Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 February ; 36(2): e9–16. doi:10.1161/ATVBAHA.115.306607.

Deletion of hyaluronan synthase 3 inhibits neointimal hyperplasia

Lena S. Kiene[1,2], **Susanne Homann**[1,2], **Tatsiana Suvorava**[1], **Berit Rabausch**[1,2], **Julia Müller**[1,2], **Georg Kojda**[1], **Inga Kretschmer**[1,2], **Sören Twarock**[1,2], **Guang Dai**[1,2], **René Deenen**[3], **Sonja Hartwig**[4], **Stefan Lehr**[4], **Karl Köhrer**[3], **Rashmin C. Savani**[5], **Maria Grandoch**[#1,2], and **Jens W. Fischer**[#1,2]

[1]Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

[2]Cardiovascular Research Institute Düsseldorf (CARID), Universitätsklinikum der Heinrich-Heine Universität, Düsseldorf, Germany

[3]Biologisch-Medizinisches Forschungszentrum (BMFZ), Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

[4]Deutsches Diabetes Zentrum, Düsseldorf, Germany

[5]Divisions of Pulmonary & Vascular Biology and Neonatal-Perinatal Medicine, Department of Pediatrics, The University of Texas Southwestern Medical Center, Dallas, Texas, USA.

[#] These authors contributed equally to this work.

Abstract

Objective—Hyaluronan (HA) is a polymeric glucosaminoglycan that forms a provisional extracellular matrix in diseased vessels. HA is synthesized by three different HA-synthases (HAS1, -2, -3). Aim of this study was to unravel the role of the HAS3 isoenzyme during experimental neointimal hyperplasia.

Approach and Results—Neointimal hyperplasia was induced in *Has3*-deficient mice by ligation of the carotid artery. HA in the media of *Has3*-deficient mice was decreased 28 days after ligation and neointimal hyperplasia was strongly inhibited. However, medial and luminal areas were unaffected. Cell density, proliferation, and apoptosis were not altered, suggesting a proportional decrease of both the number of cells and extracellular matrix. In addition, endothelial function as determined by acetylcholine-induced relaxation of aortic rings and immunoblotting of endothelial nitric oxide synthase and arterial blood pressure were not affected. Furthermore, the oxidative stress response was not affected as determined in total protein extracts from aortae. Transcriptome analysis comparing control versus ligated carotid arteries hinted towards a mitigated differential regulation of various signaling pathways in *Has3*-deficient mice in response to ligation that were related to VSMC migration including focal adhesions, integrins, MAPK, and

Correspondence: Jens W. Fischer, PhD, Institut für Pharmakologie und Klinische Pharmakologie, Universitätsklinikum der Heinrich-Heine-Universität Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany, Phone: +49 211 81-12500, Fax: +49 211 81-14781, jens.fischer@uni-duesseldorf.de.

Disclosures: none

phosphatidylinositol signaling system. Lentiviral overexpression of *HAS3* in vascular smooth muscle cells (VSMC) supported the migratory phenotype of VSMC in response to PDGF-BB in vitro. Accordingly, knock down of *HAS3* reduced the migratory response to PDGF-BB and in addition decreased the expression of *PDGF-B* mRNA.

Conclusion—HAS3-mediated HA synthesis after vessel injury supports seminal signaling pathways in activation of VSMC, increases PDGF-BB-mediated migration, and in turn enhances neointimal hyperplasia in vivo.

Keywords

restenosis; extracellular matrix; hyaluronan; smooth muscle cells; neointimal hyperplasia

Introduction

Hyaluronan (HA) is considered to be a key constituent of “permissive” extracellular matrices (ECM) that supports tissue remodeling in response to physiologic and pathophysiologic stimuli. HA is composed of repeating non-sulfated disaccharides [-β (1,4)-glucuronic acid – β (1,3)-N-acetylglucosamine-]$_n$ assembled into polymers of high molecular weight ranging from 1-10 × 10^6 Da by HA synthase (HAS1-3) isoenzymes.

HA activates RHAMM (receptor for HA-mediated motility)- and CD44-mediated signaling[1] in vascular smooth muscle cells (VSMC) which in turn causes increased VSMC proliferation and migration in vitro and in vivo.[2, 3] Recently, RHAMM has been attributed an important role in the control of division fidelity during neointimal hyperplasia.[4] Lack of RHAMM prevents constrictive vascular remodeling after vessel injury.[5] Therefore, inhibition of interstitial HA synthesis by VSMC appears to be a promising target to inhibit restenosis.[6-8]

Studying the regulation of HAS isoenzyme expression in vitro in human VSMC revealed that *HAS1* and *HAS2* transcription are strongly responsive to cAMP-activated signaling by prostaglandins/prostaglandin receptors.[9] Furthermore, *HAS2* is known to be responsive to platelet-derived growth factor BB (PDGF-BB), transforming growth factor beta 1 and thrombin-mediated protease activated receptor 1 signaling.[10] In addition, *HAS1* is upregulated by adenosine A2A- and A2B receptors in VSMC.[11]

Functionally, it was shown in these studies that HAS2-mediated HA synthesis increases proliferation and migration of VSMC. Recently, it was discovered that HAS1 is involved in migratory responses of VSMC as well.[11] In addition, overexpression of *HAS1* promotes adhesion of monocytes to the pericellular HA matrix suggesting that the HA matrix formed by HAS1 is involved in inflammatory responses.[12] Interestingly, in the study by Wilkinson et al. (2006) overexpression of all HAS isoforms led to decreased proliferation and migration rates, which is in contrast with the results of knocking down endogenous *HAS1* and *HAS2* that also inhibited proliferation and migration.[9, 10, 12]

Has2 knockout mice die at midgestation due to disturbed cardiac and vascular morphogenesis and defects in endothelial mesenchymal transition, suggesting that HAS2

fulfills critical physiological functions.[13] A fascinating example of physiological HA-driven neointimal hyperplasia is the closure of the ductus arteriosus Botalli. Here, prostaglandin E (PGE)-mediated activation of the prostaglandin E receptor 4 promotes induction of *Has2* and subsequently VSMC migration and the formation of a HA-rich intimal cushion.[14] On the other hand, the dilatory effect of PGE2 keeps the ductus open. This remodeling process is essential in preparing the timely and rapid closure of the ductus arteriosus after cessation of PGE synthesis. In contrast to the lethal genetic deletion of *Has2*, deletion of *Has1* or *Has3* did not result in an obvious phenotype without pathophysiological challenge.

