Neurofibromin is a novel regulator of RAS-induced signals in primary vascular smooth muscle cells

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Neurofibromatosis type I (NF1) is a genetic disorder caused by mutations in the NF1 tumor suppressor gene. Neurofibromin is encoded by NF1 and functions as a negative regulator of Ras activity. NF1 patients develop renal artery stenosis and arterial occlusions resulting in cerebral and visceral infarcts. Further, NF1 patients develop vascular neurofibromas where tumor vessels are invested in a dense pericyte sheath. Although it is well established that aberrations in Ras signaling lead to human malignancies, emerging data generated in genetically engineered mouse models now implicate perturbations in the Ras signaling axis in vascular smooth muscular cells (VSMCs) as central to the initiation and progression of neointimal hyperplasia and arterial stenosis. Despite these observations, the function of neurofibromin in regulating VSMC function and how Ras signals are terminated in VSMCs is virtually unknown. Utilizing VSMCs harvested from Nf11/2 mice and primary human neurofibromin-deficient VSMCs, we identify a discrete Ras effector pathway, which is tightly regulated by neurofibromin to limit VSMC proliferation and migration. Thus, these studies identify neurofibromin as a novel regulator of Ras activity in VSMCs and provide a framework for understanding cardiovascular disease in NF1 patients and a mechanism by which Ras signals are attenuated for maintaining VSMC homeostasis in blood vessel walls.

INTRODUCTION

Mutations in the NF1 tumor suppressor gene cause neurofibromatosis type I, an autosomal dominant disorder with an incidence of 1 in 3000 (1,2). Neurofibromin, the protein encoded by NF1, functions as a GTPase activating protein (GAP) for Ras by accelerating the conversion of active Ras-GTP to inactive Ras-GDP (3–7). One of the least studied complications of NF1 involves disorders of the cardiovascular system and the initiation of angiogenesis in cutaneous and plexiform neurofibromas.

Some NF1 patients develop vascular lesions including renal artery stenosis, arterial aneurysms and arterial occlusions resulting in cerebral and visceral infarcts (8–12). The vessels of an affected NF1 patient are often characterized by lumen occlusion and intimal wall hyperplasia (10–12). In addition to a genetic predisposition to premature cerebrovascular disease, ~95% of NF1 patients develop neurofibromas, which are highly vascular, and the endothelial cells within the tumors are densely coated with pericytes and vascular smooth muscle cells (VSMCs) (13–16). Consistent with this histologic analysis, a recent study demonstrated that Nf1+/− pericytes have increased proliferation in vivo though the biochemical mechanism for this observation was not determined (16). Despite these observations, the biochemical mechanism by which neurofibromin potentially regulates VSMC or pericyte function is unknown.

Mice harboring genetic mutations that increase signaling through the platelet-derived growth factor (PDGF)-Ras signaling axis develop an exaggerated neointimal hyperplasia and arterial occlusive disease reminiscent of the cerebrovascular complications, which develop in some NF1 patients (17,18). In addition, PDGF activates multiple Ras effector pathways, which controls both VSMC proliferation and migration, but the mechanisms by which these Ras-induced signals are attenuated are not completely understood (19–22). Based on these
prior clinical observations and the importance of Ras in coordinating signals in VSMC to control proliferation and migration, we hypothesized that neurofibromin functions as a negative regulator of Ras activity in VSMCs. Utilizing VSMCs harvested from \( Nf1^{+/−} \) mice and primary human neurofibromin-deficient VSMCs, we identify a discrete Ras effector pathway, which is tightly regulated by neurofibromin to limit VSMC proliferation and migration in response to PDGF.

RESULTS

\( Nf1^{+/−} \) VSMCs have increased migration and proliferation in response to PDGF-BB compared with WT controls

PDGF-BB stimulates VSMC proliferation and migration and is an important regulator of VSMC homeostasis in the blood vessel wall (19–22). Therefore, we tested whether heterozygous inactivation of \( Nf1 \) alters either the proliferation and/or migration of VSMCs in response to PDGF-BB. We isolated VSMCs from the aortas of \( Nf1^{+/−} \) and WT mice as previously described (19). The cells isolated from both experimental genotypes expressed \( α \)-smooth muscle actin (\( α \)-SMA) and calponin (Fig. 1A and B), but did not express CD31, which is an endothelial cell antigen (Fig. 1C). These studies confirmed that we isolated VSMCs but not endothelial cells.

