NF1 Regulates a Ras-Dependent Vascular Smooth Muscle Proliferative Injury Response

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Background—Neurofibromatosis type I (NF1) is a common autosomal dominant disorder with a broad array of clinical manifestations, including benign and malignant tumors, osseous dysplasias, and characteristic cutaneous findings. In addition, NF1 patients have an increased incidence of cardiovascular diseases, including obstructive vascular disorders. In animal models, endothelial expression of the disease gene, NF1, is critical for normal heart development. However, the pathogeneses of the more common vascular disorders are not well characterized.

Methods and Results—To examine the role of NF1 in vascular smooth muscle, we generated mice with homozygous loss of the murine homolog Nf1 in smooth muscle (Nf1smKO). These mice develop and breed normally. However, in response to vascular injury, they display a marked intimal hyperproliferation and abnormal activation of mitogen-activated protein kinase, a downstream effector of Ras. Vascular smooth muscle cells cultured from these mice also display enhanced proliferation and mitogen-activated protein kinase activity. Smooth muscle expression of the NF1 Ras-regulatory domain (GTPase activating protein–related domain) rescues intimal hyperplasia in Nf1smKO mice and normalizes vascular smooth muscle cell Ras effector activity and proliferation in vitro, similar to blockade of downstream effectors of Ras.

Conclusions—In this in vivo model of NF1 obstructive vascular disease, we have shown that Nf1 regulation of Ras plays a critical role in vascular smooth muscle proliferation after injury. These results suggest opportunities for targeted therapeutics in the prevention and treatment of NF1-related vascular disease and in the treatment of neointimal proliferation in other settings. (Circulation. 2007;116:2148-2156.)

Key Words: muscle, smooth | neurofibromatosis | stenosis | vasculature

Neurofibromatosis type I (NF1), an autosomal dominant disorder that affects 1 in 3500 individuals, has a broad array of clinical manifestations, including tumors, osseous dysplasia, learning disorders, and cardiovascular disease.1 The gene responsible for this disease, NF1, encodes neurofibromin, which can function as a Ras GTPase activating protein (GAP) to downregulate Ras activity2 and to modulate epithelial-mesenchymal transformation and cell proliferation.3 Mutations within NF1 result in inactivated forms of neurofibromin and increased cellular proliferation that underlies tumor formation.

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NF1 vasculopathy, first described in 1945, is a significant complication among NF1 patients.4 Increasing evidence indicates that NF1 vasculopathy can be extremely serious, even causing sudden death.5–7 Analysis of US death certificates showed that vascular disease was a cause of death >7 times more often than expected among NF1 patients <30 years of age.8 Despite this, many individuals with NF1-related vascular disease are not brought to clinical attention, so its frequency probably is underestimated.9

One of the more common vascular lesions in NF1 is renal artery stenosis with consequent hypertension.10–13 NF1 vasculopathy is most frequently characterized by lumen occlusion with intimal wall and smooth muscle hyperplasia.4 The pathogenesis of these vascular lesions in NF1 is not clear. However, because NF1 is normally expressed in endothelial and vascular smooth muscle cells14 and can regulate cell growth through Ras regulation, its loss from one or both of these tissues could contribute to the pathogenesis of obstructive vascular disease in NF1.

Previous work from our laboratory has shown that loss of endothelial Nf1 leads to gross cardiac developmental malformations.15 These defects result in midgestational lethality for the vast majority of these mice.15 However, some endothelium-specific knockouts survive to adulthood.16 In these animals, we have not noted vascular abnormalities. Until now, the significance of the in vivo loss of Nf1 in smooth muscle has not been examined, although cell culture
studies analyzing haploinsufficient Nf1<sup>+/−</sup> cells have implicated neurofibromin in smooth muscle cell homeostasis. To gain insight into the functions of Nf1 in smooth muscle, we generated mice with tissue-specific homozygous loss of Nf1 in smooth muscle (Nf1<sub>smKO</sub>). We then analyzed its role in a mouse model of arterial injury and in cultured arterial smooth muscle cells obtained from these animals and littermate controls.