HAS2 likely represents an attractive target to inhibit neointimal hyperplasia because in vivo studies revealed that overexpression of *Has2* in VSMC led to increased atherosclerosis in apolipoprotein E-deficient mice and to increased cuff-mediated neointimal hyperplasia in C57BL/6 mice.[15, 16] However, a limitation that needs to be considered is that those results reflect increased HA synthesis in medial VSMC due to *Has2* overexpression in differentiated VSMC, and do not specifically reflect the physiologic function of HAS2 during neointimal hyperplasia.

Due to the embryonic lethality of *Has2*-deficient mice and the importance of HAS2 for proper development of the cardiovascular system as well as for endothelial mesenchymal transition it is doubtful that HAS2 represents a suitable therapeutic target. Therefore, the other HAS isoenzymes are of great interest with respect to their specific role in vascular pathologies. Whether HAS1 and HAS3 play a role in neointimal hyperplasia is not known. Similarly, other than the knowledge that *HAS3* is upregulated in aged VSMC and that oxLDL and cholesterol induce *HAS3* in human VSMC little is known about its regulation and function in VSMC.[17, 18] Hence, the aim of the current study was to test the effect of loss of HAS3 during neointimal hyperplasia in vivo using mice with targeted deletion of *Has3* and to study the underlying mechanisms in vitro.

Material and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Inhibition of neointimal hyperplasia in *Has3*-deficient mice

Has3-deficient mice displayed no differences with respect to body weight, blood glucose and total cholesterol (Supplemental Figure I). To address the role of HAS3 for VSMC phenotype in vivo, left carotid arteries of *Has3*-deficient mice and wildtype (WT) mice were ligated to induce neointimal hyperplasia driven by VSMC proliferation and migration.[19] In *Has3*-deficient mice the neointimal area and neointimal/medial ratio were strongly reduced compared to WT mice (Figure 1A, B). In contrast, the medial and luminal areas were not changed (Figure 1C). A small reduction of the circumference of the external elastic lamina of ligated carotid arteries was observed (Figure 1C) indicating constrictive remodeling. Determination of cell density in the neointima suggested that both cell number and ECM declined proportionally in *Has3*-deficient mice after ligation (Figure 1D). Analysis of HA staining in control carotid arteries versus ligated WT carotids revealed an accumulation of

HA both in the media and in the neointima after 28 days (Figure 2A). Quantitative image analysis revealed less HA accumulation in the whole vessel (Figure 2A, B) in *Has3*-deficient mice after ligation. Interestingly, the difference was pronounced in the media (Figure 2B). Additionally, after ligation expression of *Has1* and *Has2* were not further upregulated in *Has3*-deficient mice compared to WT, thereby excluding a compensatory counter regulation in response to *Has3*-deficiency (Figure 2C, D). *Has3* mRNA was not detected in *Has3*-deficient mice (not shown).

To gain further mechanistic insight, ligated carotid arteries were investigated five days after ligation. As evidenced by BrdU staining, the proliferation rate was not affected in the neointima of *Has3*-deficient compared to WT controls (Figure 3A). In addition, neither apoptotic rate (Figure 3B) nor immunostaining of alpha smooth muscle actin were affected (Figure 3C) five days after ligation. Furthermore, mRNA of *Acta2* and *SM22alpha* were determined. Both markers of differentiated VSMC were strongly downregulated in response to ligation as expected. However, the extent of this downregulation was not different between genotypes (Figure 3D).

Next, it was addressed if *Has3* deficiency might have general effects on endothelial function that might result in changes of ligand-induced vasodilatation and blood pressure. First, expression of endothelial nitric oxide synthase (eNOS) was found to be unaffected in aortas of WT versus *Has3*-deficient mice (Figure 4A). Likewise, acetylcholine-induced vasorelaxation was not affected (Figure 4B) as well as relaxation in response to an exogenous NO-donor (S-nitroso-N-acetyl-D,L-penicillamine, SNAP) and contraction in response to phenylephrine (Figure 4C, D).

Further, arterial blood pressure of *Has3*-deficient mice was similar to WT mice (Figure 4E). To address the question whether the observed phenotype might involve changes in the oxidative stress response, Oxyblot™ analysis was performed. No differences in oxidative protein modifications were observed between genotypes (Figure 4F).

All things considered, an effect of *Has3*-deletion on vascular tone and reagibility can be excluded as the mechanism underlying reduced neointimal hyperplasia in *Has3*-deficient mice.

Attenuated transcriptional response in *Has3*-deficient mice after carotid artery ligation

In search of the underlying mechanisms, genome wide transcriptome analysis of control and ligated carotids was performed five days after ligation. In WT mice 8006 transcripts were differentially regulated when comparing ligated carotid arteries to non-ligated control carotids (Supplemental Figure II). In contrast, employing identical experimental settings in *Has3*-deficient mice only 3635 transcripts were affected by the ligation suggesting a strong influence of *Has3*-deficiency on transcriptional responses to vessel injury. The dampened response in *Has3*-deficient mice can also be seen in the hierarchical clustering and the volcano plots shown in Supplemental Figure III. Next, the transcriptome data were analyzed for KEGG pathways that were only significantly regulated in response to ligation in carotid arteries from WT and not in carotids from *Has3*-deficient mice (Supplemental Table I). Of note, various pathways related to the migratory SMC phenotype, ECM signaling and general

signaling leading to phenotypic activation were identified such as regulation of actin cytoskeleton (mmu04810), vascular smooth muscle contraction (mmu04270), calcium signaling pathway (mmu04020), focal adhesion (mmu04510), ECM-receptor interaction (mmu04512), phosphatidylinositol signaling system (mmu04070), inositol phosphate metabolism (mmu00562), and MAPK signaling pathway (mmu04010). Expression of genes representing the oxidative stress response did not appear to be differentially regulated between the genotypes confirming the results from the Oxyblot™ analysis as indicated above (Figure 4F).