To compare the proliferation of WT and \( Nf1^{+/−} \) VSMCs in response to PDGF-BB, we serum-starved WT and \( Nf1^{+/−} \)-VSMCs and performed thymidine incorporation assays. VSMCs were cultured in either 20 ng/ml PDGF-BB or media alone without growth factors or serum, and DNA synthesis was measured utilizing thymidine incorporation assays. Although there were no differences in baseline proliferation between the two experimental genotypes, \( Nf1^{+/−} \) VSMCs had a 2-fold increase in proliferation in response to PDGF-BB when compared with WT controls (Fig. 2A). Fluorescence-activated cells sorting (FACS) analysis of VSMCs did not detect differences in the expression of the PDGF-BB receptor between the two experimental genotypes to account for differences in proliferation (data not shown).

We next compared the migration of \( Nf1^{+/−} \) and WT VSMCs to PDGF-BB utilizing transwell haptotaxis assays. \( Nf1^{+/−} \) and WT VSMCs were serum-starved and placed in the upper chamber of a gelatin-coated transwell. Either culture media without serum and growth factors or 20 ng/ml PDGF-BB was placed in the lower well of the transwell to stimulate migration. After 3 h, the cells were stained with hematoxylin and counted to identify migrated VSMCs. Although there were no differences in baseline migration between the two experimental genotypes, \( Nf1^{+/−} \) VSMCs had a 2-fold increase in migration to PDGF-BB when compared with WT controls (Fig. 2B). These experiments demonstrate that heterozygous inactivation of \( Nf1 \) increases both the proliferation and migration of VSMCs in response to PDGF-BB.

Increased migration and proliferation of \( Nf1^{+/−} \) VSMCs is mediated via hyperactivation of Erk

PDGF-BB binding to its receptor activates both the Ras-Erk and PI-3 kinase-Akt signaling pathways, which regulate the proliferation and migration of VSMCs (19–22). Neurofibromin is expressed in VSMCs (23) and functions as a GAP for Ras in some cell lineages (24–26). However, it remains unclear whether heterozygosity of \( Nf1 \) alters activation of the Ras-Erk or PI-3 kinase pathways in VSMCs to alter their proliferation and migration.

To address this question, serum-starved \( Nf1^{+/−} \) and WT VSMCs were stimulated with 20 ng/ml PDGF-BB and assayed for changes in Ras-GTP levels. Even though \( Nf1^{+/−} \) VSMCs had detectable, but reduced levels of neurofibromin as determined by western blot (Fig. 3A), \( Nf1^{+/−} \) VSMCs had increased PDGF-BB-stimulated Ras activity when compared with WT cells (Fig. 3B). However, there were no differences in baseline Ras activity between the two experimental genotypes (Fig. 3B). We next tested whether there were differences in Erk and Akt activation between \( Nf1^{+/−} \) and WT VSMCs in response to PDGF-BB. \( Nf1^{+/−} \) and WT VSMCs were serum-starved and stimulated with 20 ng/ml PDGF-BB. Cells were lysed and Akt and Erk activation were measured by western blot. \( Nf1^{+/−} \) VSMCs demonstrated increased Erk activation in response to PDGF-BB when compared with WT controls (Fig. 3C). Interestingly, we did not detect differences in Akt activation between the two experimental genotypes (data not shown), even though PI-3 kinase is hyperactivated in other neurofibromin-deficient cell types (26–31).

