Human Cancer Biology

Clinical Cancer Research

The Activation of the WNT Signaling Pathway Is a Hallmark in Neurofibromatosis Type 1 Tumorigenesis

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Abstract

Purpose: The hallmark of neurofibromatosis type 1 (NF1) is the onset of dermal or plexiform neurofibromas, mainly composed of Schwann cells. Plexiform neurofibromas can transform into malignant peripheral nerve sheath tumors (MPNST) that are resistant to therapies.

Experimental Design: The aim of this study was to identify an additional pathway in the NF1 tumorigenesis. We focused our work on Wnt signaling that is highly implicated in cancer, mainly in regulating the proliferation of cancer stem cells. We quantified mRNAs of 89 Wnt pathway genes in 57 NF1-associated tumors including dermal and plexiform neurofibromas and MPNSTs. Expression of two major stem cell marker genes and five major epithelial–mesenchymal transition marker genes was also assessed. The expression of significantly deregulated Wnt genes was then studied in normal human Schwann cells, fibroblasts, endothelial cells, and mast cells and in seven MPNST cell lines.

Results: The expression of nine Wnt genes was significantly deregulated in plexiform neurofibromas in comparison with dermal neurofibromas. Twenty Wnt genes showed altered expression in MPNST biopsies and cell lines. Immunohistochemical studies confirmed the Wnt pathway activation in NF1-associated MPNSTs. We then confirmed that the knockdown of *NF1* in Schwann cells but not in epithelial cells provoked the activation of Wnt pathway by functional transfection assays. Furthermore, we showed that the protein expression of active β-catenin was increased in *NF1*-silenced cell lines. Wnt pathway activation was strongly associated to both cancer stem cell reservoir and Schwann–mesenchymal transition.

Conclusion: We highlighted the implication of Wnt pathway in NF1-associated tumorigenesis. *Clin Cancer Res; 20(2); 358–71. ©2013 AACR.*

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurocutaneous disorder affecting 1 in 3,000 individuals worldwide (1). The *NF1* gene is located in the chromosome region 17q11.2. Its protein product, neurofibromin, functions as a negative regulator of RAS proteins. The main clinical features of NF1 are *café-au-lait* macules, skinfold freckling, iris Lisch nodules, as well as skeletal and cardiovascular abnormalities. NF1 patients have an increased risk of both benign and malignant tumors, therefore, NF1 is classified as a tumor predisposition syndrome. The most common tumors are benign peripheral nerve

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Translational Relevance

The commonest neurofibromatosis type 1 (NF1)-associated tumors are nerve sheath tumors (NSTs: dermal or plexiform neurofibromas), mainly composed of Schwann cells. Plexiform neurofibromas can undergo malignant transformation to malignant peripheral nerve sheath tumors with a poor vital prognosis. Little is known of the genetic events required for neurofibromas formation or transformation. Here, we found that the Wnt pathway was significantly activated by quantifying mRNAs of 89 Wnt pathway genes in 57 NF1-associated NSTs. We then confirmed that the knockdown of *NF1* in Schwann cells provoked the activation of Wnt pathway by functional transfection assays. We showed that active β-catenin protein expression was increased in *NF1*-silenced cell lines. Wnt pathway activation was strongly associated to both putative cancer stem cell reservoir and Schwann–mesenchymal transition. We highlighted the implication of Wnt pathway in NF1 peripheral nerve sheath tumorigenesis. Elucidation of the role of cellular signaling pathways tumorigenesis will enable to establish molecular targeted therapeutics in NF1.

sheath tumors (neurofibromas), which vary greatly in both number and size, and may be dermal or plexiform (2).

Dermal neurofibromas are typically small and grow as discrete lesions in the dermis whereas plexiform neurofibromas can develop internally along the plexus of major peripheral nerves and become quite large. Plexiform neurofibromas are probably derived from embryonic Schwann cell lineage (3). During development, neural-crest cells migrate along the peripheral nerves, and a subset of cells commit to the Schwann cell lineage. Zheng and colleagues demonstrated that plexiform neurofibromas could originate from *Nf1* deficiency in fetal nonmyelinating Schwann cells (4). Le and colleagues identified a population of progenitor cells residing in the dermis, termed "skin-derived precursors" that form dermal neurofibromas through loss of *Nf1* (5). These differences between dermal and plexiform neurofibromas suggest that the type of progenitor but also the timing of somatic *NF1* mutations may determine the clinical course of these tumors (6).