HAS3-mediated HA synthesis promotes migration and proliferation

Analysis of the neointima as presented in Figure 1-3 revealed decreased neointimal hyperplasia in *Has3*-deficient mice due to proportional decrease in cells and matrix. Since neither BrdU incorporation nor apoptosis were changed in *Has3*-deficient mice, it is likely that primarily migratory responses are affected. In line with this assumption was the finding that *Pdgf-b* mRNA, known to promote VSMC migration[20,21], was upregulated WT after carotid artery ligation but not in *Has3*-deficient mice as determined by gene array analysis (NCBI GEO accession number GSE70410). In order to complement the results obtained in *Has3*-deficient mice in vitro, lentiviral shRNA was employed to specifically knockdown *HAS3* (sh*HAS3*, Figure 5A) in human VSMC. Lentiviral sh*HAS3* efficiently reduced *HAS3* mRNA expression and reduced HA secretion into the cell culture medium (Figure 5A). As observed in the gene array analysis, *PDGF-B* mRNA expression was significantly reduced in sh*HAS3* cells (Figure 5A). Importantly, migration was diminished in sh*HAS3* as determined in a microchemotaxis chamber (Figure 5B). Neither the mitogenic response to PDGF-BB (Figure 5B) nor the apoptotic rate (data not shown) was significantly altered in sh*HAS3*. The gene array and the in vitro migration data strongly suggested a role of HAS3-mediated HA synthesis in migration. In line with this assumption, the expression of a panel of genes involved in the migratory response was down regulated after knock down of *HAS3* in VSMC as determined by qPCR (Figure 5C). Accordingly, the migratory response of VSMC to PDGF-BB was strongly increased in HAS3 overexpressing (*HAS3*oe) cells (Supplemental Figure IV). Proliferation was quantified by DNA-synthesis, as determined by [^{3}H]-thymidine incorporation, and was slightly increased in *HAS3*oe cells compared to controls (Supplemental Figure IV).

Finally, it was considered whether the inflammatory response was affected in *Has3*-deficient mice. Importantly, neointimal hyperplasia in normocholesteremic mice is considered mainly a model of VSMC-driven neointimal hyperplasia.[22]

In the gene array analysis, expression of genes representing the inflammatory response was upregulated in both genotypes and also inflammatory KEGG pathways were regulated in both genotypes.

mRNA expression of *Tnfa*, *Ccl2* (MCP-1), and *Il1b* as determined by qPCR in ligated carotid arteries, showed a strong increase compared to unligated controls. However, no differences between genotypes were observed (data not shown). Likewise, circulating cytokines were mostly unaffected except increased IL-13 and GM-CSF (Figure 6). Importantly, IL-13-primed macrophages are characterized by a more anti-inflammatory M2

phenotype and suppress pro-inflammatory type 1 macrophages[23] which in turn have been shown to substantially promote neointima formation via promotion of VSMC proliferation and migration.[24] In contrast, GM-CSF is thought to promote inflammation so that both, pro- and primarily anti-inflammatory cytokines seem to be induced in *Has3*-deficiency and the net outcome remains unknown. Taken together, reduced inflammation is unlikely the mechanism underlying reduced neointimal hyperplasia in *Has3*-deficient mice.

Discussion

This study is the first to address the specific role of HAS3 during experimental neointimal hyperplasia in vivo and shows a substantial reduction of neointimal hyperplasia in *Has3*-deficient mice. In response to the arterial injury, strong accumulation of HA was observed within the neointimal layer, in line with earlier results.[25, 26] In healthy vessels HA-matrix is mainly limited to the adventitial layer and the endothelial glycocalyx. Since HAS2 is pivotal for development of the cardiovascular system as well as endothelial mesenchymal transition, it is likely not a suitable therapeutic target with favorable benefit/risk ratios. Therefore, therapeutic strategies specifically inhibiting HAS3-mediated HA expression might be considered in the prevention of restenosis after surgical intervention. Based on the unaltered cell density in the neointima in vivo, a proportional decrease of both cell number and ECM is suggested in *Has3*-deficient mice. Gene expression of *Acta2* and *SM22alpha* was reduced five days after ligation indicating dedifferentiation of VSMC. However, *Has3*-deficiency did not affect the expression of differentiation markers. In addition, immunostaining of alpha smooth muscle actin and determination of proliferation and apoptosis revealed no changes between the genotypes five days after ligation. In search of the underlying mechanisms, effects of *Has3*-deficiency on endothelial function, blood pressure, inflammation, and the oxidative stress response were excluded.

Importantly, HA accumulation in the ligated vessels was reduced in *Has3*-deficient mice. Analysis of medial and intimal HA-accumulation revealed that reduction of HA occurred also in the media of ligated carotids from *Has3*-deficient mice. In this context it is important to consider that migration of VSMC from the media into the intima is one of the first steps during neointimal hyperplasia. PDGF-BB has been shown to promote formation of pericellular HA-matrix in VSMC that in turn strongly promotes migration.[27] Therefore, HAS3 may be involved in the induction of the provisional HA-rich ECM in the media and neointima of ligated carotid arteries and in turn promotes VSMC migration. In this context it may be important that *Pdgf-b* mRNA expression as assessed by gene array analysis was significantly upregulated in ligated WT carotids but not in Has3-deficient carotids in response to ligation. Further, *PDGF-B* was reduced in VSMC after lentiviral knockdown of *HAS3*. This may represent a feed forward mechanism that further inhibits migratory responses in the absence of HAS3. Based on the present findings and the literature it is likely that HAS3 expression in VSMC promotes neointimal hyperplasia as opposed to HAS3 expression by other cell types such as endothelial cells. This is also supported by the differentially regulated KEGG pathways that point towards VSMC but not endothelial cells, and the in vitro results. In future studies, VSMC-specific deletion of *Has3* may be used to further confirm the role of VSMC.