**Methods**

**Mouse Breeding and Genotyping**

We used 8- to 12-week-old, 20- to 30-g, wild-type and Nf1<sub>smKO</sub> mice of a mixed C57BL/6 and 129sv background (bred in our laboratory). We generated Nf1<sub>smKO</sub> mice using the Nf1<sup>−/−</sup> (Nf1<sup>+/−</sup>)<sup>18</sup> and Nf1<sup>−/−</sup>-flox (Nf1<sup>−/−</sup>-flox)<sup>19</sup> alleles and the SM22α-Cre transgenic mouse, which expresses Cre recombinase in vascular smooth muscle.<sup>20</sup> We used Rosa–HA–GAP-related domain (GRD) mice as previously described.<sup>21</sup> We performed genotyping of Nf1<sup>−/−</sup>-null, Nf1<sup>+/−</sup>-null, Nf1<sup>−/−</sup>-flox, SM22α-Cre, and Rosa–HA–GRD as previously described.<sup>20,21</sup> All protocols conformed to the guidelines established by the Association for the Assessment and Accreditation of Laboratory Animal Care and were approved by the University of Pennsylvania Animal Care and Use Committees.

**Carotid Artery Ligation**

Mice were anesthetized with intraperitoneal injection of ketamine 100 mg/kg. The left common carotid artery was ligated at the distal bifurcation with a 6-0 silk suture, as described previously.<sup>22–24</sup> Twenty-eight days later, mice were killed, and the left and right carotid arteries were harvested. To assess cell proliferation, BrdU (Sigma Chemical Co, St Louis, Mo) 100 mg/kg was injected intraperitoneally daily beginning 24 hours after ligation until the day of harvest. Intimal proliferation was determined by examinations of 2 sections per sample obtained 600 to 1000 μm from the ligation site.

**Histology and Immunostaining**

Tissue collection, fixation, and staining were performed as previously described.<sup>25</sup> Briefly, arteries were collected in ice-cold PBS, fixed overnight in 4% paraformaldehyde at 4°C, washed with PBS, and dehydrated through an ethanol series before paraffin embedding. Hematoxylin and eosin stain was used for routine histology, and Verhoeff’s elastin stain was used to delineate elastic fiber. Immunostaining with anti-smooth muscle α-actin, anti-phospho-p44/42, and anti-BrdU was performed as previously described.<sup>15</sup>

**Isolation of Aortic Smooth Muscle Cells**

Smooth muscle cells from the aortas of adult mice (AoSMCs) were used for experiments from passages 2 through 5.

**Western Blot Analysis**

Cell lysates were prepared in magnesium lysis buffer consisting of 2.5 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% Igepal CA-630, 10% glycerol, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 nmol/L sodium orthovanadate. Samples were separated by SDS-PAGE and transferred to polyvinyl difluoride membranes. Antibodies for p44/42 (ERK), phosphorylated ERK (pERK), mammalian target of rapamycin (mTOR), phospho-mTOR (serine 2448 and serine 2481), s6K, and phospho-s6K (threonine 389) (Cell Signaling Technologies, Beverly, Mass) were used at the manufacturer’s suggested concentrations.

**Cell Proliferation Assay**

AoSMCs (10<sup>4</sup>) were cultured for 8 hours in 96-well plates in serum-containing media as noted above. For our experiments, the cells were serum starved for 24 hours for cell cycle synchronization. AoSMCs were treated with vehicle control (dimethyl sulfoxide), 100 nmol/L rapamycin,<sup>26</sup> 10 μmol/L U0126,<sup>27</sup> or 20 μmol/L LY-294002.<sup>28</sup> Rapamycin, U0126, and LY294002 were purchased from LC Laboratories (Woburn, Mass). The cell proliferation rate was determined with use of the WST-1 cleavage-based cell proliferation (Chemicon International, Temecula, Calif) as described.<sup>29</sup>