Previous experiments have demonstrated that Erk activation controls VSMC migration and proliferation in response to PDGF-BB (21). Given that \( Nf1^{+/−} \) VSMCs have increased Erk activation, we tested whether chemical inhibition of Erk activity with a Mek inhibitor (PD98059) would reduce the proliferation and migration of \( Nf1^{+/−} \) VSMCs. We compared the migration and proliferation of \( Nf1^{+/−} \) and WT VSMCs in the presence or absence of PD98059 utilizing the migration and proliferation assays described earlier. The proliferation and migration of both WT and \( Nf1^{+/−} \) VSMCs was inhibited by PD98059 (Fig. 3D and E).
siRNA reduction of neurofibromin expression in human VSMCs increases their proliferation and migration via hyperactivation of Erk

Species divergence in the activation of Ras effector pathways has been observed between some murine and human cell lineages (32). To test whether our observations in Nf1+/− VSMCs are valid in human cells, we obtained primary human VSMCs and transfected them with a siRNA directed against neurofibromin (Nf1 siRNA) or a control siRNA encoding a scrambled oligonucleotide sequence. VSMCs transfected with Nf1 siRNA express decreased levels of neurofibromin when compared with cells transfected with control siRNA (Fig. 5A). Importantly, p120GAP protein levels were not altered (data not shown). Utilizing the cell migration and proliferation assays outlined earlier, we demonstrated that VSMCs transfected with Nf1 siRNA have both increased migration and proliferation (Fig. 5B and C) in response to PDGF-BB when compared with cells transfected with control siRNA. However, there were no differences in baseline proliferation or migration between the two experimental genotypes (Fig. 5B and C). Importantly, pre-incubation of VSMCs transfected with Nf1 siRNA with PD98059 inhibited their migration and proliferation in response to PDGF-BB (Fig. 5B and C). Consistent with this observation, VSMCs transfected with Nf1 siRNA displayed increased Erk activation in response to PDGF-BB when compared with VSMCs transfected with control siRNA (Fig. 5D). Thus, these data verify that genetic reduction of neurofibromin in human VSMCs enhances Erk activation to increase VSMC migration and proliferation in response to PDGF-BB.

Nf1+/− fibroblast conditioned media (FCM) is a potent stimulus for Nf1+/− VSMC migration

Fibroblasts are a major cellular constituent of neurofibromas (33). In solid tumors, fibroblasts secrete growth factors, which recruit pericytes and VSMCs to tumor microenvironments (34). Given that Nf1+/− VSMCs have increased migration and proliferation in response to PDGF-BB and that Nf1+/− fibroblasts are a potential cellular source of PDGF in neurofibromas, we tested whether Nf1+/− fibroblasts secrete soluble factors to stimulate Nf1+/− VSMC migration. Though phenotypically distinct, the migration and proliferation of pericytes and aortic VSMCs are controlled by conserved signaling pathways, which are activated by the PDGF receptor (35). Therefore, since isolation of sufficient numbers of pericytes for in vitro experiments is problematic, we utilized aortic VSMCs for the following experiments.

We isolated and cultured fibroblasts from day 13.5 WT and Nf1+/− murine embryos and obtained FCM. To test whether WT or Nf1+/− FCM would stimulate either WT or Nf1+/− VSMC migration, we performed migration assays in response to WT or Nf1+/− FCM. WT and Nf1+/− VSMCs migrated to FCM as a potent stimulus for Nf1+/− VSMC migration (Fig. 6A). Although the migration of both VSMC genotypes was augmented in response to Nf1+/− FCM, the absolute number of Nf1+/− VSMCs migrating to Nf1+/− FCM was higher when compared with WT VSMCs (Fig. 6A). These data demonstrate that Nf1+/− fibroblasts secrete soluble factor(s) that stimulate both WT and Nf1+/− VSMC migration. However, Nf1+/− VSMCs had a 2-fold increase in migration to Nf1+/− FCM when compared with WT controls.