In order to further understand the underlying cellular mechanisms, mRNA expression in the uninjured and ligated carotids was analyzed in an unbiased genome wide approach using gene array technology. This analysis revealed that in WT mice about twice as many transcripts are regulated after ligation as compared to *Has3*-deficient mice (8006 versus 3635). Furthermore, a variety of KEGG pathways were identified that were only differentially regulated in WT mice in response to carotid ligation but not in *Has3*-deficient mice. Among these pathways those related to the migratory VSMC phenotype (contractility, actin cytoskeleton), ECM signaling (focal adhesion kinase, ECM-receptor interactions), and general signaling (MAPK, phosphatidylinositol signaling system) appeared particularly interesting because they could represent avenues of phenotypic activation of VSMC in WT that were dampened in *Has3*-deficient mice. In line with these effects on transcriptional programs in vivo, we detected corresponding functional differences in VSMC after knock down of *HAS3* in vitro. Lentiviral knockdown of *HAS3* inhibited migration in VSMC and proliferation was not altered. Conversely, overexpression of *HAS3* also revealed that HAS3-mediated HA synthesis augmented mainly migration and to a lesser extent proliferation in human VSMC.

The in vivo role of RHAMM and CD44 in neointimal hyperplasia has been demonstrated before. With respect to RHAMM, a role in both migration but also proliferation and cell division fidelity has recently been proposed in vivo.[4, 28] In *Rhamm*-deficient mice, constrictive artery wall remodeling was reduced after carotid artery ligation improving the lumen caliber, but surprisingly, did not alter the neointimal area itself.[5] Similarly, a role of CD44 in promoting neointimal hyperplasia has been proposed.[29] Though, this has not directly been shown by use of knockout mice. Strong evidence however suggests that CD44 orchestrates also inflammatory responses in vivo e.g. during atherosclerosis.[30, 31] Interestingly, it is known that inflammatory stimuli induce CD44 expression in VSMC.[32, 33] Toll-like receptors (TLR) are also involved in neointimal hyperplasia.[34, 35] However, from the data presented here it cannot be concluded which of the receptors CD44 or RHAMM or even alternative HA receptors such as TLR2, -4, LYVE1, and HARE are involved in the in vivo response and the inhibition of neointimal hyperplasia. For this purpose, future in vivo studies employing the respective receptor knockouts or pathway inhibitors are needed.

Collectively, it is demonstrated here (i) that *Has3* deletion inhibits neointimal hyperplasia and (ii) that loss of HAS3-dependent synthesis of HA decreases PDGF-BB-mediated migration of VSMC. We conclude that HAS3 is a critical player in the phenotypic activation of medial VSMC during neointimal hyperplasia and may represent a suitable therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The excellent technical assistance by Petra Rompel and Annika Zimmermann is gratefully acknowledged.

Sources of funding:

This study was funded by Deutsche Forschungsgemeinschaft (DFG FI 682/3-1, IRTG 1902), NIH grants HL073896 and HL075930, as well as the William Buchanan Chair in Pediatrics to RCS.

Abbreviations

BrdU	5-bromo-2'-deoxyuridine
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HA	hyaluronic acid
HA-BP	hyaluronan binding protein
HAS	hyaluronan synthase
H&E	hematoxylin and eosin
IL-13	interleukin 13
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	mitogen-activated protein kinases
MCP-1	monocyte chemotactic protein 1
PARP	Poly (ADP-ribose) polymerase
PDGF-BB	platelet-derived growth factor BB
PGE	Prostaglandin E
RHAMM	receptor for HA-mediated motility
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VSMC	vascular smooth muscle cell
WT	wildtype

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Significance

Intimal thickening causes luminal narrowing after stent implantation, balloon catheter-based revascularization, by-pass grafting, and during atherosclerosis. Accumulation of extracellular matrix is critically involved in these pathologies because it directly contributes to neointimal volume expansion and regulates smooth muscle cell responses. As therapeutic targets hyaluronan synthases (HAS) appear attractive because three different isoenzymes exist which rapidly generate the hyaluronan matrix and might be targeted specifically in the future. Here we show that genetic deletion of *Has3* inhibits neointimal hyperplasia and that HAS3-dependent HA-synthesis increases PDGF-BB-mediated migration. These results suggest that inhibition of HAS3-mediated HA synthesis may be used to limit neointimal hyperplasia.