**Reverse-Transcriptase and Real-Time Polymerase Chain Reaction**

Total RNA was extracted from AoSMCs with Rneasy Mini (Qiagen, Valencia, Calif) and from endothelial cell preparations and aorta with Trizol (Invitrogen, Carlsbad, Calif). RNA was quantified by ultraviolet absorption at 260 nm. Total RNA (1 μg) was reverse transcribed using high-capacity cDNA reverse transcription (Applied Biosystems, Foster City, Calif). Transcripts of interest were then amplified with specific primers. The following primers and predicted sizes were used: Nf1 (194 bp), 5′-GTA TTG ATT TGA AGC ACC TTG GTT TGG C′ (forward) and 5′-CGT CCC AAG GCT CCC CCA G′ (reverse); Cre (400 bp), 5′-ATC CTC CCA CCA TCA CGT CG′ (forward) and 5′-CGT TTG CTG AGC ATA CCT GGA G′ (reverse); GAPDH (306 bp), 5′-CGG AGT CAA CGG ATT TGG TCG TAT 3′ (forward) and 5′-AGC CTT CTC CAT GGT GAG GAC GAC 3′ (reverse). Amplification products were visualized on 1.5% agarose gels and visualized by ethidium bromide staining. For real-time polymerase chain reaction (PCR), PCR products were synthesized from cDNA with TaqMan probe sets for Nf1 and GAPDH (Applied Biosystems, Foster City, Calif). Relative gene expression levels were quantified by the use of the comparative threshold method with GAPDH serving as the endogenous reference gene.

**Statistical Analysis**

All data are expressed as mean±SEM. One-way ANOVA was used to compare intimal area, intima-to-media ratio, percentage of luminal narrowing, and cellular proliferation between wild-type, Nf1<sub>smKO</sub>, and Nf1<sub>smKO</sub>+GRD mice. Unpaired Student’s t test was used to compare BrdU-positive and pERK-positive cells between wild-type and Nf1<sub>smKO</sub> mice. Values of P<0.05 were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Arteries in Nf1<sub>smKO</sub> Mice Appear Structurally Normal**

To elucidate the role of Nf1 in smooth muscle, we generated Nf1<sub>smKO</sub> mice using the Nf1<sup>−/−</sup>-null allele (Nf1<sup>+/−</sup>),<sup>18</sup> Nf1<sup>−/−</sup>-flox allele (Nf1<sup>+/−</sup>),<sup>19</sup> and the SM22α-Cre mouse, which expresses Cre recombinase in smooth muscle.<sup>20</sup> Specifically, Nf1<sup>+/−</sup>, SM22α-Cre<sup>−</sup> and Nf1<sup>+/−</sup><sup>−</sup> SM22α-Cre<sup>−</sup> mice were crossed with Nf1<sup>+/−</sup> and SM22α-Cre<sup>−</sup> to generate Nf1<sup>+/−</sup><sup>−</sup>, SM22α-Cre<sup>−</sup> and Nf1<sup>+/−</sup><sup>−</sup> SM22α-Cre<sup>−</sup> mice, referred to herein as Nf1<sub>smKO</sub>. For our in vivo experiments, we used Nf1<sup>+/−</sup><sup>−</sup>, SM22α-Cre<sup>−</sup> mice to better model the environment of clinical disease. Nf1<sub>smKO</sub> mice were born alive at the expected mendelian ratios (Table) and without any apparent abnor-
malities. Adult Nf1smKO mice were fertile and appeared grossly normal. To evaluate the structure of the blood vessels, hematoxylin and eosin and Verhoeff’s elastin stains were performed on samples of large arteries in adult mice, including the aorta, renal arteries, and carotid arteries. Verhoeff’s stain was used to delineate the elastic lamina and intimal structures. No obvious abnormalities were seen in sections of descending aorta and renal artery in adult mice (data not shown). Likewise, in the carotid artery, wild-type and Nf1smKO mice exhibited similar-appearing overall structure and elastic laminae (Figure 1A and 1B). Smooth muscle α-actin expression was similar in the smooth muscle layers in the arteries from both wild-type (Figure 1C) and Nf1smKO (Figure 1D) mice.