In contrast to dermal neurofibromas, plexiform neurofibromas can undergo malignant transformation to malignant peripheral nerve sheath tumors (MPNST) in about 10% of NF1 patients (7). Roughly half of all MPNSTs are sporadic; they are found in patients who do not carry any known genetic predisposition for cancer. The remaining MPNSTs are found in patients who are diagnosed with NF1. MPNSTs are resistant to conventional therapies, and their deep-seated position and locally invasive growth hinder complete surgical resection. The 5-year survival rate among patients with MPNSTs ranges from 30% to 50%. In parallel to the double inactivation of the *NF1* gene, additional genetic lesions seem necessary for malignant progression of plexiform neurofibromas. *TP53* mutations have been identified in MPNSTs but not in benign neurofibromas, indicating that the p53-mediated pathway is involved in malignant progression (6, 8). Alterations of other genes (*p16/CDKN2A*, *p14/ARF*, *p27/KIP1*, *EGFR*, *mTOR*) were also detected in MPNST (9–12).

Although we have now identified some of the genes that when mutated initiate tumor formation and drive progression, the identity of the cell population(s) susceptible to such transforming events remains undefined in the majority of human cancers. Recent studies indicate that a small population of cells (called cancer stem cells) endowed with unique self-renewal properties and tumorigenic potential is present in some, and perhaps, all tumors (13, 14). Many signaling pathways involved in the maintenance of normal stem cells are found to be altered in several human cancers (i.e., Wnt, Hedgehog, and Notch pathways). Moreover, several authors addressed the possible association between the formation of stem cells and the epithelial–mesenchymal transition (EMT). Regulation of the EMT is a crucial step in cancer development (15). Genetic alterations that impair the differentiation program could endow such dedifferentiated cells with attributes that mimic stem cell behavior.

In NF1 tumorigenesis, the question whether these tumors arise from neural crest stem cells or differentiates glia remains very controversial (4, 5, 16, 17). In this regard, we have previously shown a dedifferentiation of Schwann cells, as well as an activation of the Hedgehog signaling pathway, during malignant transformation of plexiform neurofibromas (18). *TWIST1*, an important transcription factor involved in embryonic development and in EMT, was shown to be upregulated in NF1-associated MPNST (19). Taken together, these findings suggest a genetic-driven Schwann–mesenchyme transition (SMT) that could generate cancer stem cells during NF1 tumorigenesis.

Wnt pathway is a major developmental pathway involved in maintenance of normal stem cells (20), and altered in several human cancers (21). Wnt signaling is a complex process that requires interplay of many different proteins. In addition to a large cohort of Wnt ligands, and frizzled receptors, some Wnt pathways also require the presence of coreceptors. Wnt ligands may activate 3 different pathways (Supplementary Fig. S1): (i) the canonical pathway: involving β-catenin and LEF/TCF transcription factor; (ii) the planar cell polarity pathway; and (iii) the Wnt/calcium pathway (20, 22). All 3 pathways have different consequences and drive specific processes for the cells in which they signal.

Little is known of the additional cooperating genetic events potentially required for full plexiform neurofibroma formation. Furthermore, the respective tumorigenic mechanisms of dermal neurofibromas and plexiform neurofibromas have rarely been compared (6). In this manuscript we addressed the potential role of Wnt signaling in NF1 tumorigenesis. We here quantified the gene expression profiles of 89 actors of Wnt pathway in NF1-associated tumors. We identified specific genes involved in Wnt signaling pathway whose expressions significantly differed between

3 NF1-associated tumor types: dermal and plexiform neurofibromas and MPNSTs. We functionally confirmed the crosslink between *NF1* alteration and Wnt pathway activation, using siRNA strategy in a cell line model. Finally, we investigated the link between Wnt signaling pathway activation and the altered expression of stemness and SMT genes.