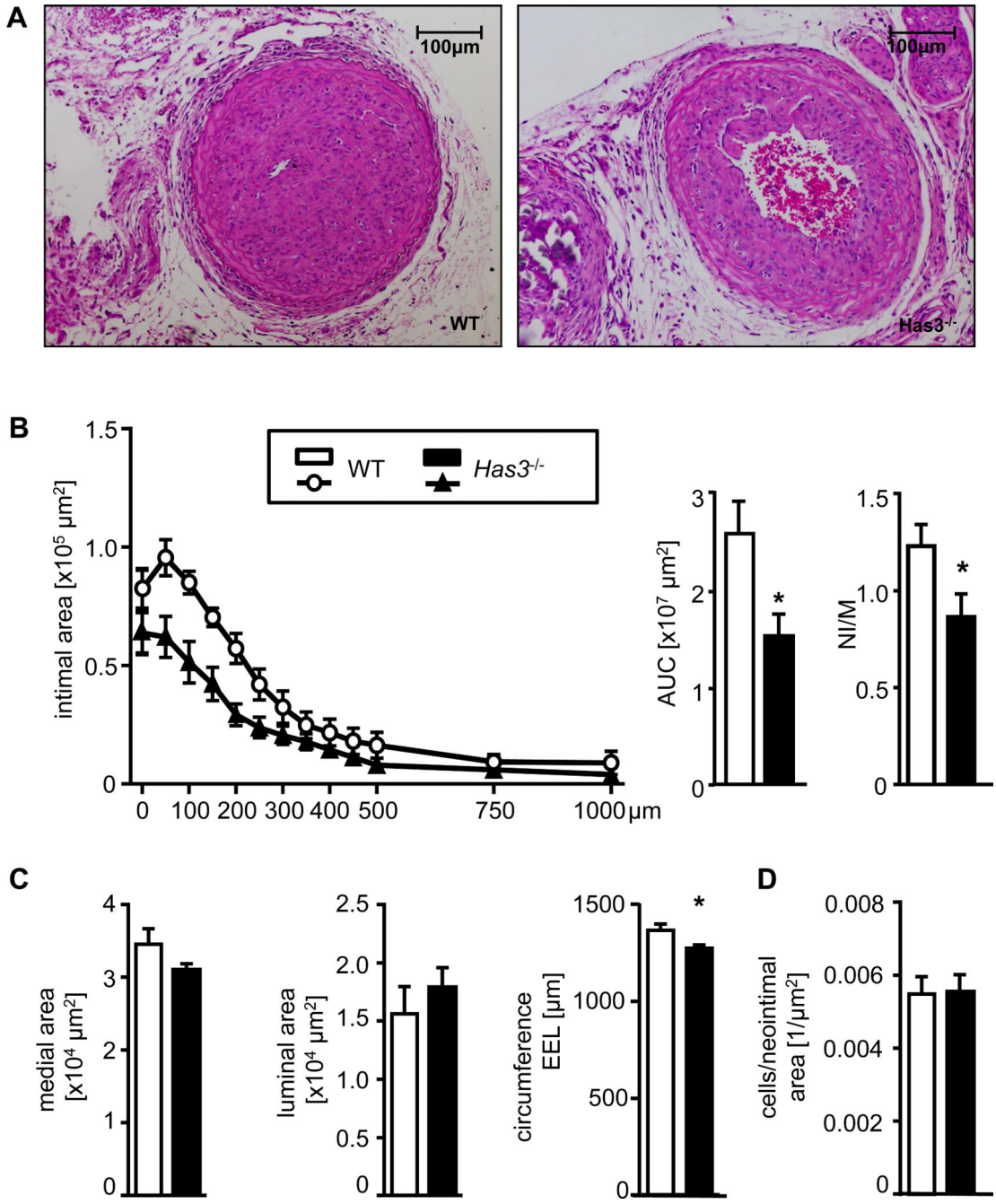

Figure 1. Neointimal hyperplasia is reduced in *Has3*-deficient mice

Neointimal hyperplasia was induced by carotid artery ligation in WT and *Has3*-deficient mice and analyzed after 4 weeks. **A**, Representative images of H&E stained sections of WT (left) and *Has3*-deficient (right) mice. **B**, Neointimal hyperplasia was characterized by morphometry. The neointimal area is plotted as function of the distance to ligation (left). Mean area under the curve (AUC) and the neointimal/medial (NI/M) ratio are given as well (right). **C**, Mean medial and luminal area, mean circumference of the external elastic lamina (EEL), and **D**, cellular density in the neointima. Data are means ± SEM, n = 9 *, $p < 0.05$ vs. WT.

