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ORIGINAL PAPER

Angiogenic Expression Profile of Normal and Neurofibromin-Deficient Human Schwann Cells

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Abstract Peripheral nerve sheath tumors from individuals with Neurofibromatosis Type 1 (NF1) are highly vascular and contain Schwann cells which are deficient in neurofibromin. This study examines the angiogenic expression profile of neurofibromin-deficient human Schwann cells relative to normal human Schwann cells, characterizing both pro-angiogenic and anti-angiogenic factors. Conditioned media from neurofibromin-deficient Schwann cell lines was pro-angiogenic as evidenced by its ability to stimulate endothelial cell proliferation and migration. Using gene array and protein array analysis, we found increased expression of pro-angiogenic factors and decreased expression of anti-angiogenic factors in neurofibromin-deficient Schwann cells relative to normal human Schwann cells. Neurofibromin-deficient Schwann cells also showed increased expression of several growth factor receptors and decreased expression of an integrin. We conclude that neurofibromin-deficient Schwann cells have dysregulated expression of pro-angiogenic factors, anti-angiogenic factors, growth factor receptors, and an integrin. These dysregulated molecules may contribute to the growth and progression of NF1 peripheral nerve sheath tumors.

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Introduction

Neurofibromatosis type I (NF1) is a genetic disorder of the nervous system in which there is a loss-of-function of the *NF1* gene [1]. The *NF1* gene codes for the protein neurofibromin [2, 3] which contains a GAP-related domain (GRD) [4, 5] and functions as a negative regulator of Ras [6]. Individuals with NF1 have an increased incidence of developing peripheral nerve sheath tumors including benign neurofibromas (NFB) and malignant peripheral nerve sheath tumors (MPNST). Peripheral nerve sheath tumors are highly vascular [7, 8], and MPNST tissue and NF1 tumor-derived human Schwann cells can stimulate angiogenesis in vivo [7, 9–11]. Previous studies have reported increased pro-angiogenic factor expression in NF1 tumor tissue or neurofibromin-deficient mouse Schwann cells [8, 12–16]. However, it is the balance between pro-angiogenic and anti-angiogenic factor expression that regulates the process of angiogenesis [17]. Therefore, we carried out a comprehensive analysis of neurofibromin-deficient human Schwann cells relative to normal human Schwann cells with respect to pro-angiogenic and anti-angiogenic factor expression.

This study determined which pro-angiogenic and anti-angiogenic factors have dysregulated expression in neurofibromin-deficient human Schwann cells derived from NF1 tumor tissue. The angiogenic profile of dermal NFB-derived Schwann cells and MPNST-derived Schwann cell lines were studied relative to

primary normal human Schwann cells (used as a baseline). Endothelial cell proliferation and migration in response to Schwann cell conditioned media was used to determine the overall in vitro angiogenic potential of neurofibromin-deficient Schwann cells. Gene array and protein array analysis were performed to identify factors with dysregulated expression in the neurofibromin-deficient Schwann cells. Neurofibromin-deficient human Schwann cells were found to have a dysregulated angiogenic profile, involving both pro-angiogenic and anti-angiogenic factors, leading to an in vitro angiogenic potential that favors angiogenesis.

Materials and methods

Isolation and culture of schwann cells

Human peripheral nerve (cauda equina and sciatic nerve) was obtained from Dr. Patrick Wood of the Miami Project to Cure Paralysis. Human Schwann cells were isolated as described previously [18] from tissue donated by four different individuals. Human Schwann cells were grown on 100 mm collagen type I-coated dishes (BD Biocoat, Becton Dickinson, Bedford, MA) in DMEM (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (NovaCELL I, Nova-Tech Inc., Grand Island, NE), 10 nM NRG-1β (provided by Genentech Inc., San Francisco, CA), and 0.03 mg/ml gentamycin (Invitrogen Corp., Carlsbad, CA). Human Schwann cells were used through passage 5.

Dermal neurofibroma-derived Schwann cells, designated 36 L, were isolated in our laboratory from a dermal neurofibroma resected from a patient with NF1 as previously described [19]. These NF1-derived Schwann cells were shown to be neurofibromin-deficient by Western blot analysis [19]. Neurofibroma-derived Schwann cells were grown on 100 mm collagen type I-coated dishes in DMEM supplemented with 10% FBS and 10 nM NRG-1β. Dermal neurofibroma-derived Schwann cells were used through passage 10.

MPNST-derived Schwann cell lines, sNF02.2 and sNF94.3, were kindly provided by Dr. David Muir (University of Florida, Gainesville, FL). The sNF94.3 cell line has a constitutional *NF1* microdeletion, but does not have p53 loss of heterozygosity [10]. The genotype of the sNF02.2 has not been determined. These NF1-derived Schwann cell lines were shown to be neurofibromin-deficient by Western blot analysis [19]. MPNST Schwann cell lines were grown on 100 mm collagen type I-coated dishes in DMEM supplemented with 10% FBS. MPNST-derived Schwann cells were used through passage 10.

Conditioning medium

To condition media, Schwann cells were grown in 60 mm collagen type I-coated dishes with serum containing growth media until the cells were approximately 80% confluent. The media was removed and the cells were washed twice with PBS. Since each cell type is grown in different media conditions, all cells were grown in DMEM supplemented with 5% FBS for 48 h before beginning to collect conditioned media. Cells were washed again with PBS and 1.5 ml serum-free endothelial basal media (EBM-2) (Clonetics Inc., Walkersville, MD) supplemented with 0.1% BSA was added to the cells. The cells were incubated for 20 h at 37°C. After incubation, the conditioned media was collected and centrifuged to remove any cellular debris. To determine the number of cells conditioning the media, the cells on the plate were trypsinized and counted using a hemocytometer. The total number of cells was calculated and then divided by the total number of milliliters of conditioned media to determine the number of cells conditioning one ml of media. The DC protein assay (Bio-Rad, Hercules, CA) was used to determine the amount of protein in the conditioned media.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) from Glycotech (Gaithersburg, MD) were purchased from the National Cancer Institute. HUVECs were grown on 100 mm dishes in EGM-2 growth media (Clonetics Inc., Walkersville, MD). HUVECs were used through passage 6.

Endothelial cell proliferation assay

HUVECs were seeded 3,000 cells per well into a 96-well plate and cultured for 24 h in EGM-2 after which the media was removed and the cells were washed twice with PBS. The media was replaced with either serum-free EBM-2 supplemented with 0.1% BSA (untreated condition) or Schwann cell conditioned media diluted to a concentration of 260,000 cells conditioning 1 ml of media. The cells were then cultured for 24, 48, or 72 h followed by media removal and 2 washes with PBS. The plates were frozen at –80°C until all plates were ready to be analyzed for DNA content. To obtain a DNA reading for the 3,000

cells per well plated at time 0 h, cells were allowed to adhere to the plate for 6 h followed by 2 washes with PBS. The plate was then frozen at –80°C. Nucleic acid content was assessed using the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Briefly, cells were thawed at room temperature and then lysed with a buffer containing the CyQUANT GR dye which emits fluorescence when bound to nucleic acids. The plates were read with a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems, Framingham, MA) at the excitation/emission wavelengths of 485/530 nm.

