases in the differentiated SMC markers as well as in components of basement membrane and elastic fibers suggest that ASMCs can be reprogrammed to actively synthesize the ECM components present in healthy arteries while maintaining a differentiated and contractile phenotype. Intriguingly, proteomic profiling by mass spectrometry has identified V3 as part of the secretome of primary human skeletal muscle cells (51), suggesting it may play an intrinsic role in promoting muscle cell differentiation.

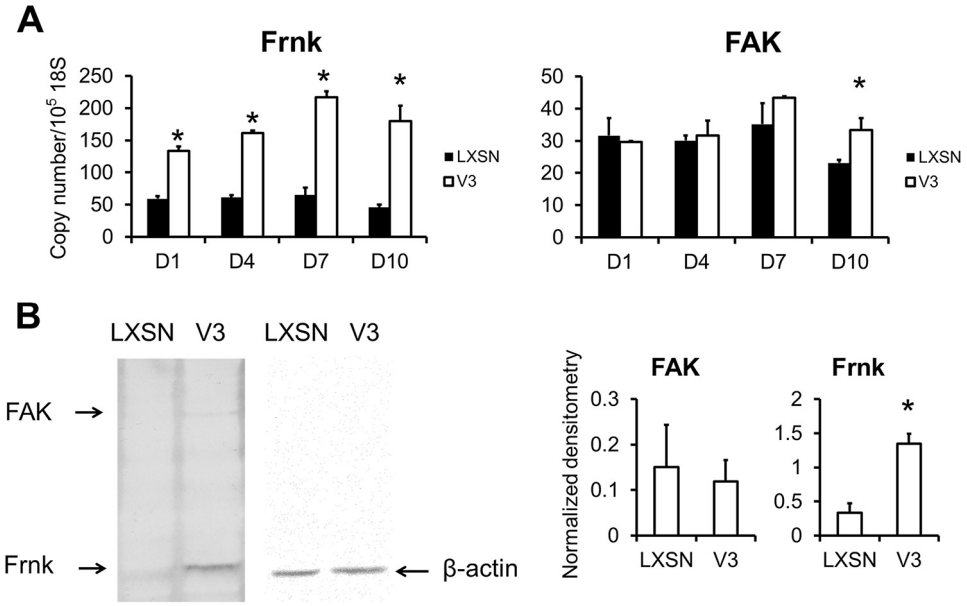

FIGURE 5. *A*, to validate whether the changes in *Ptk2* detected by microarray analysis were specific for Fak or Frnk, we used one set of primers against regions common for both Fak and Frnk (*Frnk* primers), and another set of primers for kinase domain that is present only in Fak but not in Frnk (*Fak* primers). V3-expressing cells had significantly elevated transcripts for Frnk but not Fak at day 1, which persisted up to day 10. $n = 3$, *, $p < 0.05$ in unpaired *t* test. *B*, when probed by a polyclonal antibody, which detects both Fak and Frnk, we found a significant increase in Frnk but not Fak. $n = 4$, *, $p < 0.05$ in unpaired *t* test.

Our studies have previously demonstrated that V3-expressing ASMCs bind fewer monocytes *in vitro* and *in vivo* (26, 27). This was due to the generation of an ECM enriched in elastin and depleted in hyaluronan, as well as a decrease in Vcam1, a key monocyte-binding cell surface molecule (27). Our present findings demonstrate that V3 expression also reduces other inflammatory mediators, including chemokines and cytokines, such as lipopolysaccharide-binding protein, angiogenin, *Wnt4, Cxcl1, Ccl20, Ccl2, Cxcl6, Cxcl12*, and macrophage migration inhibitory factor. This is most likely due to down-regulation of the proinflammatory transcription factors, CCAAT/enhancer-binding protein β and NFκB1, shown in this study. Such results indicate that V3 expression reprograms ASMCs to have an anti-inflammatory phenotype.