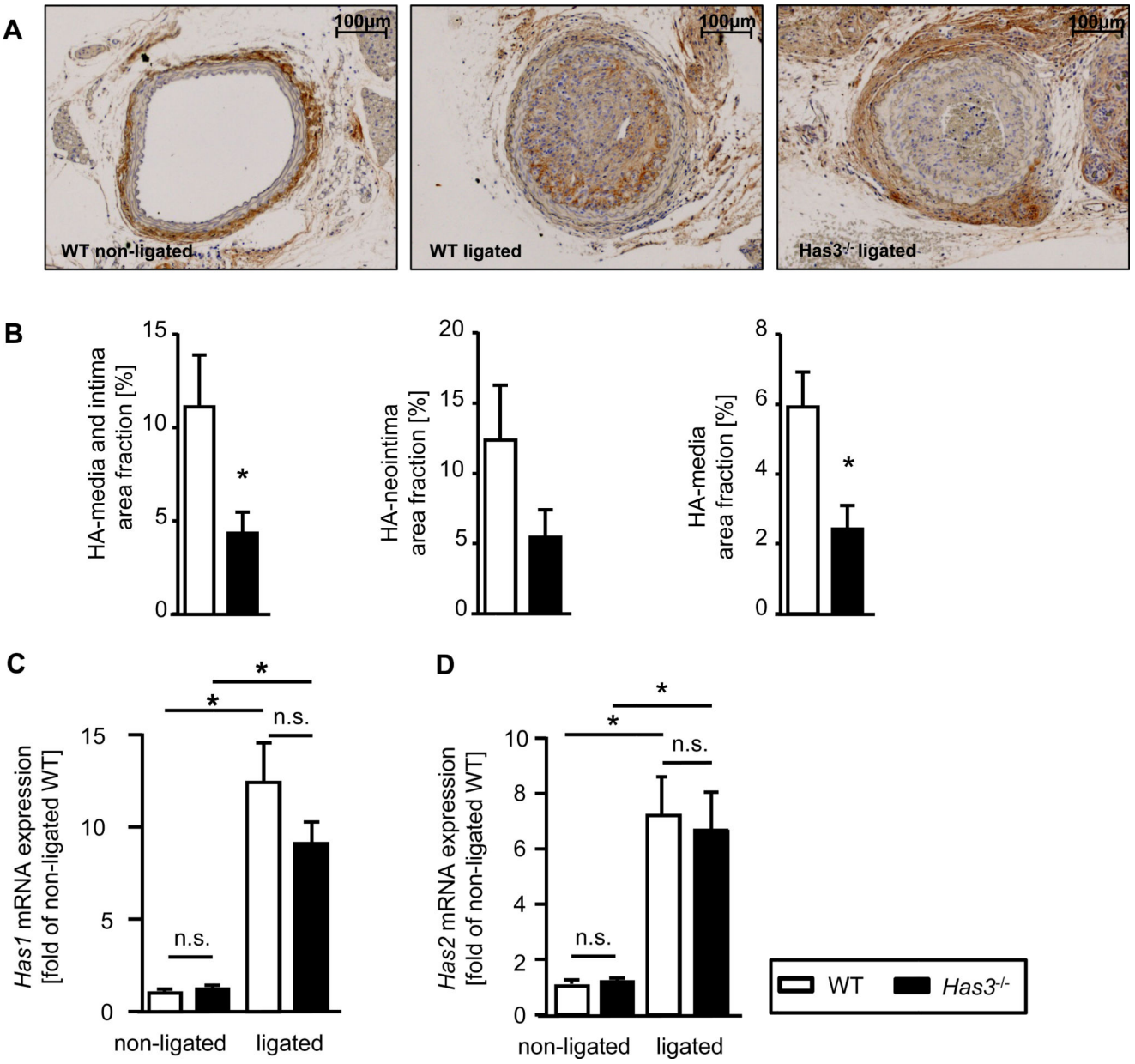

Figure 2. Carotid arteries from *Has3*-deficient mice show reduced HA accumulation and no compensatory regulation of *Has1* and *Has2*

Neointimal hyperplasia was induced by carotid artery ligation in WT and *Has3*-deficient mice. Histological sections were analyzed for HA content after 4 weeks; **A**, representative images of uninjured vessel (left) and WT (middle) and *Has3*-deficient (right) carotid arteries after ligation stained for HA-BP. **B**, Area fraction quantified in media and intimal layer, and in media and intima separately, n = 9 *, $p < 0.05$ vs. WT. **C**, **D**, *Has1* and *Has2* mRNA expression were analyzed after five days. **C**, *Has1* gene expression; **D**, *Has2* mRNA expression; n = 7-9 *, $p < 0.05$ vs. respective non-ligated controls analyzed using Kruskal-Wallis test with Dunn's post hoc test. Data are means ± SEM.

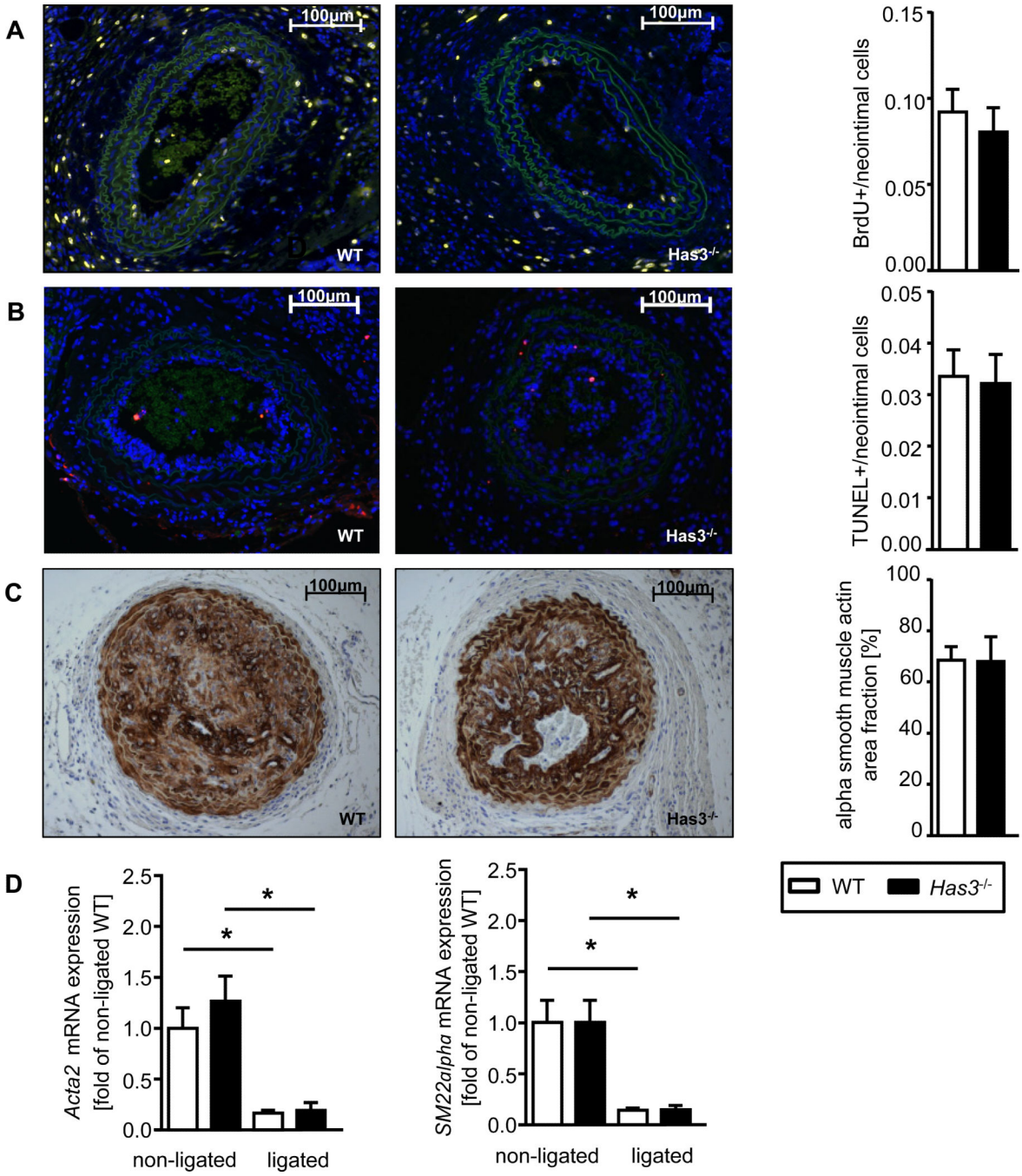

Figure 3. Characterization of VSMC proliferation, apoptosis, and differentiation in the neointima of *Has3*-deficient mice

Five days after ligation BrdU incorporation, TUNEL staining, and mRNA expression were analyzed. **A**, Representative images of immunostaining of incorporated BrdU (yellow), proliferative index calculated as the ratio of BrdU+ nuclei to total nuclei within the neointimal layer; n = 11. **B,** Representative images of TUNEL staining (red) and quantification of apoptosis as TUNEL positive cells per total neointimal nuclei; n = 13. Original magnification 200 fold, nuclei are stained in blue, elastic laminae are indicated by autofluorescence (FITC channel, green), pictures from WT are shown on the left, images

from *Has3*-deficient mice on the right. **C,** Representative images of alpha smooth muscle actin stained sections of WT (left) and *Has3*$^{-/-}$ (right) mice harvested four weeks after ligation and quantification of intimal area fraction, n = 9,8.
D, mRNA expression of alpha smooth muscle actin (*Acta2*) and *SM22alpha*; n = 8-9 *, $p < 0.05$ vs. respective non-ligated controls analyzed using Kruskal-Wallis test with Dunn's post hoc test. Data are presented as mean ± SEM.