Endothelial cell migration assay

BD Biocoat Angiogenesis System for endothelial cell migration (Becton Dickinson, Bedford, MA) was used according to the manufacturer's protocol with minor modifications. HUVECs were serum-starved in EBM-2 supplemented with 0.1% BSA for 18–20 h prior to the start of the migration assay. Cells were counted and seeded on top of the membrane insert at 100,000 HUVECs in 250 μl serum-free EBM-2 supplemented with 0.1% BSA. Cells were allowed to adhere to the membrane for 1 h at 37°C followed by the addition of 750 μl of conditioned media, 1% FBS (positive control), or EBM-2 supplemented with 0.1% BSA (untreated) into the well below the membrane insert. The plate was then incubated at 37°C for 22 h. After migration, the membrane inserts were transferred to a new plate containing the cell permeant fluorescent dye Calcein AM (Molecular Probes, Eugene, OR) at a concentration of 4 μg/ml. Cells were incubated at 37°C with Calcein AM for 90 min. The plate was read from the underside of the wells to detect only the cells that had migrated to the underside of the membrane. The plate was read with a Cytofluor Series 4000 (PerSeptive Biosystems Inc., Framingham, MA) fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

Gene expression array analysis

Normal human Schwann cells and neurofibromin-deficient Schwann cells were grown on 100 mm dishes until the cells reached approximately 80% confluence. At this point, the normal growth media was removed, the cells were washed twice with PBS, and DMEM/F12 media supplemented with 5% FBS was added to the cells. After 48 h incubation with DMEM/F12 + 5% FBS, cells are washed twice with PBS and total RNA was extracted using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RNA samples were tested for purity by the 260 nm/280 nm absorbance ratio (greater than 1.8 considered acceptable) and tested for integrity by agarose gel electrophoresis to assess 28S and 18S ribosomal RNA band appearance. RNA (3 μg) for each cell type was reverse transcribed using the GEArray TrueLabeling-RT kit (SuperArray Bioscience Corp., Frederick, MD) which produces biotin-16-dUTP labeled cDNA probes. The cDNA probes were hybridized to an angiogenesis gene array (GEArray Q Series Human Angiogenesis Gene Array) purchased from SuperArray Bioscience Corp. Gene arrays were performed according to the manufacturer's protocol. Chemiluminescent images were obtained with a Kodak Image Station 440 CF (Eastman Kodak, Rochester, NY). Kodak 1D Image Analysis Software was used to make a region of interest (ROI) template that could be applied to the images to collect signal intensity data. The intensity data were background subtracted (average of negative control and blank values) and then normalized to the signal intensity for GAPDH (background subtracted gene of interest intensity/background subtracted GAPDH intensity $\times$ 100). Gene arrays were repeated three times and analyzed for statistical significance of gene expression between normal human Schwann cells and neurofibromin-deficient Schwann cells.

Protein expression array analysis

Media conditioned for 20 h from normal human Schwann cells and neurofibromin-deficient Schwann cells was analyzed for the expression of 19 angiogenic factors using a custom human antibody array purchased from RayBiotech Inc. (Norcross, GA). The proteins included on the array were angiogenin, bFGF, FGF4, FGF6, FGF7, GROa, HGF, IGF-1, IL-8, IL-10, PlGF, TGF-b1, TGF-b2, TIMP-1, TIMP-2, TNFa, uPAR, VEGF, and VEGFD. Conditioned media was concentrated to 1,000 μg of protein per 1.5 ml media for incubation with the array membrane. The protein array was carried out according to the manufacturer's protocol. Briefly, the sample was incubated with the immobilized capture antibody array, washed, and incubated with biotinylated detection antibodies followed by incubation with HRP labeled strepavidin. Following incubation with a chemiluminescent substrate, the intensity of the signal related to a given protein was measured. Chemiluminescent images were obtained with a Kodak Image Station 440 CF (Eastman Kodak, Rochester, NY). Kodak 1 D Image Analysis Software was used to make a region of interest (ROI) template that could be applied to the

images to collect signal intensity data. The intensity data background was subtracted (negative control) and then normalized to the positive control (biotinylated protein). Arrays were repeated 3 times and analyzed for statistical significance of protein expression between normal Schwann cells and neurofibromin-deficient Schwann cells.

Enzyme linked immunosorbent assay (ELISA)

Media conditioned for 20 h by normal human Schwann cells and neurofibromin-deficient Schwann cells was analyzed for expression of the proteins HGF, bFGF, GROa, IL-8, TIMP-2, and SPARC. Conditioned media was diluted to a concentration of 100,000–300,000 cells conditioning 1 ml of media depending on the protein being analyzed. To analyze HGF, GROa, IL-8, and TIMP-2, ELISA development DuoSets were purchased from R&D Systems (Minneapolis, MN) and used according to the manufacturer's protocol with minor modifications. SPARC was analyzed using the osteonectin ELISA kit from Heamatologic Technologies (Essex Junction, VT) and bFGF was analyzed using a Quantikine ELISA from R&D Systems (Minneapolis, MN). The conditioned media was added in triplicate to wells. ELISA assays were repeated 3–4 times and analyzed statistically.

Statistical analysis

For studies comparing neurofibromin-deficient Schwann cells to normal human Schwann cells, analysis of variance (ANOVA) followed by Dunnett's t post hoc test was used to analyze the data and determine significance.

Results

In vitro angiogenic potential of neurofibromin-deficient Schwann cells

To determine if neurofibromin-deficient Schwann cells have an aberrant in vitro angiogenic potential, media conditioned by the Schwann cells was evaluated for the ability to induce endothelial cell migration and proliferation. Media conditioned by normal human Schwann cells (nhSC), dermal neurofibroma-derived Schwann cells (36 L), and malignant peripheral nerve sheath tumor-derived Schwann cells (sNF02.2 and sNF94.3) was analyzed in these studies. As seen in Fig. 1A, media conditioned by neurofibromin-deficient Schwann cells stimulates the proliferation of endothelial cells, whereas media conditioned by normal human Schwann cells is not able to stimulate endothelial cell proliferation. Endothelial cell number decreases over time for both cells treated with normal Schwann cell conditioned media and serum-free media alone (untreated). The ability of neurofibromin-deficient Schwann cell conditioned media to induce endothelial cell proliferation is apparent by 24 h. By 72 h, there are more cells in the wells treated with media conditioned by MPNST-derived Schwann cells than in the wells treated with media conditioned by NFB-derived Schwann cells. In addition, endothelial cell migration is significantly increased in response to media conditioned by neurofibromin-deficient Schwann cells compared to media conditioned by normal human Schwann cells (Fig. 1B). The neurofibromin-deficient Schwann cells 36 L, sNF02.2, and sNF94.3 show a 2-fold, 5-fold, and 3-fold respective increase in migration of endothelial cells over the migration stimulated by normal Schwann cell conditioned media. Furthermore, media conditioned by MPNST-derived Schwann cells stimulates endothelial cell migration to a greater degree than the endothelial cell migration stimulated by the positive control 1% FBS.