V3-expressing ASMCs exhibited a marked increase in cell spreading and larger areas of close contact (24). In this study, we found that V3 expression increased *Ptk2*, which encodes for Fak or Frnk. Fak mediates key signaling pathways elicited by extracellular stimuli activating integrins or growth factors, and it further regulates cell proliferation, survival, and migration via PI3K, MAPK, and/or NFκB (38–42). Down-regulation of Fak activation was correlated with increased expression of differentiated SMC markers when cells were plated on laminin, as opposed to fibronectin, which increased proliferation while decreasing differentiated SMC marker expression (38). Frnk is an endogenous inhibitor of Fak, and it has been shown to be selectively expressed by SMCs and large conduit blood vessels and negatively regulates proliferative and migratory phenotypes (62) while reinforcing expression of differentiated SMC markers (63). Recently, it has been shown that bone marrow mononuclear cells of multiple myeloma patients had elevated transcript levels of versican, β-catenin, β1 integrin, as well as Fak when compared with healthy control populations (64), suggesting that versican may have functional roles in β1 integrin and Fak signaling downstream of β-catenin. In our study, V3 expression significantly increased expression of Frnk, but not Fak, at mRNA and protein levels. Frnk has a transcriptional start site within an intron of the Fak gene, which is positively regulated by TGFβ (63). This is concomitant with the aforementioned TGFβ signaling pathways affected in V3-expressing ASMCs. Moreover, we have previously shown that activation of PI3K and NFκB induced by EGF was significantly down-regulated in V3-expressing ASMCs (27). Based on these findings and our present finding that V3 expression significantly up-regulated Frnk, but not Fak, the effect of V3 on reprogramming ASMC phenotype may be due to altered Fak signaling pathways leading to increases in differentiated smooth muscle marker expression and decreases in activation of PI3K and NFκB.

A significant aspect of our observations is that expression of a single ECM gene, V3, elicits a wide range of phenotypic reprogramming exhibited by expression changes in multiple genes. However, the molecular mechanism(s) that causes these changes is not understood. One possibility is that changes in cell shape caused by V3 expression induce such phenotypic reprogramming. Our earlier work showed that forced expression of V3 resulted in a dramatic cell shape change creating a more flattened, highly spread cell with large areas of close contacts potentially altering cell adhesion through Fak signaling (24). Such shape changes most likely came about as a result of the dramatic changes in the composition of the ECM deposited by the V3-expressing cells (23–25). There is extensive evidence that compositional changes in the ECM can lead to changes in cell shape and micromechanical properties, which in turn will alter gene expression and associated cell phenotypes (65–68). Alternatively, V3 may directly interact with β1 integrins and EGF receptor via the C-terminal G3 domain (15), which will in turn affect Fak signaling downstream of integrins and EGF receptors. We suspect that V3 may modulate Fak signaling pathways by either directly interacting with these cell surface receptors or by altering the micromechanical environment around the cells.

Overall, these findings demonstrate that V3 expression reprograms ASMCs promoting anti-inflammatory and differentiated SMC phenotypes via altering cell-ECM interactions by regulating focal adhesion signaling pathways. This work provides novel insight into the mechanisms by which specific components of the ECM regulate SMC phenotypes, which may offer potential therapeutic targets in the treatment of vascular and other diseases.

Author Contributions—I. K. participated in the conception and design of the study, acquired, analyzed, and interpreted data, and wrote and revised the paper. J. L. B., acquired, analyzed, and interpreted microarray data and participated in drafting and revising the paper. E. P. S. acquired, analyzed, and interpreted microarray data and participated in drafting the paper. D. W. Y. acquired, analyzed, and interpreted qPCR and Western blotting data. G. A. W. acquired, analyzed, and interpreted qPCR data. K. R. B. contributed to the conception and design of the study, and acquired, analyzed, and interpreted microarray and qPCR data. W. S. A. participated in the conception and design of the study, analyzed and interpreted microarray data, and contributed to the writing of the paper. T. N. W. participated in the conception and design of the study, interpreted data, and contributed to the writing and revising of the paper. All authors reviewed the results and approved the final version of the manuscript (with the exception of W. S. A. who passed away before the final version was completed).

Acknowledgments—We thank the Bioinformatics Core of the Benaroya Research Institute for their assistance with the microarray analysis and Dr. Virginia M. Green for editing and preparation of the manuscript.

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