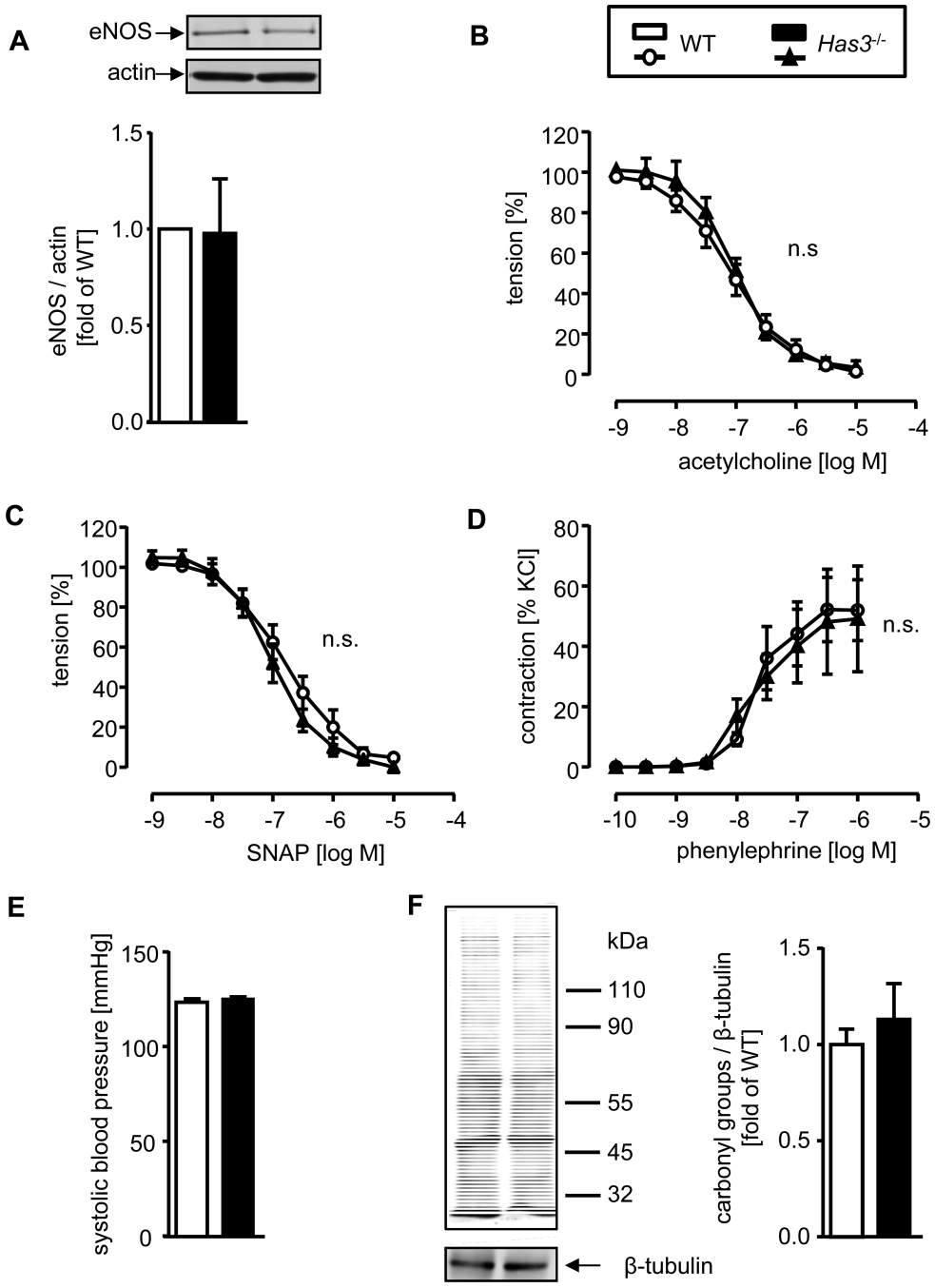

Figure 4. eNOS expression and endothelial function is not altered by *Has3*-deficiency

Thoracic aortae were harvested from *Has3*-deficient mice and WT controls. **A**, eNOS protein expression in aortas of *Has3*-deficient and WT mice. The upper panel shows representative Western blots for eNOS in aortic tissue, the lower panel represents a densitometric quantification of the signals normalized to actin; n = 4-5. **B**, Endothelium-dependent relaxation to acetylcholine, **C**, endothelium-independent relaxation to the NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and **D**, contractile response to phenylephrine in aortic rings of *Has3*-deficient and WT mice, not significant (n.s.), n = 3-4.

E, systolic blood pressure; n = 5. **F**, Representative Western blot of carbonyl groups as a marker for oxidative protein modifications and densitometric quantification of the signals normalized to β-tubulin; n = 4. Data are means ± SEM.