Gene expression array analysis

Since neurofibromin-deficient Schwann cells are more angiogenic than normal human Schwann cells as measured by the potential to induce endothelial cell proliferation and migration in vitro, an angiogenesis gene expression array and a protein array were used to determine which pro-angiogenic and anti-angiogenic factors have dysregulated expression in neurofibromin-deficient Schwann cells. The gene array analysis revealed that 79 of the 96 genes represented on the array were expressed by at least one type of Schwann cell using 1% of GAPDH signal intensity as the cutoff for expression (Table 1). As seen in Table 1, 15 genes were found to have statistically significant changes in expression in neurofibromin-deficient Schwann cells compared to normal Schwann cells. Some genes have a several fold change in expression in neurofibromin-deficient Schwann cells, but these changes were not statistically significant due to the variability in gene expression in the four normal human Schwann cell samples as shown in Table 1. Of the 15 aberrantly expressed genes, 8 are pro-angiogenic factors and 2 are anti-angiogenic soluble factors that are secreted by Schwann cells. Of the 8 aberrantly expressed pro-angiogenic factors, 6 have increased expression in neurofibromin-deficient Schwann cells (ANGPT1, bFGF, GROa, HGF, uPA, VEGFC), whereas 2 of

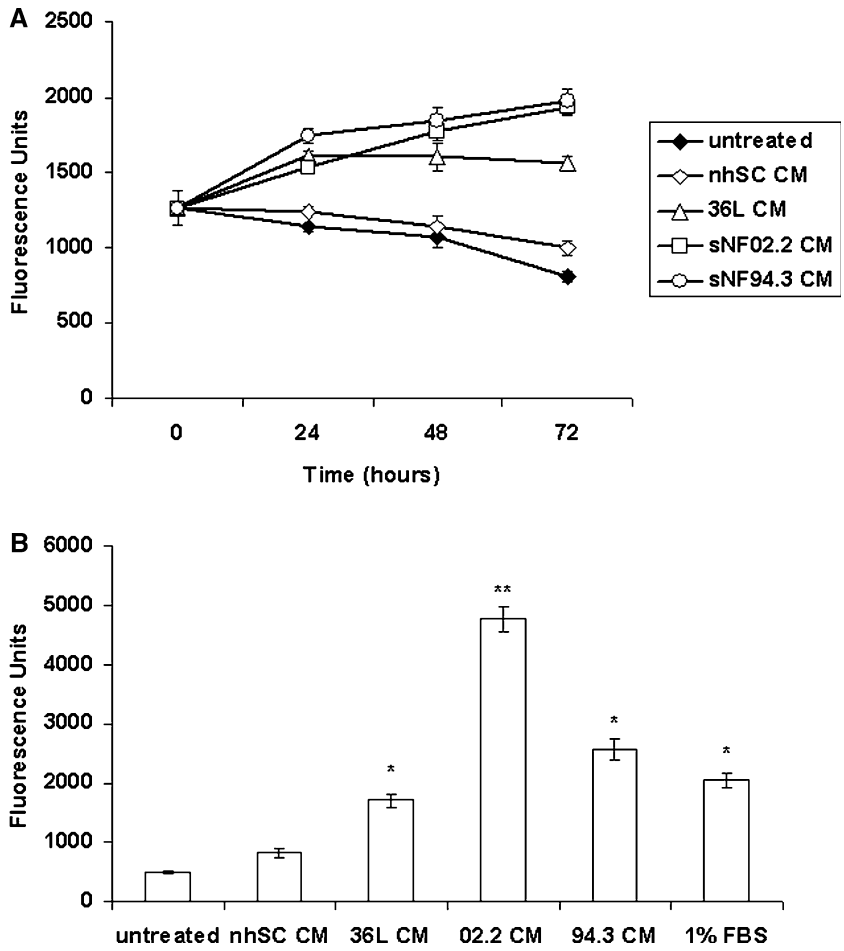

Fig. 1 Induction of endothelial cell proliferation and migration in response to neurofibromin-deficient Schwann cell conditioned media (CM). (**A**) Endothelial cells were cultured with normal and neurofibromin-deficient Schwann cell conditioned media for 24, 48 or 72 h followed by analysis of DNA content. (**B**) Endothelial cells migrated through a fibronectin-coated porous membrane toward Schwann cell conditioned media, endothelial cell basal media (untreated), or 1% fetal bovine serum (positive control). Assays were repeated 3 times with 3 different samples for each cell type. Data is significantly different from nhSC at $*P < .05$ or $**P < .01$

the pro-angiogenic factors have decreased expression (osteopontin, PDGFa) (Fig. 2A). The anti-angiogenic factor SPARC has decreased gene expression in neurofibromin-deficient Schwann cells compared to normal Schwann cells, whereas the anti-angiogenic factor Meth1 has increased expression in neurofibromin-deficient Schwann cells (Fig. 2B). The other 5 genes with significant changes in gene expression in neurofibromin-deficient Schwann cells compared to normal Schwann cells are growth factor receptors (EGFR, FGFR3, PDGFRa, neuropilin-1) and an integrin (integrin aV) (Table 1).