Identification of PDGF in Nf1+/− FCM as a potent stimulus for Nf1+/− VSMC migration

To identify chemotactic factors secreted by Nf1+/− fibroblasts, we performed protein and ELISA assays on WT and Nf1+/− FCM. These studies identified several chemokines and growth factors in both WT and Nf1+/− FCM (data not shown). Given our observations that Nf1+/− VSMCs have increased migration in response to PDGF-BB, we tested whether PDGF was a growth factor in Nf1+/− FCM, which promotes Nf1+/− VSMC migration. Although PDGF was detected in WT and Nf1+/− FCM, the concentration of PDGF was 5-fold higher in Nf1+/− FCM when compared with WT controls (Fig. 6B).
Imatinib mesylate (Gleevec) is a therapeutic agent that diminishes signaling through the PDGF receptor and is used to inhibit pericyte/VSMC recruitment to tumor microenvironments (34,36). Thus, we tested whether pre-treatment of WT and Nf1+/− VSMCs with 4 μM Gleevec would inhibit their migration to either WT or Nf1+/− FCM. Pre-incubating WT and Nf1+/− VSMCs with a therapeutic dose of Gleevec inhibited the migration of both VSMC genotypes to either WT or Nf1+/− FCM (Fig. 6C). Importantly, pre-incubation of Nf1+/− VSMCs with PD98059 inhibited their migration to Nf1+/− FCM (Fig. 6C). Thus, this observation links activation of the PDGF-Ras-Erk signaling pathway to the migration of Nf1+/− VSMCs to Nf1+/− FCM.

DISCUSSION

NF1 is a complex genetic disorder with diverse clinical manifestations including premature development of...
cerebrovascular disease in some patients and nearly universal progression of cutaneous and plexiform neurofibromas arising in different anatomical locations (8–12). In this study, we identified a neurofibromin-deficient VSMC phenotype, which offers mechanistic and potentially therapeutic insights into both the development of NF1 vasculopathies and the recruitment of pericytes and VSMCs into the neurofibroma microenvironment.

Vasculopathy is a recognized manifestation of NF1 and can produce significant cerebrovascular complications and mortality, particularly in young patients (8–12). Specifically, NF1 patients are at increased risk for renal artery stenosis with consequent hypertension, arterial occlusion resulting in cerebral and visceral infaracts, and vessel aneurysms (8–12). In support of these observations, a recent report showed that the median age of death reported on death certificates of 3253 individuals with NF1 was approximately 15 years less than expected when compared with control subjects (37). Strikingly, a diagnosis suggestive of NF1 vasculopathy was listed 7.2 times more often than expected among NF1 patients less than 30-years-old and 2.2 times more often than expected among those who were 30–40-years-old (37). Further, another study demonstrated that 2.5% of children with NF1, who underwent a brain MRI, had an abnormality of the cerebrovascular system including narrowed or ectatic vessels, vascular stenosis, aneurysms or moy-a-moya (8). Despite these clinical observations, the molecular mechanism for the development and increased incidence of vasculopathies in NF1 patients is completely unknown.

A hallmark of diseased vessels in NF1 patients is luminal occlusion and intimal wall hyperplasia (11). Based on the microscopic analysis of the affected vessels, it has been hypothesized that NF1 vasculopathy results from abnormal neurofibromin function in VSMCs resulting in excessive VSMC proliferation during normal vessel maintenance. This hypothesis is congruent with a recent study, which showed that conditional deletion of the NF1 gene in endothelial cells did not result in vasculopathies, but rather congenital heart disease (38,39). However, while neurofibromin is known to be expressed in VSMCs, the function of neurofibromin in controlling VSMC proliferation or migration is not known.

Utilizing VSMCs harvested from NF1+/− mice and primary human neurofibromin-deficient VSMCs, we determined that neurofibromin functions as a novel GAP for Ras in murine and human VSMCs. Specifically, neurofibromin regulates signaling through the canonical Ras-Raf-Mek-Erk pathway to control VSMC proliferation and migration in response to PDGF. In addition, we also showed that expression of the NF1 GRD alone was sufficient to restore the increased proliferation and migration of NF1+/− VSMCs to WT levels via restoration of Ras-Erk signaling. These studies provide formal genetic proof that neurofibromin functions as a GAP for Ras in VSMCs.