Materials and Methods

Patients and samples

NF1-related samples were obtained by laser excision (dermal neurofibromas) or surgical excision (plexiform neurofibromas and MPNSTs) from patients with NF1 at Henri Mondor Hospital (Creteil, France). Sample handling and processing was identical for all NF1-related tumors. After clinical examination, the surgical removal of dermal neurofibromas was proposed in case of esthetical burden. Resection or removal of plexiform neurofibromas was proposed for repair or in case of symptoms, pain or compression. The plexiform neurofibromas (deep lesions involving a plexus of nerves) were large, had a nodular aspect, and severely deformed the affected tissues. They were all S100-positive by immunostaining. Four patients with plexiform neurofibromas had developed MPNSTs. The main clinical and histological characteristics of the 16 patients with MPNSTs (validation set) are shown in Supplementary Table S1. Immediately after surgery the tumor samples were flash frozen in liquid nitrogen and stored at −80°C until RNA extraction. NF1-related MPNSTs all arose from plexiform neurofibromas and showed very weak S100 immunostaining (data not shown). MPNST samples from non-NF1 patients were obtained by surgical excision at Cochin Hospital (Paris, France). Dermal neurofibromas were used as "normal" control samples as they never undergo malignant transformation in MPNSTs. Indeed, neurofibromas are heterogeneous benign tumors composed of Schwann cells, fibroblasts, mast cells, and other cells, and have no "normal" tissue equivalent. Gene expression levels determined by real time reverse transcription (RT)-PCR analysis in plexiform neurofibromas and MPNSTs were thus expressed relative to expression levels in dermal neurofibromas.

Cell lines

We analyzed 7 human MPNST cell lines, namely NMS-2, NMS-2PC, 88-3, ST88-14, 90-8, S462, and T265. NMS-2 and NMS-2PC were kind gifts from Dr. Akira Ogose (Niigata University School of Medicine, Niigata, Japan). 88-3, ST88-14, and 90-8 were kind gifts from Dr. Nancy Ratner (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). S462 was a kind gift from Dr. Lan Kluwe (University Hospital Eppendorf, Hamburg, Germany). T265 was a kind gift from Dr. G De Vries (Loyola University, Chicago, IL). MPNST cell lines were grown in RPMI medium supplemented with 10% heat-inactivated FBS, 10 IU/mL penicillin, and 10 μg/mL streptomycin. Normal Schwann cells and fibroblasts were obtained by primary cell culture and differential isolation from normal sciatic nerve biopsies and skin, respectively, and using cell culture and isolation conditions as previously described (23–25). Normal mast cells were obtained by means of cell culture and various specific purification steps from cord blood-derived human cells, as previously described (26).

We functionally analyzed the crosslink between *NF1* alteration and the Wnt pathway using siRNA strategy in the mouse Schwann cell line MSC80. MSC80 cells exhibit normal Schwann cell characteristics (S100, myelin protein zero, peripheral myelin protein 22 expression) and have retained the capacity to myelinate axons (27). The mouse Schwann cell line MSC80 was maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% decomplemented fetal calf serum (Hyclone-Perbio), 1% penicillin, 1% streptomycin (Gibco), and 1% glutamine. Culture cells were grown at 37°C in a humidified atmosphere of 5% CO_2. A siRNA strategy was also used in 2 human non-Schwann cell lines: the breast cancer cell line, namely MCF-7 (Michigan Cancer Foundation-7), and one embryonic kidney cell line, namely HEK293 (Dr. François Lallemand, Institut Curie, Saint-Cloud, France). We also analyzed mouse embryonic fibroblasts (MEF) derived from wild-type mice and from $Nf1^{-/-}$ mice (Dr. Thomas De Readt, Harvard Medical School, Boston, MA).