Protein expression array analysis

Since aberrant gene expression does not necessarily correlate with aberrant protein expression, a custom protein array composed of 19 factors that were included on the gene array was used to compare with the results of the gene array. From the results of the protein array, it was found that 5 pro-angiogenic factors and 3 anti-angiogenic factors have statistically significant changes in expression in media conditioned by neurofibromin-deficient Schwann cells compared to media conditioned by normal human Schwann cells (Table 2, Fig. 3). The protein array results confirmed the gene array results for HGF in which expression is increased in neurofibromin-deficient Schwann cells compared to normal Schwann cells with the highest expression in MPNST-derived Schwann cells (Fig. 3A). The pro-angiogenic factor FGF7 is not detected in media conditioned by normal human Schwann cells, but is secreted by neurofibromin-deficient Schwann cells. In addition, the pro-angiogenic factor PlGF has increased secretion from NFB-derived Schwann cells compared to normal human Schwann cells. Other pro-angiogenic factors, TNFa and IL-8, were found to have decreased expression by neurofibromin-deficient Schwann cells. Another interesting finding from the protein array is that the anti-angiogenic factors TIMP-1, TIMP-2, and uPAR have decreased expression in neurofibromin-deficient Schwann cells compared to normal Schwann cells (Fig. 3B). Thus, protein array analysis identified several proteins

Table 1 List of genes compared between neurofibromin-deficient and normal human Schwann cells

Accession #	Gene Description	% of GAPDH			
		nhSC	36 L	sNF02.2	sNF94.3
Growth Factors and Receptors					
Ephrin Family					
5	Ephrin A2	0.000	0.000	0.000	1.293
NM_001962	Ephrin A5 receptor	0.243	0.100	0.000	1.593
NM_004093	Ephrin B2	2.213	0.267	0.777	1.773
NM_004444	Ephrin B4	0.677	0.947	0.727	1.737
Fibroblast Growth Factors and Receptors					
X51943	Fibroblast growth factor 1 (aFGF/FGF1)	0.750	0.390	0.000	0.593
NM_002006	Fibroblast growth factor 2 (bFGF/FGF2)	2.653	**39.280****	17.460	14.133
J02986	Fibroblast growth factor 4 (FGF4/HST1)	0.000	0.050	0.437	0.750
NM_020996	Fibroblast growth factor 6 (FGF6/HST2)	0.000	0.000	0.000	0.633
NM_002009	Fibroblast growth factor 7, keratinocyte growth factor (FGF7/KGF)	5.567	18.440	44.390	51.430
M34185	Fibroblast growth factor receptor 1 (FGFR1/FLG)	0.747	11.110	13.260	19.013
M55614	Fibroblast growth factor receptor 2 (FGFR2/Bek/KGFR)	0.000	0.453	0.000	0.820
M64347	Fibroblast growth factor receptor 3 (FGFR3)	2.530	**23.767****	9.500	9.120
L03840	Fibroblast growth factor receptor 4 (FGFR4)	0.867	0.263	0.623	0.100
Platelet-Derived Growth Factors and Receptors					
X06374	Platelet-derived growth factor alpha (PDGFa)	27.563	**0.753****	**0.013****	**0.697****
X63966	Platelet-derived growth factor-BB (PDGFb)	0.193	0.357	0.000	0.000
M21574	Platelet-derived growth factor receptor, alpha (PDGFRa)	0.527	13.247	13.073	**18.777***
NM_002609	Platelet-derived growth factor receptor, beta (PDGFRb)	3.987	6.967	3.663	7.303
NM_002619	Platelet factor 4 (PF4)	0.000	0.000	0.000	2.013
Transforming Growth Factors and Receptors					
NM_003236	Transforming growth factor, alpha (TGFa)	0.000	1.353	0.000	3.033
X02812	Transforming growth factor, beta 1 (TGFb1)	20.983	16.697	8.563	13.230
M19154	Transforming growth factor, beta 2 (TGFb2)	12.263	0.000	0.560	8.437
NM_003239	Transforming growth factor, beta 3 (TGFb3)	0.183	0.870	0.000	0.000
L11695	Transforming growth factor beta receptor I (TGFbR1/ALK-5)	2.167	2.257	1.487	4.020
D50683	Transforming growth factor, beta receptor II (TGFbR2)	19.620	26.157	31.543	45.353
NM_003243	Transforming growth factor, beta receptor III (TGFbR3)	2.003	2.930	0.010	1.293
Vascular Endothelial Growth Factors and Receptors					
M32977	Vascular endothelial growth factor (VEGF)	19.123	8.560	16.803	14.333
U48801	Vascular endothelial growth factor B (VEGFB)	0.647	4.017	1.460	4.620
X94216	Vascular endothelial growth factor C (VEGFC)	1.493	**24.797****	**17.537***	15.173
D89630	Vascular endothelial growth factor D (VEGFD/FlGF)	0.000	0.940	0.000	1.633
NM_002632	Placental growth factor (PGF/PlGF)	0.000	1.260	0.000	0.000
AF035121	Kinase insert domain receptor (FLK1/VEGFR2)	0.000	0.000	0.000	0.313
NM_002019	Fms-related tyrosine kinase 1 (FLT1/VEGFR1)	0.493	0.177	0.000	0.000
NM_003873	Neuropilin 1 (NRP1)	6.153	**46.023***	**41.740***	**39.267***
Other Growth Factors and Receptors					
NM_000072	CD36 antigen, collagen type I receptor, thrombospondin receptor (CD36)	2.730	2.750	0.453	2.010
NM_001400	Endothelial differentiation (EDG1)	0.000	0.840	0.000	1.597
X04571	Epidermal growth factor (EGF)	0.033	0.220	0.000	0.590
X00588	Epidermal growth factor receptor (EGFR)	1.163	**24.887***	**24.523***	**24.200***
X12510	GRO1, melanoma growth stimulating activity alpha (GROa/MGSA)	2.950	**27.410***	2.043	1.277
X57574	Hepatocyte growth factor, scatter factor (HGF)	0.020	1.483	5.347	**9.847****
M27544	Insulin-like growth factor 1, somatomedin C (IGF-1)	1.307	2.843	0.000	1.067
NM_000459	TEK tyrosine kinase (TEK/Tie-2)	1.553	2.043	0.130	2.910
NM_005424	Tyrosine kinase, immunoglobulin & EGF homology (Tie)	0.000	1.203	0.013	0.000
Cytokines and Chemokines					
X03438	Granulocyte colony stimulating factor 3 (G-CSF)	0.000	0.143	0.000	0.413
NM_024013	Interferon, alpha 1 (IFNa1)	0.000	0.000	0.000	0.480
M28622	Interferon, beta 1 (IFNb1)	1.360	5.267	0.043	0.013
X13274	Interferon, gamma (IFNr)	0.000	0.677	0.000	0.000
M57627	Interleukin 10 (IL-10)	310.020	266.113	206.583	223.100
M65271	Interleukin 12A (IL-12A)	2.083	4.750	1.153	3.650
M17017	Interleukin 8 (IL-8)	10.893	26.897	0.000	7.147
NM_002391	Midkine, neurite growth-promoting factor 2 (MDK)	16.460	13.833	11.240	15.900
M29386	Prolactin (PRL)	2.313	1.220	0.000	0.160
X52946	Pleiotrophin, neurite growth-promoting factor 1 (PTN)	8.340	2.240	7.253	14.397