These observations are intriguing and provide insights into NF1 vasculopathy given the emerging paradigm in vascular biology where tight control of the PDGF-Ras-Erk signaling axis in VSMC is critical for maintaining VSMC homeostasis in blood vessel walls and preventing premature development of vascular occlusive disease. Prior animal studies indicate that either increased or prolonged Ras activation augments VSMC proliferation and migration, and the subsequent development of vascular lesions (17,40–47). These vascular lesions are characterized by intimal wall hyperplasia that ultimately leads to occlusive vascular disease (17,40–47). In support of the importance of Ras in development of occlusive vascular disease, adenoviral-mediated transfer of a dominant negative H-Ras into VSMCs of vessel walls inhibits the development of stenotic lesions in rats after mechanical arterial injury by inhibiting the proliferation and migration of VSMCs (42–45). Similar results were obtained when animals were treated with a chemical Ras farnesyltransferase inhibitor, which blocks Ras activation, prior to arterial mechanical injury (46,47).

The importance of Ras activation in neointima formation was further highlighted in recent studies utilizing Grb2+/− mice (17). Grb2 is a critical signaling protein that facilitates Ras activation by receptor tyrosine kinases including the PDGF-β receptor (PDGF-βR) (17). PDGF-βR is released locally by platelets, and other cells in arteries after injury (48). Similar to prior observations utilizing Ras inhibitors, Grb2+/− mice were resistant to neointima development following vessel injury when compared with WT control mice (17). Further, VSMCs harvested from Grb2+/− mice...
demonstrated decreased proliferation and Erk activation in response to PDGF-BB in vitro (17).

Finally, when genetically engineered mice harboring a constitutively active PDGF receptor in smooth muscle cells were intercrossed with low-density lipoprotein receptor knockout mice (Ldlr<sup>-/-</sup>), which have a predisposition to developing atherosclerosis, the mutant progeny developed aneurysms, and a marked susceptibility to cholesterol-induced atherosclerosis (18). The mutant mice also showed hyperproliferation of VSMCs and increased Erk activation in vivo (18). Consistent with the central pathogenic role of hyperactivation of the PDGF-receptor and Erk signaling pathway in controlling VSMC proliferation in vivo, mutant mice treated with Gleevec, which is an inhibitor of PDGF receptor signaling, did not develop premature atherosclerosis or aneurysms (18). Collectively, these genetic studies demonstrate that hyperactivation of PDGF-Ras signaling pathway activates a discrete set of biochemical effectors, which potentiates VSMC proliferation and migration in vivo. Given the phenotype of neurofibromin-deficient VSMCs described in our current study, it is likely that abnormal activation of the PDGR-Ras-Erk pathway contributes at least in part to NF1 vasculopathies. To extend the current studies, we are developing mice where Nf1 is conditionally deleted in VSMCs to further enhance our mechanistic understanding of NF1 vasculopathies in vivo.

Although these studies offer significant insights into the development of vasculopathies in NF1 patients, they also identify potential therapeutic targets in treating neurofibromas, which are pathognomonic for NF1. Neurofibromas are highly vascular and endothelial cells within the tumors are densely coated with pericytes and VSMCs (13–16). We and others have recently demonstrated that Nf1<sup>+/−</sup> endothelial cells have increased migration and proliferation in response to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor via hyperactivation of the Ras-Erk pathway (Munchhof et al., submitted) (49,50). In addition, a recent study demonstrated that Nf1<sup>+/−</sup> pericytes have increased proliferation in vivo though the biochemical mechanism for this observation was not determined (16).