To establish the loss of Nf1 from the smooth muscle of Nf1smKO mice, we compared the smooth muscle expression of Nf1 by reverse-transcriptase PCR using cultured AoSMCs from these mice. In Nf1smKO AoSMCs, Nf1 mRNA levels were decreased markedly compared with wild-type AoSMCs (Figure 1E). Quantification using real-time PCR indicated that Nf1smKO AoSMCs displayed 14% of wild-type transcript levels (Figure 1F). This dramatic reduction in Nf1 transcript still likely represents an overestimate of the presence of Nf1 transcript resulting from contamination from non–smooth muscle cells in the primary culture preparations.

### Table. Loss of Smooth Muscle Nf1 Does Not Lead to Embryonic or Perinatal Lethality

<table>
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<tr>
<th>Genotype</th>
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<tr>
<td>Nf1+/−</td>
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<td>5</td>
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<tr>
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<td>19</td>
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<tr>
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<td>16.5</td>
<td>4</td>
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<td>4</td>
</tr>
<tr>
<td>Nf1+/−, SM22α-Cre+</td>
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</tr>
<tr>
<td>Total</td>
<td>88</td>
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SM22αCre+ indicates 1 or 2 copies of SM22αCre. Crosses between Nf1+/− SM22αCre+ × Nf1+/− SM22αCre− generate live-born offspring at the expected mendelian ratios. Offspring lacking Nf1 in smooth muscle were born at the expected numbers without any apparent abnormalities and were able to develop and breed normally.

We used this model because of the consistency of injury we could achieve, simplifying subsequent analysis compared with other models in which endothelial injury is more diffuse and less demonstrably uniform.

We compared the vascular injury response of wild-type and Nf1smKO mice, harvesting both carotid arteries for histological analysis (1 ligated and 1 contralateral control) 28 days after unilateral ligation. Compared with wild-type mice, the Nf1smKO mice displayed an exaggerated vascular remodeling response that was marked by abnormally prolific neointimal formation (Figure 2A, 2B, 2D, and 2E). There was a robust, 2.5-fold increase in intimal thickening in Nf1smKO mice (9561 ± 344 versus 3792 ± 64 μm² in wild type; 6 mice in each group; P < 0.001; Figure 2G). Nf1smKO mice also showed an increased intima-to-media ratio (2.4-fold; P < 0.001) and intima-to-lumen ratio (3.1-fold; P < 0.001) compared with wild-type mice (Figure 2H and 2I). Most cells in the neointima were identified as smooth muscle cells by staining for anti–smooth muscle α-actin (Figure 2D and 2E).

In summary, loss of Nf1 from smooth muscle was associated with a consistent, profound hyperproliferation of the intima after vascular injury. These changes, consistent with the types of vasculopathy seen in NF1, suggest an important role for Nf1 in the regulation of the vascular injury response.

### Loss of Nf1 From Smooth Muscle Is Associated With Increased Cell Proliferation

#### In Vivo and In Vitro

Growth and proliferative abnormalities in tissues lacking Nf1 have been well documented.16,30 To understand the anatomic abnormalities that developed in injured vessels in the Nf1smKO mice, we examined the proliferation of smooth muscle in vivo and in vitro. To evaluate cellular proliferation in vivo, we quantified incorporation of BrdU in the injured arteries. Injured vessels from both wild-type and Nf1smKO mice had BrdU-positive cells, indicating cellular proliferation after vascular injury. However, there was a marked increase in the number of BrdU-labeled cells in the arteries of Nf1smKO mice. Loss of Nf1 from smooth muscle in vivo led to a significant increase in BrdU-positive cells in the neointima (27 ± 3 versus 7 ± 1; n = 6 mice; P < 0.005; Figure 3A through 3C). There was not, however, an appreciable difference in the number of cells undergoing apoptosis in these samples (data not shown). Thus, the vascular anatomic abnormalities that developed after injury seen with the loss of Nf1 from smooth muscle are associated with a conjugate increase in cellular proliferation in vivo.