Table 1 continued

Accession #	Gene Description	% of GAPDH nhSC	36 1	sNF02.2	sNF94.3
X14768	Small inducible cytokine A2, monocyte chemotactic protein 1 (SCYA2)	25.073	11.810	23.630	26.187
NM_003118	Secreted protein acidic and rich in cysteine, osteonectin (SPARC)	370.480	**175.507***	**118.447****	**92.457****
X01394	Tumor necrosis factor alpha (TNFa)	1.490	0.587	0.000	2.403
NM_005118	Vascular endothelial cell growth inhibitor (VEGI/TNFSF15)	2.037	3.403	0.930	3.573
Matrix Proteins, Proteases, and Inhibitors					
AF060152	A disintegrin-like metalloprotease with thbs1 motif (Meth1/ADAMTS1)	8.747	24.113	35.933	**50.823***
NM_007037	A disintegrin-like metalloprotease with thbs1 motif (Meth2/ADAMTS8)	0.513	0.000	1.743	9.437
NM_016214	Type XVIII collagen (fragment is endostatin)	0.000	0.397	0.000	0.130
X02761	Fibronectin 1 (FN1)	399.827	377.507	251.127	184.047
AF084467	Heparanase	1.043	1.110	0.000	1.960
J03210	Matrix metalloproteinase 2, gelatinase A, 72 kD collagenase IV (MMP-2)	83.450	97.833	43.523	57.313
J05070	Matrix metalloproteinase 9, gelatinase B, 92 kD collagenase IV (MMP-9)	0.510	2.137	0.000	2.960
NM_002445	Macrophage scavenger receptor 1 (MSR1/SR-A)	13.957	9.457	9.873	16.860
D00244	Urokinase-type plasminogen activator (uPA/PLAU)	0.000	2.390	13.890	**19.203***
NM_002639	Protease inhibitor 5 (maspin)	0.000	0.977	0.000	0.000
M90439	Pigment epithelium-derived factor (PEDF)	0.353	11.550	9.190	2.037
NM_003246	Thrombospondin 1 (THBS1/TSP-1)	232.193	282.397	115.657	101.147
L12350	Thrombospondin 2 (THBS2/TSP-2)	19.967	40.277	33.047	28.033
L38969	Thrombospondin 3 (THBS3/TSP-3)	1.067	2.367	0.983	4.157
NM_003248	Thrombospondin 4 (THBS4/TSP-4)	0.000	0.087	0.060	2.430
NM_003254	Tissue inhibitor of metalloproteinase 1, collagenase inhibitor (TIMP1)	38.057	57.917	101.630	108.677
NM_003255	Tissue inhibitor of metalloproteinase 2 (TIMP2)	216.583	105.620	101.143	113.800
Adhesion Molecules					
X59796	Cadherin 5, VE-cadherin	10.310	8.727	7.750	11.657
X06256	Integrin alpha 5, fibronectin receptor alpha	26.187	12.963	11.430	14.460
NM_002210	Integrin alpha V, vitronectin receptor alpha	39.820	**6.223****	**6.577****	**9.850***
J02703	Integrin beta 3, platelet glycoprotein IIIa	12.863	2.610	0.233	0.007
NM_000442	Platelet/endothelial cell adhesion molecule (PECAM1/CD31)	0.000	0.407	0.000	1.353
Transcription Factors					
M11730	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (erb-b2)	0.540	0.607	0.367	0.890
L16510	v-ets avian erythroblastosis virus E26 oncogene homolog 1 (c-ets1)	13.350	4.043	3.160	4.500
NM_001530	Hypoxia-inducible factor 1 alpha (HIF1A)	60.860	90.067	64.477	103.800
D13889	Inhibitor of DNA binding 1 (ID1)	24.027	14.497	3.030	8.903
X66924	Inhibitor of DNA binding 3 (ID3)	7.427	0.233	0.523	2.427
NM_005900	Mothers against decapentaplegic homolog 1 (MADH1/Smads)	3.773	2.233	0.130	1.340
Other Related Genes					
NM_000118	Endoglin (ENG)	17.413	37.777	36.713	37.237
J00307	Prothrombin kringle 1	0.637	0.433	0.000	1.050
NM_000603	Nitric oxide synthase 3 (NOS3)	0.000	0.533	0.000	0.000
NM_000962	Prostaglandin-endoperoxide synthase 1 (COX-1/PTGS1)	0.330	4.667	0.877	7.917
NM_000963	Prostaglandin-endoperoxide synthase 2 (COX-2/PTGS2)	1.337	2.417	0.000	2.717
NM_002956	Restin—intermediate filament—associated protein (RSN)	27.067	19.250	20.410	25.223
M83248	Secreted phosphoprotein 1 (SPP1), osteopontin	46.237	**1.557****	**0.00****	**0.230****
Specific Promoters and Inhibitors					
AF286598	Angiostatin binding protein 1, angiomotin	0.413	0.733	0.093	0.110
M11567	Angiogenin, ribonuclease, RNase A family 5 (ANG)	0.620	1.577	1.303	2.690
NM_001146	Angiopoietin-1 (ANGPT1)	0.000	1.430	7.180	**20.747***
NM_001147	Angiopoietin-2 (ANGPT2)	0.000	0.000	0.000	0.807
NM_001275	Chromogranin A, parathyroid secretory protein 1 (vasostatin precursor)	15.237	11.407	9.430	18.200

Data is average of 3 gene arrays. Numbers in bold print are significantly different from nhSC at $^{*}P < .05$ or $^{**}P < .01$

with significantly dysregulated expression in neurofibromin-deficient Schwann cells, although gene array analysis did not find the expression of these genes to be dysregulated (Table 2). Therefore, either the protein array is more sensitive than the gene array or the expression of the protein persists after gene expression has stopped.

ELISA confirmation of aberrant angiogenic profile

Some of the gene and protein array results were confirmed by ELISA analysis to quantitate the amount of protein secreted into the conditioned media by normal and neurofibromin-deficient Schwann cells. As seen in Fig. 4, the pro-angiogenic factor