Although pharmacologic targeting of endothelial cells to inhibit the growth and metastasis of solid tumors is a well-established concept, an emerging paradigm in tumor
angiogenesis is the functional importance of pericytes and VSMCs, which cover blood vessels and provide microvascular stability (34,51). The vascular wall is composed of two principal cell types, endothelial and mural cells (35). Depending on the morphology and density, mural cells are referred to as either pericytes or VSMCs, though the migration and proliferation of both cell types is controlled by conserved signaling pathways, which are activated by the PDGF receptor (35). Pericytes and VSMCs are recruited to and maintained in tumor microenvironments in response to local concentrations of PDGF produced by cells within the tumor stroma, including fibroblasts (34,36). In support of this concept, several studies have shown that tumor vessels lacking pericytes are more dependent on VEGF for their survival than are vessels invested by pericytes (52). Further, treatment of mice genetically engineered to mimic human tumors with both an anti-VEGF drug and Gleevec, which inhibits pericyte recruitment and proliferation by directly interfering with PDGF signaling, was highly efficacious (36).

In the current studies, we demonstrate that both human and murine neurofibromin-deficient VSMCs have increased migration and proliferation to PDGF via increased activation of the Ras-Erk pathway. Consistent with other tumor models (34), we also show that Nf1-/- fibroblasts secrete increased concentrations of PDGF to increase the migration of Nf1-/- VSMCs. Importantly, we show that migration of Nf1-/- VSMCs to Nf1/-/- FCN is completely inhibited by Gleevec. Although our data cannot eliminate the possibility that Gleevec reduces the migration of Nf1-/- VSMCs to Nf1/-/- FCN via inhibition of the c-kit receptor tyrosine kinase signaling pathway, which is a known target of this drug (34), it is unlikely given that kit ligand is not a potent proliferative or chemotactic stimulus for VSMC. Finally, it is possible that within the neurofibroma microenvironment, Nf1-/- mast cells, which are the major immune cell resident in these tumors (26,27), secrete increased concentrations of PDGF to increase the migration and proliferation of Nf1-/- VSMCs. Given that mast cells can secrete PDGF (53), we are actively testing this hypothesis in the laboratory in order to identify other therapeutic targets in neurofibromas. Nevertheless, based on these observations, it is possible that treating NF1 patients with both Gleevec and an FDA-approved anti-VEGF inhibitor may be a particularly attractive strategy for treating plexiform neurofibromas. However, it is imperative to test the effect of Gleevec and anti-VEGF inhibitors on other Nf1-/- cell lineages, which exist outside the neurofibroma microenvironment, prior to initiating human clinical trials given the potential toxicities of these drugs on these cells.

In summary, we have identified a previously unrecognized biochemical and cellular phenotype in neurofibromin-deficient VSMCs. These observations should provide the initial framework for understanding cerebrovascular disease in NF1 patients and the development of mouse model of NF1 vasculopathy, which is currently in progress. Finally, given the conservation of signaling pathways in VSMCs and pericytes and our current experimental data, we are actively testing whether Gleevec and an anti-VEGF drug prevents or delays plexiform neurofibroma development in validated mouse models of NF1 (54).

MATERIALS AND METHODS

Animals

Nf1-/- mice were obtained from Dr Jacks at MIT in a C57BL/6.129 background and backcrossed for 13 generations into the C57BL/6J strain. The Nf1 allele was genotyped by polymerase chain reaction as previously described (28). Experiments were conducted in accordance with a protocol approved by the Indiana University Animal Use and Care Committee.

Murine VSMC isolation and culture

VSMCs were obtained by outgrowth from explants of Nf1-/- and WT thoracic aortas. VSMCs were cultured in Dulbecco’s Modified Eagles Medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 1.5% N-(2-hydroxy ethyl) pipperazine-N’-(2-ethane sulfonic acid) (HEPES) (Cambrex, Walkersville, MD, USA), 100 U/ml streptomycin (Cambrex) and 100 µg/ml penicillin (Cambrex).
in a 37°C, 5% CO₂-humidified incubator. Media was replaced every 2 days. Cultures were passed by exposure to trypsin (0.5 mM)-EDTA (0.5 mg/ml, Invitrogen) and washed with Hank’s Balanced Salt Solution (Invitrogen) before re-plating at a density of 3500 cells/cm². Experiments were performed using cells of passage 1–3.