To address the role of Nf1 in smooth muscle cell proliferation in vitro, we examined cell proliferation in isolated vascular smooth muscle cells. Specifically, we isolated AoSMCs from wild-type and Nf1smKO mice and compared their proliferation under a variety of conditions (Figure 4A). Under serum-free conditions, Nf1smKO AoSMCs had a marked increase in proliferation over wild-type AoSMCs by 2 hours, which continued up to 8 hours. Taken together, our results from in vivo and in vitro analyses of smooth muscle cell growth strongly suggest a cell-autonomous role for Nf1 in the regulation of smooth muscle proliferation.
Intimal Hyperplasia in \(Nf1\)smKO Mice Is Associated With an Upregulation of Mitogen-Activated Protein Kinase and mTOR Signaling

Loss of \(Nf1\) expression and consequent loss of neurofibromin protein are associated with elevations in activated Ras and in Ras downstream effectors in a number of cell types. In particular, loss of \(Nf1\) has been associated with activation of elements of the mitogen-activated protein kinase (MAPK) signaling pathway. Ras stimulation of the MAPK/extracellular regulated kinase (ERK) pathway often is required for cell proliferation. Inhibitors of MEK have been used to block the growth of some tumors. Indeed, when we examined injured arteries for evidence of hyperactivation of downstream pathways of Ras activation, we found a marked increase in the arteries from \(Nf1\)smKO compared with wild-type mice. We first looked at pERK, an element of the MAPK signaling pathway. In \(Nf1\)smKO mice, we detected significantly more pERK-positive cells than wild-type mice (50/11006 versus 9/11006; \(n=6\); \(P<0.0001\); Figure 3D, 3E, and 3F), suggesting a role for upregulated MAPK in the intimal hyperplasia of \(Nf1\)smKO mice. This in vivo finding was reinforced by our examination of MAPK signaling in AoSMCs (Figure 4B). We found an increase in pERK in \(Nf1\)smKO AoSMCs relative to wild-type cells.

In addition to alterations in MAPK signaling, the loss of \(Nf1\) has been shown to lead to abnormalities in the mTOR
signaling pathway.\textsuperscript{21,31,32} TOR signaling has been shown to be important in many aspects of cell behavior, including sensitivity to nutrients and growth factors,\textsuperscript{36–38} and has been implicated in a number of diseases with abnormal cellular growth and tumor formation.\textsuperscript{39,40} We found that \textit{Nf1} ablation was associated with upregulation of phosphorylated mTOR and its downstream signaling effector S6 kinase (S6K) in cultured AoSMCs (Figure 4B). These findings are consistent

Figure 2. Abnormal proliferative response to vascular injury with the loss of \textit{Nf1} from smooth muscle. Representative low-power (40×) photomicrographs of cross sections from the carotid arteries of adult wild-type (A and D), \textit{Nf1}smKO (B and E), and \textit{Nf1}smKO+GRD (C and F) mice collected 28 days after carotid artery ligation. \textit{Nf1}smKO (B and E) arteries show a marked increase in tissue growth versus wild type (A and D) and \textit{Nf1}smKO+GRD (C and F). Although there is increased tissue in all layers of the artery, there is a particular increase in tissues on the luminal side of the inner elastic lamina (blue arrowheads), the inner-most ring of elastin-stained tissue (A through C). Smooth muscle α-actin staining (D through F) demonstrates that the cells making up the new growth are largely smooth muscle. In particular, the neointima of \textit{Nf1}smKO (E) stains quite strongly for smooth muscle α-actin. Quantitative morphometric image analysis of intimal area (μm²) (G), the intima/media ratio (H), and the intima-to-lumen ratio (I) all demonstrate significant increases for \textit{Nf1}smKO over wild type and \textit{Nf1}smKO+GRD. For each of these measures, there was no significant difference between wild type and \textit{Nf1}smKO+GRD. For each value, n=6 animals, with 2 sample sections each at prescribed distances from the ligation.
with the phenotype of increased growth of the Nf1smKO AoSMCs and the increased injury-induced intimal proliferation in the Nf1smKO carotid arteries we observed. Collectively, these data indicate a mechanism for the increased proliferation in the smooth muscle of Nf1smKO that is likely Ras dependent.