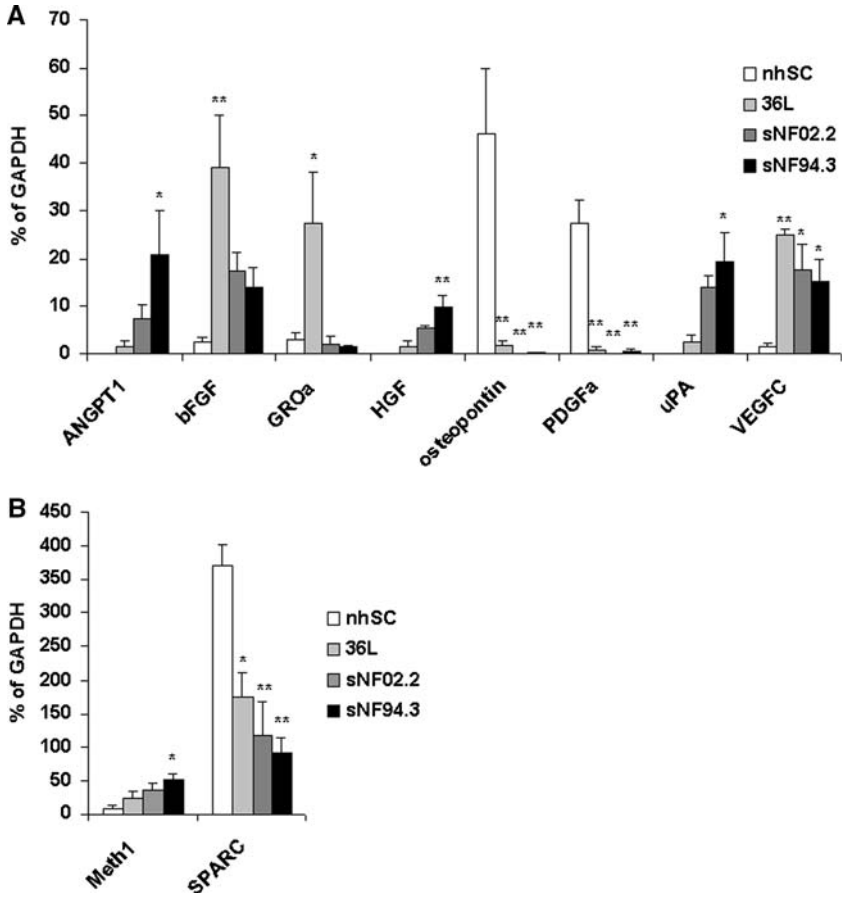

Fig. 2 Gene expression array using RNA extracted from normal human Schwann cells and neurofibromin-deficient Schwann cells. For each cell type, 3 μg of RNA was reverse transcribed and hybridized to an array membrane. The signal intensity for each gene of interest was normalized to the signal intensity for GADPH and reported as a percentage of the GADPH signal intensity. Panel **A** shows pro-angiogenic factor gene expression, whereas panel **B** shows anti-angiogenic factor gene expression. Assay was repeated 3 times with 3 different samples for each cell type. Data is significantly different from nhSC at $^{*}P < .05$ or $^{**}P < .01$

Table 2 List of proteins compared between neurofibromin-deficient and normal human Schwann cells

Protein Description	% of Positive Control			
	nhSC	36 L	sNF02.2	sNF94.3
Angiogenin	80.75	74.90	99.45	58.80
Fibroblast growth factor 2 (bFGF/FGF2)	2.28	3.98	1.35	1.05
Fibroblast growth factor 4 (FGF4/HST1)	0.00	0.58	0.00	0.00
Fibroblast growth factor 6 (FGF6/HST2)	0.00	0.10	0.58	0.05
Fibroblast growth factor 7 (FGF7/KGF)	0.63	**12.65****	**16.50****	7.40
Growth related oncoprotein alpha (GROa/GRO1)	1.80	5.88	4.35	4.63
Hepatocyte growth factor, scatter factor (HGF)	15.93	54.85	**310.53****	**348.88****
Insulin like growth factor 1 (IGF-1)	3.85	2.30	4.05	7.48
Interleukin 8 (IL-8)	433.18	539.20	**173.65****	297.20
Interleukin 10 (IL-10)	10.18	13.53	1.78	2.60
Placental growth factor (PGF/PlGF)	4.10	**15.90***	1.18	5.83
Transforming growth factor beta 1 (TGFb1)	1.05	1.30	0.68	0.18
Transforming growth factor beta 2 (TGFb2)	2.50	1.70	1.60	2.70
Tissue inhibitor of metalloproteinase 1, collagenase inhibitor (TIMP-1)	128.78	118.68	**91.58****	**102.95***
Tissue inhibitor of metalloproteinase 2 (TIMP-2)	172.53	**90.55****	**107.03****	**119.43***
Tumor necrosis factor alpha (TNFa)	8.03	**2.60***	**1.78***	**2.55***
Urokinase-type plasminogen activator receptor (uPAR)	235.58	**64.88****	**29.25****	**18.33****
Vascular endothelial growth factor (VEGF/VEGFA)	12.68	6.68	8.75	7.30
Vascular endothelial growth factor D (VEGFD)	0.30	1.05	0.55	0.10

Data is average of 3 protein arrays. Numbers in bold print are significantly different from nhSC at $^{*}P < .05$ or $^{**}P < .01$

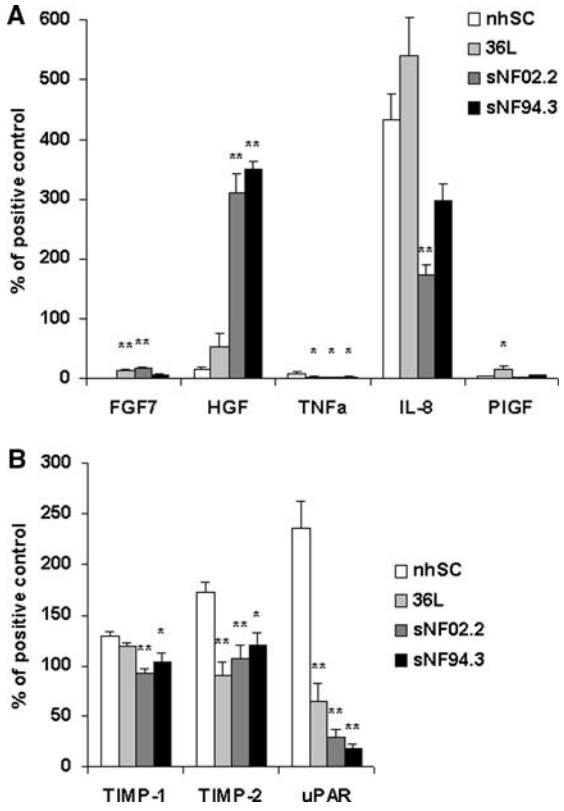

Fig. 3 Protein expression array using media conditioned by normal human Schwann cells and neurofibromin-deficient Schwann cells. Serum-free media was conditioned for 20 h and then conditioned media containing 1000 μg of protein was incubated with the array membrane. The intensity of the chemiluminescence for each protein in the array was normalized to the chemiluminescence of a biotinylated IgG positive control; the results are expressed as a percentage of the positive control. Assay was repeated 3 times with 3 different samples for each cell type. Data is significantly different from nhSC at $*P < .05$ or $**P < .01$

HGF is significantly increased in neurofibromin-deficient Schwann cells with a 3-fold increase in NFB Schwann cells and a 12-fold increase in MPNST Schwann cells. The pro-angiogenic factors bFGF, GROa, and IL-8 have increased expression in NFB Schwann cells, whereas the expression of these factors in MPNST Schwann cells either does not change or decreases (Fig. 4A). The high amount of GROa secreted by the NFB Schwann cells was consistently observed. Furthermore, the anti-angiogenic factor TIMP-2 has at least a 2-fold decrease in expression in neurofibromin-deficient cells (Fig. 4B). Another anti-angiogenic factor, SPARC, also has decreased expression in neurofibromin-deficient Schwann cells compared to normal human Schwann cells (Fig. 4B).