**Human VSMC culture**

Human aortic VSMCs were obtained from Cambrex at passage 3. Cells were seeded in 75 cm² tissue culture flasks and cultured with Smooth muscle Growth Medium (SmGM-2, Cambrex) in a 37°C, 5% CO₂-humidified incubator. The SmGM-2 media contained Smooth muscle Basal Medium (SmBM-2, Cambrex) supplemented with a smooth muscle cell bullet kit (Cambrex) and 100 U/ml streptomycin and 100 μg/ml penicillin. The culture medium was changed every 2 days. The cells were harvested by exposure to trypsin–EDTA. Experiments were conducted with cells of passage 4 and 5.

**Immunophenotyping of murine VSMCs**

VSMCs were grown on glass coverslips, washed with phosphate-buffered saline (PBS) and fixed in methanol at −20°C for 10 min. Following blocking of non-specific binding by pre-incubation with PBS containing 10% goat serum for 1 h, the cells were stained with a monoclonal anti-α-SMA (Sigma, St Louis, MO, USA) antibody directly conjugated to fluorescein isothiocyanate (FITC) at a dilution of 1:100, a monoclonal anti-calponin antibody (Sigma) at a dilution of 1:200, or anti-CD31 antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:50 at 25°C for 1 h. Cells incubated with anti-calponin or anti-CD31 were then incubated with goat-anti-mouse IgG FITC secondary antibody (BD Pharmingen, San Jose, CA, USA). Purified mouse IgG<sub>2a</sub> (BD Pharmingen) was used for an isotype control, and D4T, a murine endothelial cell line (a kind gift from Dr Gordon Keller, Mt Sinai School of Medicine), were used as a positive control for anti-CD31 staining. Coverslips were mounted in 90% glycerol/10% PBS, pH 8.0, containing, 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) to permit nuclear identification. Immunofluorescence labeling was examined using a Zeiss LSM510-Meta confocal microscopy system with a 40 × C-Apochromat/1.2NA water immersion lens (Zeiss, Thornwood, NY, USA). Images were acquired with the manufacturer’s software and assembled in Adobe Photoshop CS version 8.0.

**Murine fibroblast culture and generation of FCM**

Fibroblasts were generated from day 13.5 WT and Nf1<sup>−/−</sup> embryos. Embryo organs were removed, and the remaining tissue was incubated in DMEM containing 0.05% trypsin–EDTA for 10 min. The cell suspension was filtered after adding DMEM medium containing 10% FBS and seeded into tissue culture dishes. The cells were replated on day 5 as passage 1, and medium was replaced every 5 days. Fibroblasts used in the experiments were passage 2 and 3. FCM was harvested as previously described from cell cultures on passage 2 after 24 h of culture in serum-free DMEM (55), and the FCM was not concentrated prior to conducting experiments.

**Quantification of PDGF in murine FCM**

Quantitative concentration of murine PDGF in FCM was determined by ELISA using Quantikine M according to the manufacturer’s protocol (R&D Systems Inc., Minneapolis, MN, USA).

**Generation of recombinant retroviral plasmids**

Recombinant retrovirus constructs of Nf1 GRD were developed using the murine stem cell virus (MSCV) backbone developed by Dr Robert Hawley (56). The internal sequences of these constructs are under the transcriptional control of the myeloproliferative sarcoma retrovirus promoter. The construct contains a puromycin resistance gene, pac, which is under the transcriptional control of the phosphoglycerate kinase promoter. Using standard cloning techniques as previously described, two viruses were developed for use in these experiments as follows: (i) a virus expressing pac (MSCV-NF1 GRD-pac); (ii) a construct encoding the selectable marker gene alone (MSCV-pac) (24).

**Retroviral infection of VSMCs**

To express Nf1 GRD and its control vector in VSMCs, a clone of GP+ E86 packaging cell line, which produced retrovirus containing recombinant plasmids of Nf1 GRD and pac, and another clone of GP+ E86 packaging cell line producing retrovirus including only pac gene were used. VSMCs were transduced with the retroviruses in the presence of 8 μg/ml polybrene (Sigma) four times in a row for 2 days. Transduced cells were selected in medium containing 2 μg/ml puromycin for 7 days. Cells were used at 90% confluency.