Human mRNAs quantification by real-time RT-PCR: studied panels

We first quantified the mRNA expression level of the 89 Wnt pathway human genes in a series of 7 dermal neurofibromas, 7 plexiform neurofibromas, and 8 MPNSTs (screening set; $N = 22$ tumors). The 89 Wnt pathway quantified genes encode 19 Wnt ligands, 13 Wnt receptors, 32 proteins involved in canonical Wnt signal transduction, 4 Wnt transcription factors, 10 LEF/TCF–inducible proteins (http://www.stanford.edu/~rnusse/pathways/targets.html), and 11 proteins involved in noncanonical Wnt signal pathways including 4 proteins involved in the planar cell polarity pathway and 7 proteins in the Wnt/calcium pathway (Supplementary Table S2). The mRNA expression of the 32 markedly differentially expressed genes was then determined in a larger tumor series including 10 dermal neurofibromas, 31 plexiform neurofibromas, and 16 MPNSTs (validation set: 57 tumors). Expression of 2 major stem cell marker genes (*PROM1* and *NKX2.2*) and 5 major EMT marker genes (*TWIST1*, *SLUG*, *VIM*, *SNAIL*, and *CDH1*) was also assessed in the validation set ($N = 57$ tumors).

We then selected for further study the 24 genes whose expression in the MPNST group and/or in the plexiform neurofibroma group markedly differenced (>2-fold) from that in the dermal neurofibroma group. Expression of the 24 genes was studied in normal human Schwann cells, fibroblasts, endothelial cells, and mast cells, in 7 NF1-related MPNST cell lines, in 4 sporadic MPNSTs, and in $Nf1^{+/+}$ and $Nf1^{-/-}$ MEFs. Expression of the 24 genes was also studied in the 2 human non-Schwann cell lines (HEK293 and MCF-7) and one mouse Schwann cell line (MSC80) with siRNAs-mediated *NF1* knockdown.

mRNAs quantification by real-time RT-PCR: method

We first used real-time quantitative RT-PCR to quantify the mRNA expression of 89 selected Wnt pathway associated-genes in a series of MPNSTs and plexiform neurofibromas, by comparison with dermal neurofibromas. Quantitative RT-PCR is a major alternative to microarrays for molecular tumor profiling, being far more precise, reproducible, and quantitative (28). Furthermore, it is more appropriate for analyzing weakly expressed genes such as the Wnt genes in this study. Finally, RT-PCR requires smaller amounts of total RNA (about 2 ng per target gene), which is more adapted for analyzing small-sized tumors.

The theoretical and practical aspects of real-time quantitative RT-PCR using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere (28). We quantified transcripts of 2 endogenous RNA control genes involved in 2 cellular metabolic pathways, namely *TBP*, which encodes the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) and *RPLP0* (also known as *36B4*), which encodes acidic ribosomal phosphoprotein P0. *TBP* and *RPLP0* were selected as endogenous controls because the levels of their transcripts in the tumor samples were low (Ct values between 24 and 26) and high (Ct values between 18 and 20), respectively. Each sample was normalized on the basis of its *TBP* (or *RPLPO*) content. Results, expressed as *N*-fold differences in target gene expression relative to the *TBP* (or *RPLPO*) gene, and termed "N*target*," were determined as $N\mathit{target} = 2^{\Delta Ct_{sample}}$, where the ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *TBP* (or *RPLP0*) gene (29). The N*target* values of the tumor samples were subsequently normalized such that the mean of the dermal neurofibroma N*target* values was 1. Primers for *TBP*, *RPLP0*, and the target genes were chosen with the assistance of the computer programs Oligo 6.0 (National Biosciences). For each primer pair, we performed no-template control (NTC) and no-reverse-transcriptase control (RT-negative) assays, which produced negligible signals (usually >40 in Ct values), suggesting that primer–dimer formation and genomic DNA contamination effects were negligible. RNA extraction, cDNA synthesis, and PCR reaction conditions have been described elsewhere (29).

Immunohistochemical study

Formalin fixed, paraffin embedded skin samples were retrieved from the archive material of the department of Pathology of Henri Mondor Hospital. Hematoxylin–eosin–saffron (HES) staining and immunohistochemistry were applied to 3-μm-thick dewaxed sections. Immunostaining of β-catenin (BD Biosciences Pharmingen) was done using the Bond max device (Menarini diagnostics, France), at a 1:4,000 dilution after antigen retrieval by heat at pH 9. In all samples, we checked the staining of epithelial (epidermis or adnexae) and/or endothelial cells as internal positive controls.