Discussion

The neurofibromin-deficient Schwann cells used in this study have a shift in angiogenic profile that favors angiogenesis in vitro. Media conditioned by neurofibromin-deficient Schwann cells stimulated the proliferation and migration of endothelial cells, whereas media conditioned by normal human Schwann cells did not stimulate endothelial cell proliferation or migration. In addition, media conditioned by the more rapidly dividing malignant peripheral nerve sheath tumor (MPNST)-derived Schwann cells was a more potent inducer of endothelial cell proliferation and migration than media conditioned by the more slowly dividing neurofibroma (NFB)-derived Schwann cells. These findings are not unexpected since the more rapidly growing MPNST cells would require more rapid angiogenesis. These findings agree with previous reports that both human and mouse neurofibromin-deficient Schwann cells induce angiogenesis in vivo [11, 20] and conditioned media from mouse *NF1*–/– Schwann cells induces proliferation of endothelial cells in vitro [14].

Analysis of gene and protein expression demonstrates increased expression of pro-angiogenic factors in neurofibromin-deficient Schwann cells compared to normal human Schwann cells. These pro-angiogenic factors may be useful targets for inhibiting angiogenesis in NF1 peripheral nerve sheath tumors. The changes in the pro-angiogenic factor expression were different for Schwann cells derived from dermal NFB compared to Schwann cells derived from MPNST. GROa, bFGF, IL-8, and PlGF were significantly increased only in NFB-derived Schwann cells, whereas ANGPT1 and uPA were significantly increased only in the MPNST-derived Schwann cells. VEGFC and FGF7/KGF were increased to the same extent in both types of neurofibromin-deficient Schwann cells, whereas HGF was increased to a greater extent in MPNST-derived Schwann cells than NFB-derived Schwann cells. Since MPNST-derived Schwann cells stimulate endothelial cell proliferation and migration more potently than NFB-derived Schwann cells, it may be the case that HGF, ANGPT1, and uPA are more potent stimulators of these effects on endothelial cells than bFGF, GROa, IL-8, and PlGF.

The pro-angiogenic profile of melanomas is very similar to the angiogenic profile we observed in dermal neurofibroma-derived Schwann cells. GROa, bFGF, and IL-8 are important autocrine factors that support tumor progression and angiogenesis in melanoma [21–24]. PlGF is also secreted by human melanoma cells

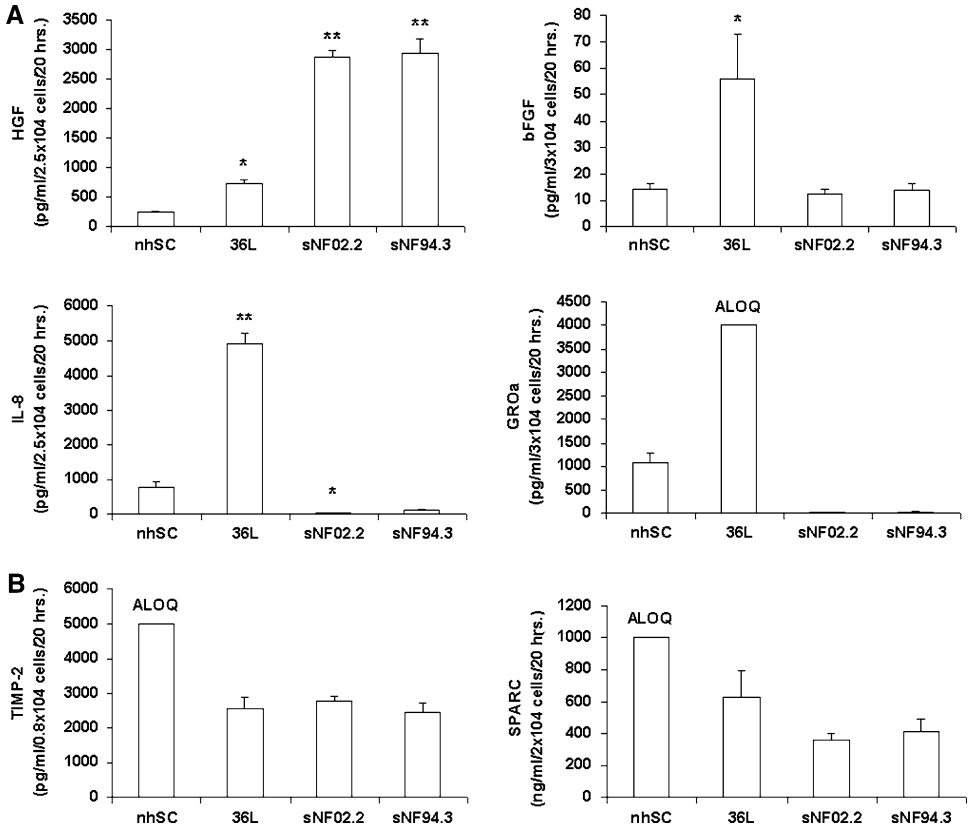

Fig. 4 ELISA analysis of protein expression in media conditioned by normal human Schwann cells and neurofibromin-deficient Schwann cells. Serum-free media was conditioned for 20 h. Panel **A** shows expression of pro-angiogenic factors, whereas Panel **B** shows expression of anti-angiogenic factors. Some of the samples were above the limit of quantitation (ALOQ). Assays were repeated 3–4 times with different samples. Data is significantly different from nhSC at $*P < .05$ or $**P < .01$

and is able to induce a proliferative response in these cells [25]. Since melanocytes and Schwann cells are both neural crest-derived cells located in the skin, these two cell types may share similar angiogenic dysregulation.

The increased expression of bFGF/FGF2 in NFB-derived Schwann cells is supported by previous studies which demonstrated that FGF2 is present in neurofibroma tissue [16, 26]. These results are consistent with the report that the FGF2 transcript is present in *NF1*–/– mouse Schwann cells but not in *NF1*+/+ Schwann cells [14]. It has also been reported that the mRNA transcript for FGF2 was upregulated in an MPNST cell line compared to normal Schwann cells [27]. Similarly, our results show a 5–6-fold increase in bFGF transcript expression in MPNST-derived Schwann cells; however, this increase was not found to be statistically significant and did not show increased expression by ELISA analysis.