Expression of NF1 GRD in Smooth Muscle Rescues Proliferation Defects, Cell Signaling Abnormalities, and Injury-Induced Intimal Hyperplasia of Nf1smKO

The role of neurofibromin as a Ras-GAP has been well documented.17,21,41–44 However, work from our laboratory and others has suggested that neurofibromin may have other functions aside from Ras regulation.21,45,46 To determine whether the abnormalities that we observed in Nf1smKO smooth muscle were due to the loss of neurofibromin Ras-GAP activity, we sought to rescue function with the isolated human NF1 GRD domain in vitro and in vivo. We have recently reported the characterization of a mouse model in which the isolated NF1 GRD can be expressed in a tissuespecific manner under the control of Cre recombinase.21 We crossed these mice with Nf1smKO mice to produce mice that lacked neurofibromin in smooth muscle but also expressed an epitope-tagged NF1 GRD in this tissue. We isolated AoSMCs from wild-type, Nf1smKO, and Nf1smKO mice that also expressed the Rosa-HA-GRD knock-in allele (Nf1smKO+GRD).21 We examined cellular proliferation and Ras-dependent signaling pathways in these AoSMCs and noted that alterations induced by loss of NF1 were markedly attenuated with NF1 GRD expression (Figure 4).

At baseline, the proliferation of Nf1smKO+GRD AoSMCs was markedly reduced compared with Nf1smKO alone. Compared with wild-type AoSMCs, the proliferation of Nf1smKO+GRD AoSMCs was only marginally elevated (Figure 4A). When we examined the cell-signaling profile of Nf1smKO+GRD AoSMCs, we found that the abnormal elevations of pERK and pS6K seen in Nf1smKO were rescued, returning close to wild-type levels, a trend observed in each of 3 independent experiments (Figure 4B and 4C). Expression of the NF1 GRD was able to rescue the cell proliferative and cell signaling effects we observed in AoSMCs resulting from the loss of NF1. In sum, these results strongly suggest a cell-autonomous, Ras-dependent role for NF1 in the regulation of smooth muscle proliferation.

We also examined the ability of the isolated NF1 GRD to rescue smooth muscle NF1 loss in vivo. We compared the carotid injury response of Nf1smKO adult littermates with and without expression of NF1 GRD in smooth muscle. As before, we compared neointimal proliferation between wild-type, Nf1smKO, and Nf1smKO+GRD mice 28 days after carotid artery ligation (Figure 2). The uninjured carotid vessels appear similar in each group (data not shown). As mentioned, carotid ligation of the Nf1smKO mice produces exaggerated neointimal formation compared with wild-type mice (Figure 2B and 2E), whereas in mice expressing NF1 GRD in smooth muscle cells lacking NF1, neointimal formation is markedly reduced compared with Nf1smKO and shows little difference from wild type.