HEK293, MCF-7, and MSC80 cells transfection with siRNA

To knockdown the *NF1* gene, human cell lines (HEK293 and MCF-7) and mouse cells (MSC80) transient transfections were carried out using Effecten reagent (Qiagen) for MSC80, and Hiperfect Transfection Reagent (Qiagen) according to HiPerFect Transfection Reagent Handbook for human cell lines. SiRNAs were purchased from Qiagen: mouse siRNA against *Nf1* (reference GS18015), human siRNA against *NF1* (reference GS4763), and nontargeted (NT) siRNAs (negative control reference 1027310). Mouse *Nf1* and human *NF1* genes were targeted with 4 different sequences of siRNA directed against 4 different regions of the cognate mRNA. We quantified gene expressions at both mRNA (using real-time RT-PCR) and protein (using Western blot) levels to assess the siRNAs efficacy.

Luciferase reporter assay of β-catenin–mediated Wnt signaling pathway activation

Luciferase reporter assay was used to determine the Wnt pathway signaling activity in *Nf1* invalidated or in control (nontargeting; NT) siRNAs MSC80 cells. SuperTOP-Flash-Luc plasmids were kindly provided by Dr. R T Moon (University of Washington School of Medicine, Seattle, WA). One day before transfection, MSC80 cells (1.5×10^5 cells per well) were seeded into 6-well plate and incubated in the DMEM culture medium containing 10% decomplemented fetal calf serum. SuperTOP-Flash-Luc plasmid (0.4 μg), the pCMV-β-galactosidase expression vector (0.1 μg), and siRNAs (25 nmol/L) were mixed with a solution containing Effecten reagents (0.85 mg/mL) in DMEM. The mixture was then added to the cells and incubated overnight. Sixteen hours after transfection, the medium was replaced by DMEM culture medium containing 10% decomplemented fetal calf serum. Luciferase activity was determined using an enzymatic method described by Massaad and colleagues (30). The β-galactosidase activity was used for the transfection efficiency normalization.

Protein extraction and Western blot analysis

Cells transfected with NT siRNA or with siRNAs against *NF1* were homogenized in 100 μL ice-cold RIPA buffer [50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 5 mmol/L EDTA, pH 8.0, 2 mmol/L phenylmethylsulfonylfluoride (PMSF; Sigma-Aldrich), 50 μg/mL leupeptin, 50 μg/mL pepstatin A, and 50 μg/mL aprotinin]. Protein concentration of the clarified homogenates (4°C, 15 minutes, 13,500 rev/min) was determined on all samples using the Bradford protein assay (Bio-Rad Laboratories). Aliquots of 30 μg of total protein extracts were used for each sample. Homogenate proteins were separated on 15% SDS-PAGE gel or on Precast XT 4% to 12% Bis-Tris Criterion Gel (Bio-Rad) for neurofibromin and blotted onto polyvinylidene difluoride membranes. Nonspecific binding sites in the transblots were blocked at 4°C overnight with 5% bovine serum albumin (BSA), 0.01% Tween 20 (Invitrogen), TBS, pH 7.4. Membranes were then incubated at room temperature

for 2 hours with the following primary antibodies diluted in a mixture of 5% BSA blocking agent and TBS-Tween 0.1%: active (dephospho) β-catenin (anti-ABC) clone 8E7 antibody (1:1,000), β-catenin antibody (1:1,000), neurofibromin antibody (1:1,000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:10,000). They were then incubated at room temperature for 1 hour 30 minutes with the appropriate secondary antibody diluted in 5% BSA blocking buffer/TBS-Tween 0.1% (anti-mouse and anti-rabbit at 1:20,000), followed by ECL Plus Western blotting detection (Amersham) before exposure to radiographic film Hyperfilm ECL (Amersham). Western blots were quantified by means of NIH Image J Software.