**Transfection with siRNA**

Nf1 siRNA oligonucleotides were purchased from Ambion (Austin, TX, USA). Sense or scrambled oligonucleotides were used as a control for every transfection experiment. VSMCs were cultured in a six-well tissue culture dish to 30–50% confluency. The siRNA oligonucleotides were diluted to 100 nM in Opti-MEM (Invitrogen) and siPORT Lipid (Ambion). Transfections were conducted as per the manufacturer’s recommendations (Ambion). After 4 h, the transfection mixture was replaced with culture medium. Cells were incubated for 48 h at 37°C before harvesting for experiments.

**Thymidine incorporation assays**

Cells were deprived of growth factors for 24 h, and quiescent cells were plated in six-well dishes at 3500 cells/cm². VSMCs were stimulated with 20 ng/ml PDGF-BB (R&D Systems) for 16 h in a 37°C, 5% CO₂-humidified incubator. Cells were pulse-labeled with 1 μCi/ml of tritiated thymidine (Perkin-Elmer Life Sciences Products, Boston, MA, USA).
for 5 h, and β emission was measured (Beckman Coulter Inc., Fullerton, CA, USA) as previously described (57). In some experiments, cells were pre-incubated with 50 μM PD98059 or its vehicle 30 min before addition of cytokines. Assays were performed in triplicate.

Haptotaxis assays

For VSMC studies, the underside of transwell cell culture inserts with 8 μM pores (BD Biosciences) were coated with 0.1% gelatin for 1 h at 37°C. Inserts were placed into the lower chamber of the transwell containing 600 μl of DMEM alone or in combination with 20 ng/ml PDGF-BB or FCM. VSMCs measuring 2 × 10^4 were suspended in 100 μl of DMEM and added to the top of each insert. In some experiments, cells were incubated with 50 μM PD98059 or 4 μM Gleevec (Novartis, Boston, MA, USA) for 30 min before exposure to cytokines. Cells were incubated for 4 h in a 37°C, 5% CO₂-humidified incubator. Non-migratory cells on the membrane’s upper surface were removed with a cotton swab, and migrated cells attached to the bottom surface of the membrane were fixed with methanol at 4°C and stained with hematoxylin (Fisher Scientific Co.) for 20 min at room temperature. The average number of migrated cells per higher-power field was counted with an inverted microscope under 20× magnification. As a control, cell migration on bovine serum albumin was determined and measured ≤0.001% of the total cell population (data not shown). Assays were performed in triplicate.

Western blotting

Erk activation was determined by depriving cells of serum and growth factors for 16–20 h, followed by stimulation with 20 ng/ml PDGF-BB. Neurofibromin expression was examined using subconfluent VSMCs. Cells were lysed in non-ionic lysis buffer (20 mM Tris–HCl (Sigma), 137 mM NaCl (Sigma), 1 mM EGTA (Sigma), 1% Triton X-100 (Sigma), 10% glycerol (Sigma), 1.5 mM MgCl₂ (Sigma), and complete lysis buffer (20 mM Tris–HCl, 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂) and complete protease inhibitors (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously (28). Lysates were normalized for protein content using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL, USA). Lysates were boiled for 5 min, subjected to SDS–PAGE, and trans-blotted to nitrocellulose. The membranes were blocked with 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA) for 1 h. Membranes were incubated overnight at 4°C with the following antibodies: anti-phospho-Erk-1/2 (Cell Signaling, Beverly, MA, USA), anti-Erk-1/2 (Cell Signaling), or anti-neurofibromin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Secondary antibodies used were either anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Proteins were visualized by ECL (Amersham Pharmacia Biotech).

Ras activation assay

VSMCs were deprived of serum and growth factors for 18–24 h and stimulated with 20 ng/ml PDGF-BB. Ras activation was subsequently determined using Ras activation assay kits (Upstate USA Inc., Charlottesville, VA, USA) according to the manufacturer’s protocol and as described previously (26).

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