Figure 3. Increased vascular neointimal formation in Nf1smKO mice is associated with an increased cell proliferation and upregulation in MAPK signaling. Representative low-power (40×) photomicrographs of sections from wild-type (A and D) and Nf1smKO (B and E) carotid arteries 28 days after ligation. Sections were stained with anti-BrdU (A and B) or anti-pERK (D and E) antibodies and quantification of positive cells for BrdU (C) and pERK (F) staining per section (n=4 per sample) in the neointima. BrdU staining is markedly increased in Nf1smKO arteries (B) vs wild type (A). Quantification (C) demonstrates a significant (P<0.005) increase in BrdU-positive cells in Nf1smKO. Staining for pERK also was increased in Nf1smKO (E) vs wild type (D). The number of pERK-positive cells in the neointima of Nf1smKO vessels was ~5 times greater than wild type (P<0.00001) (F).
data indicate that smooth muscle–specific expression of NF1 GRD expression and blockade of Ras downstream effectors. A, Proliferation of AoSMCs is increased with the loss of NF1 and is rescued by NF1 GRD expression. Cell proliferation was assessed by WST-1 cleavage to formazan and its absorbance at 440 nm. In serum-free conditions, there was a marked increase in the proliferative rate of Nf1smKO AoSMCs versus wild type. Nf1smKO+GRD, however, showed growth characteristics similar to wild type. Treatment of Nf1smKO AoSMCs with drugs blocking mTOR (rapamycin), MAPK (U0126), and PI3K (LY294002) attenuated their increased proliferation. AoSMCs not treated with drug were given vehicle control (dimethyl sulfoxide). B, Increases in Ras downstream effectors from loss of AoSMC NF1 were ameliorated by NF1 GRD expression. Western blots of pERK, p-mTOR-2448, p-mTOR-2481, and pS6K showed increases relative to total levels in Nf1smKO relative to wild type. These changes were ameliorated in Nf1smKO+GRD AoSMCs. β-Actin was used as a loading control. C, Quantification of relative intensities of pERK and pS6K derived from 3 Western blot experiments using independent protein isolates from AoSMCs collected from 2 mice of each genotype. Values for Nf1smKO and Nf1smKO+GRD are normalized to wild type (±SEM).

### Discussion

This report describes the first in vivo model for the development of obstructive vascular disease in NF1. Our model recapitulates the classic anatomic findings seen in NF1 vasculopathy, including neo-intimal proliferation of vascular smooth muscle cells.

Furthermore, our in vitro and in vivo results indicate that the loss of NF1 Ras-GAP activity from vascular smooth muscle cells is responsible for this phenotype. Loss of NF1 from vascular smooth muscle leads to an exaggerated injury response that is associated with hyperactivation of Ras and its downstream effectors, ERK, S6K, and mTOR. This effect is ameliorated both in vitro and in vivo by the expression of the NF1 GRD in vascular smooth muscle cells. Furthermore, our in vivo results are paralleled by alterations in known signaling molecules downstream of activated Ras and increases in the proliferation of isolated AoSMCs that were attenuated with the blockade of those pathways. Vascular disease is responsible for significant morbidity in NF1, and the development of an in vivo model of this condition is critical for obtaining a better understanding of the pathophysiology of the disease in humans, as well as the development and testing of novel therapeutics. In the future, it will be of interest to extend the present results to human vascular smooth muscle cells derived from NF1 patients. It is worth noting that NF1 patients are heterozygotes and that it is not known if there is loss of heterozygosity in some or all vascular smooth muscle lesions.

Although neurofibromin exhibits well-characterized Ras-GAP activity, it remains unclear which aspects of the human disease caused by loss of NF1 are directly and solely attributable to altered Ras signaling and which disease manifestations might be treatable by modulating Ras or its downstream effectors. A high degree of sequence conservation across evolution outside the GRD is consistent with empirical data that suggest additional functions of neurofibromin unrelated to Ras modulation. Hence, it is critical to define Ras-dependent and Ras-independent functions of NF1 in animal models. Our results confirm and significantly extend a
prior report that focused on haploinsufficient NFI+/− smooth muscle cells examined in culture that highlighted ERK activation.17 The Ras signaling pathway also has been previously implicated in vascular smooth muscle cell proliferation and the vascular injury response. For example, mitofusin-2 (also known as the hyperplasia suppressor gene) modulates vascular smooth muscle cell proliferation and vascular injury in an ERK-dependent manner.48 Likewise, the MAPK pathway is activated in vascular injury response. For example, mitofusin-2 (also known as the hyperplasia suppressor gene) modulates vascular smooth muscle cell proliferation and vascular injury in an ERK-dependent manner.48

It is important to establish the specific activity of neurofibromin that is responsible for vascular disease in NFI, and our results demonstrate that it is the Ras-GAP function of the protein that is critical, at least in this mouse model.