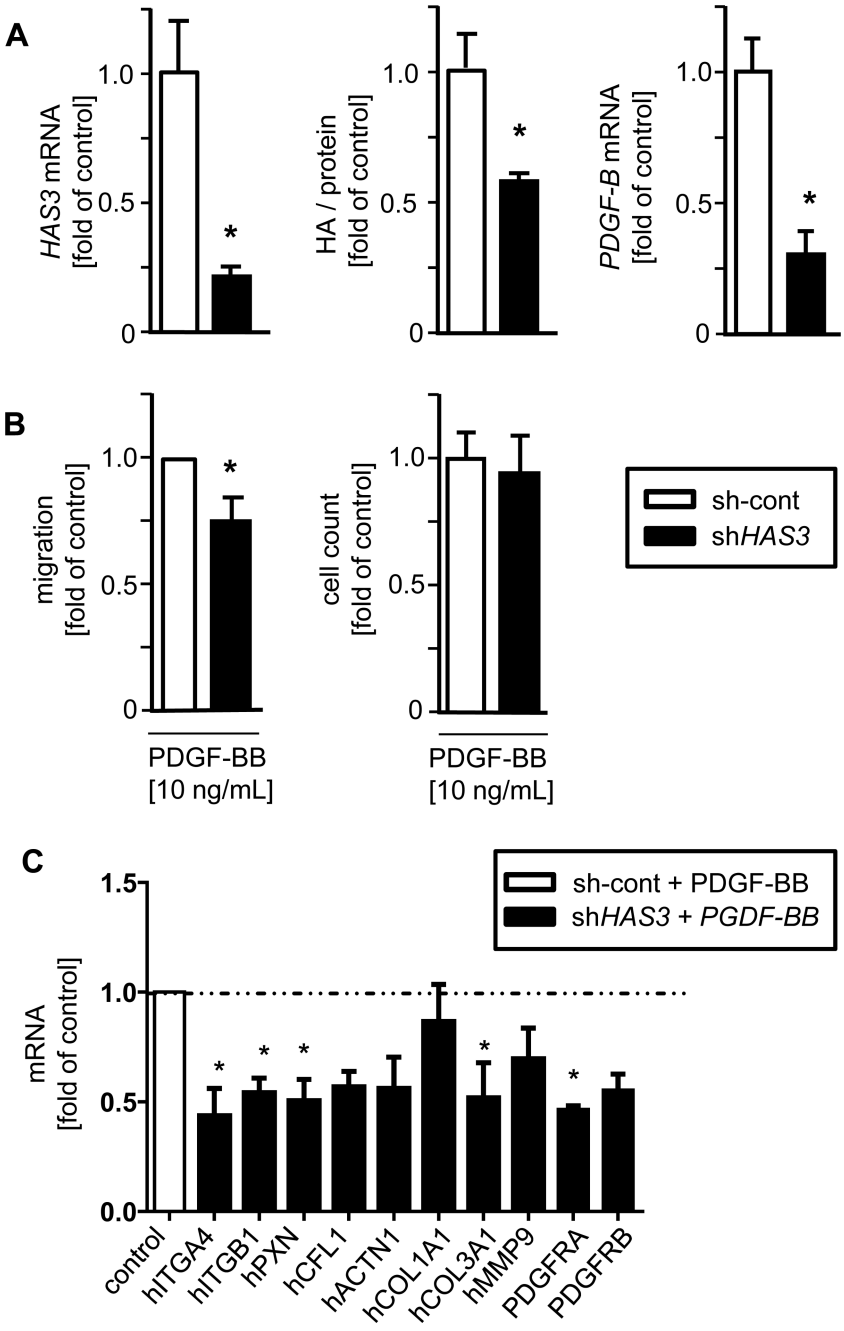

Figure 5. HAS3 supports the migratory and proliferative phenotype of human coronary VSMC

A, *HAS3* mRNA was reduced using lentiviral shRNA compared to non-targeting shRNA (sh-cont) in VSMC, leading to a diminished HA secretion, and a downregulation of *PDGF-B* mRNA. **B**, sh*HAS3* inhibited migration as determined by a modified Boyden-chamber microchemotaxis assay on collagen type-1-coated membranes and proliferation as determined by cell count. * $p < 0.05$ vs. control. **C,** sh*HAS3* reduced mRNA expression of a variety of migration-associated genes in VSMCs stimulated with PDGF-BB (10 ng/mL) for 24 hours.* $p < 0.05$ vs. sh scr (control); one-way ANOVA with Dunnett's multiple comparison test. n = 3-6. Data are means ± SEM;.

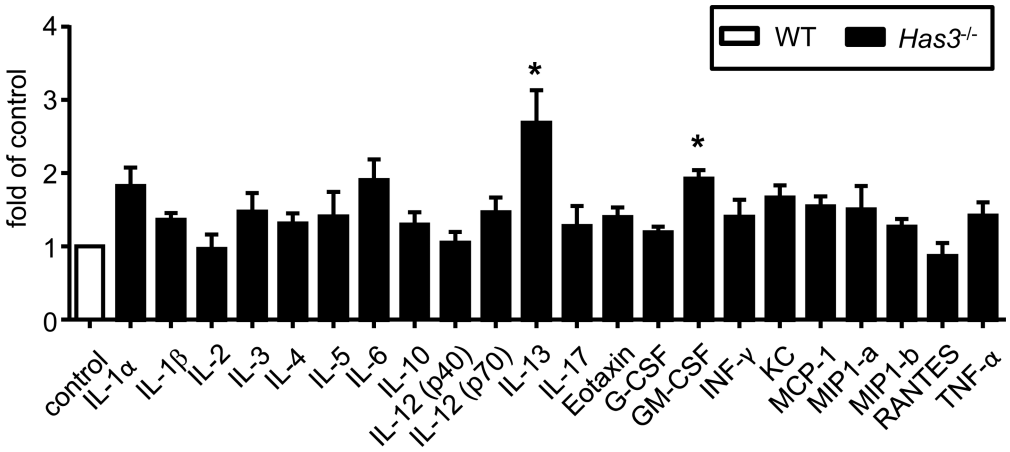

Figure 6. Multiplex cytokine immunoassay

Plasma samples were collected five days after carotid artery ligation. **A**, Determination of multiple cytokines in the plasma by a commercially available multiplex bead-based immunoassay. Expressed as fold of respective WT control; mean ± SEM vs. WT; n = 5,4 *, $p < 0.05$ Kruskal-Wallis test with Dunn's post hoc test.