Increased HGF expression in both NFB- and MPNST-derived Schwann cells is consistent with the results of previous studies. HGF immunostaining has been reported in tissue from both NFB [13] and MPNST tumors [15]. Another study examined tumor tissue in which a MPNST was developing within a NFB and found that HGFα expression was higher in the MPNST tissue than in the NFB tissue in 5 of the 8 samples analyzed [28]. Similarly, our gene and protein expression data show increased HGF expression in MPNST-derived Schwann cells (ELISA: 12-fold over nhSC) compared to NFB-derived Schwann cells (ELISA: 3-fold over nhSC). However, a previous gene expression profiling study did not find an increase in HGF transcript in MPNST cell lines and primary MPNST samples [29]. Another report found that HGF transcript was not expressed by either *NF1*+/+ or *NF1*–/– mouse Schwann cells (14). These studies are not entirely inconsistent with our gene expression data which showed a significant increase in only one of the cell lines studied, whereas protein expression analysis by ELISA showed significant increases for all NF1 samples analyzed.

Furthermore, this study does not confirm previous reports that the pro-angiogenic factor MDK has increased expression in neurofibromin-deficient Schwann cells. It was reported that MDK transcript is expressed in *NF1*–/– mouse Schwann cells but not *NF1*+/+ mouse Schwann cells (14). A gene profiling study found that MDK is up-regulated in 5 of 8 MPNST cell lines and 22 of 45 MPNST tissue samples [29]. MDK was included on the gene expression array used in our study but was not found to have differential

expression between neurofibromin-deficient Schwann cells and normal human Schwann cells. Interestingly, another study which used gene expression profiling of MPNST tissue found that a distinct molecular subset of tumors have a 4-fold average decrease in MDK expression relative to the other MPNST samples analyzed [30]. The cause for this variability in MDK expression is not clear- it could be due to staging of the disease and/or the age and location of the tumor tissue.

This is the first report of increased expression of GROa, PlGF, ANGPT1, FGF7/KGF, uPA, and VEGFC in neurofibromin-deficient Schwann cells. GROa is involved in angiogenesis, cell proliferation, protease induction, and the directed migration of immune cells (reviewed in 21, 23). Mast cell infiltration has been suggested to be important for the development of NF1 tumors [31–34] raising the possibility that GROa is involved in the migration of mast cells into NF1 tumors. Furthermore, the expression of uPA is correlated with tumor angiogenesis, metastasis, and poor prognosis in several types of cancer (reviewed in 35, 36). Many studies have shown that knocking out or inhibiting uPA results in a reduction of the angiogenic response in vivo [37–40]. It has been reported that tumorigenic Schwann cells express uPA whereas Schwann cells in normal nerve do not stain for uPA [41]. Increased expression of uPA may be important to the invasive phenotype described for both mouse and human neurofibromin-deficient Schwann cells [11, 20, 42]. Therefore, increased expression of these pro-angiogenic factors most likely contribute to a shift in the angiogenic potential in neurofibromin-deficient Schwann cells that leads to the induction of angiogenesis. Future studies will assess the importance of each of these factors individually in the induction of angiogenesis in NF1 tumors.

The anti-angiogenic factors SPARC, TIMP-1, TIMP-2, and soluble uPAR all have decreased expression in neurofibromin-deficient Schwann cells adding to the overall angiogenic potential of these cells. Our lab has previously reported that SPARC transcript is decreased in an MPNST Schwann cell line compared to normal human Schwann cells [27]. This is the first report that TIMP-1 and TIMP-2 have decreased expression in neurofibromin-deficient Schwann cells. Both SPARC and TIMP-2 are key factors responsible for the anti-angiogenic properties of conditioned media from normal Schwann cells [43, 44]. SPARC can also inhibit the proliferation of both normal and tumor cells [43, 45–49]. Therefore, decreased SPARC expression in conditioned media from neurofibromin-deficient Schwann cells is likely to make a major contribution to the pro-angiogenic nature of these cells. Additionally, the decreased TIMP expression along with decreased uPAR and increased uPA likely contribute to the increased angiogenic and invasive potential seen in NF1 Schwann cells.

Regarding dysregulated expression of growth factor receptors, we found that PDGFRa, EGFR, FGFR3, and neuropilin-1 have increased gene expression in neurofibromin-deficient Schwann cells. Our results agree with previous studies which demonstrated increased expression of PDGFRa [50, 51] and EGFR [29, 52] in human neurofibromin-deficient Schwann cells and peripheral nerve sheath tumor tissue. Increased expression of PDGFRa and EGFR is most likely important for autocrine and paracrine mitogenic signals that contribute to NF1 tumor growth. This is the first report of aberrant gene expression for FGFR3 and neuropilin-1 in neurofibromin-deficient Schwann cells. FGFR3 may be involved in an autocrine mitogenic response to bFGF in neurofibromin-deficient Schwann cells. Furthermore, FGFR3 is a negative regulator of bone growth and mutations resulting in hyperactivation of this receptor result in skeletal dysplasia [53, 54]. Given that NF1 patients have a high incidence of skeletal dysplasia, which is diagnostic of the disorder, it would be interesting to know if *NF1* haploinsufficient osteogenic progenitors overexpress FGFR3. In addition, neuropilin-1 was found to have a 6–7-fold increase in gene expression in neurofibromin-deficient Schwann cells. Neuropilin-1 functions as a receptor for $VEGF_{165}$ through an interaction with VEGFR2/KDR [55]. In this way, neuropilin may be involved in an autocrine or paracrine mitogenic response to VEGF in neurofibromin-deficient Schwann cells.

This study found that integrin αV has decreased gene expression in neurofibromin-deficient Schwann cells. This finding agrees with a previous study which found decreased integrin αV expression in an MPNST cell line using a cDNA microarray [27]. Integrin αV receptors are involved in cell adhesion, migration, survival, morphology, and angiogenesis (reviewed in 56, 57). Decreased integrin αV expression in neurofibromin-deficient Schwann cells may be involved in the changes in Schwann cell morphology, loss of extracellular matrix adhesion, and increased migration seen in these cells.

This study adds to our understanding of the mechanism by which neurofibromin-deficient Schwann cells obtain abnormal growth resulting in tumor formation. Our gene and protein expression data show that neurofibromin-deficient Schwann cells have a profile in which there is dysregulated expression of pro-angiogenic factors, anti-angiogenic factors, growth fac-

tor receptors, and an integrin. Increased pro-angiogenic factor and growth factor receptor expression along with a decrease in anti-angiogenic factor and integrin expression could provide the tumorigenic Schwann cells with an increased propensity for proliferation, survival, invasion, and induction of angiogenesis.

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