Furthermore, our data that implicate activation of the S6K/mTOR pathway in injured NFI-deficient arteries are consistent with recent reports of mTOR inhibition downstream of Ras in other NFI models31,32 and with a large body of work that confirms the vital role of this pathway in vascular disease. Potent pharmacological inhibitors of in-stent restenosis that are accepted in clinical practice, including sirolimus (rapamycin), function by inhibiting mTOR activation, and rapamycin inhibits vascular smooth muscle proliferation.49 Thus, it seems likely that sirolimus-coated stents might be especially efficacious in preventing restenosis in NFI-related vascular disease.

One interesting aspect of our model is the apparent lack of vascular disease in the uninjured animals despite a nearly complete loss of NFI from vascular smooth muscle. This is consistent with the finding that vascular disease in NFI appears to develop over time,11,52 probably as the result of a pathological response to otherwise normal stresses within the vasculature. The profound neointimal proliferation from injury that we observed suggests that loss of NFI or other perturbations that result in elevated Ras activity in vascular smooth muscle may sensitize the vasculature to minor traumas, including shear stress. An important aspect of this finding is that preventive or prophylactic therapy such as the pharmacological targeting of Ras, mTOR, or other effectors may play a central role in the prevention of the development of obstructive vascular disease in NFI. Even drugs with a moderate degree of activity against the presumptive targets, when administered early, may be able to prevent what is likely a vicious cycle of arterial injury leading to hyperplasia, stenosis, and further injury.

The study of mouse models of NFI has led investigators to propose that various aspects of the human disease are correlated with hyperactivation of Ras. For example, both human patients and mouse models exhibit learning disorders. In mouse models, these defects can be attenuated by genetic and pharmacological inhibition of Ras activity.53 Interestingly, inhibition of hydroxymethyl glutaryl coenzyme A reductase attenuates Ras signaling in the brain and improves learning defects in NFI haploinsufficient mice.54 These observations are consistent with the effects of lovastatin, a hydroxymethyl glutaryl coenzyme A reductase inhibitor, on p21Ras isoprenylation.55,56 Taken together, these observations have led to the initiation of clinical trials with hydroxymethyl glutaryl coenzyme A reductase inhibitors (statins) in NFI patients. It will be of interest to determine whether these commonly used medications will be useful for the prevention and treatment of NFI-related vascular disorders in animal models and in clinical practice.

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Disclosures

None.

References

Cardiovascular disease is a common cause of mortality in neurofibromatosis (NF1), one of the most common monogenic diseases. NF1 is thought of as a tumor and cancer predisposition syndrome, but vascular diseases such as obstructive vasculopathy and renovascular hypertension are often seen. We describe a model of NF1 vascular disease that closely mimics the human vasculopathy. Loss of smooth muscle neurofibromin, encoded by the Nf1 gene, leads to a markedly abnormal increase in smooth muscle proliferation in response to vessel injury. Neurofibromin normally attenuates Ras protooncogene activity, and loss of Nf1 from smooth muscle leads to hyperactivation of cellular pathways regulated by Ras, which normally functions to regulate cell growth and proliferation. Restoration of normal Ras activity in smooth muscle cells lacking Nf1 corrects the hyperproliferation and abnormal phenotype. This study describes a molecular mechanism for the proliferative vascular disease in NF1 and an important role for Ras activity in proliferative vascular disease in general. Knowledge of this mechanism suggests possible therapies for both of these pressing clinical conditions.
NF1 Regulates a Ras-Dependent Vascular Smooth Muscle Proliferative Injury Response
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