Primary antibodies against neurofibromin (rabbit monoclonal antibody) were purchased from Santa-Cruz (reference sc67), mouse total β-catenin (mouse monoclonal antibody) was purchased from BD Biosciences, human total β-catenin (rabbit polyclonal antibody) was purchased from Cell Signaling (reference #9562), active-β-catenin (mouse monoclonal antibody) and GAPDH (mouse monoclonal antibody) were purchased from Millipore (reference 05-665 and G8795). Secondary antibodies used for Western blotting were horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit immunoglobulin G (Millipore).

Statistical analysis

As mRNA levels did not fit a Gaussian distribution, (i) mRNA levels in each subgroup of samples were expressed as their median values and ranges, rather than their mean values and coefficients of variation; and (ii) comparison and relationships between mRNA levels of the different target genes were respectively estimated using nonparametric tests: the Mann–Whitney *U* test (link between 1 qualitative parameter and 1 quantitative parameter) and the Spearman correlation test (link between 2 quantitative parameters). Differences between the 2 populations were judged significant at confidence levels greater than 95% ($P < 0.05$). In the SuperTOP-Flash Luciferase reporter assay, 2 groups comparisons were performed by the Student *T* test. A *P*-value of <0.05 was considered to be statistically significant.

Results

mRNA expression of 89 Wnt pathway genes in 7 dermal neurofibromas, 7 plexiform neurofibromas, and 8 MPNST NF1-related samples (screening set)

Eight (9%) of the 89 genes (i.e., *WNT1*, *WNT7A*, *WNT8A*, *WNT8B*, *WNT9B*, *DKK4*, *KREMEN2*, and *TLE1*) showed very low levels of target gene in dermal neurofibromas, plexiform neurofibromas, and MPNSTs. Their mRNA levels were only detectable but not reliably quantifiable by means of real-time quantitative RT-PCR assays, mainly based on fluorescence SYBR Green methodology (Ct > 32). Thirty-two (39.5%) of the 81 remaining genes were expressed at a different level (>2-fold) in the MPNST group and/or the plexiform neurofibromas group compared with the dermal neurofibromas group: 9 genes were deregulated in the 7 plexiform neurofibromas as compared with the 7 dermal neurofibromas and 30 genes in the 8 MPNSTs as compared with the 7 dermal neurofibromas (Supplementary Table S3).

mRNA expression of 32 identified Wnt pathway genes in 10 dermal neurofibromas, 31 plexiform neurofibromas, and 16 MPNST NF1-related samples (validation set)

Expression levels of the 32 deregulated genes identified by "screening set" analysis were then determined in 10 dermal neurofibromas, 31 plexiform neurofibromas, and 16 MPNST samples. Nine (28.1%) of the 32 genes were significantly deregulated in the 31 plexiform neurofibromas as compared with the 10 dermal neurofibromas ($P < 0.05$; Fig. 1 and Supplementary Table S4): 8 genes were upregulated (*WNT9A*, *FZD1*, *SFRP4*, *SFRP5*, *TLE2*, *MYC*, *CAMK2B*, and *PRKCQ*) and 1 was downregulated (*SFRP1*) in plexiform neurofibromas. These 9 deregulated genes in plexiform neurofibromas encode 1 Wnt ligand (WNT9A), 1 Wnt receptor (FZD1), 4 proteins involved in canonical Wnt signal transduction (SFRP1, SFRP4, SFRP5, and TLE2), 1 LEF/TCF-inducible protein (MYC), and 2 proteins involved in the Wnt/calcium pathway (CAMK2B and PRKCQ).

Twenty (62.5%) of the 32 genes were significantly deregulated in the 16 MPNSTs as compared with the 10 dermal neurofibromas ($P < 0.05$; Fig. 1; Supplementary Table S4): 14 genes were upregulated (*WT5A*, *FZD1*, *FZD8*, *DKK1*, *WIF1*, *LEF1*, *ID2*, *MSX2*, *SOX9*, *WISP1*, *TWIST1*, *BMP2*, *CAMK2B*, and *PRKCQ*) and 6 were downregulated (*WNT2*, *WNT9A*, *DKK3*, *KREMEN1*, *SFRP1*, and *TCF7*) in MPNSTs.

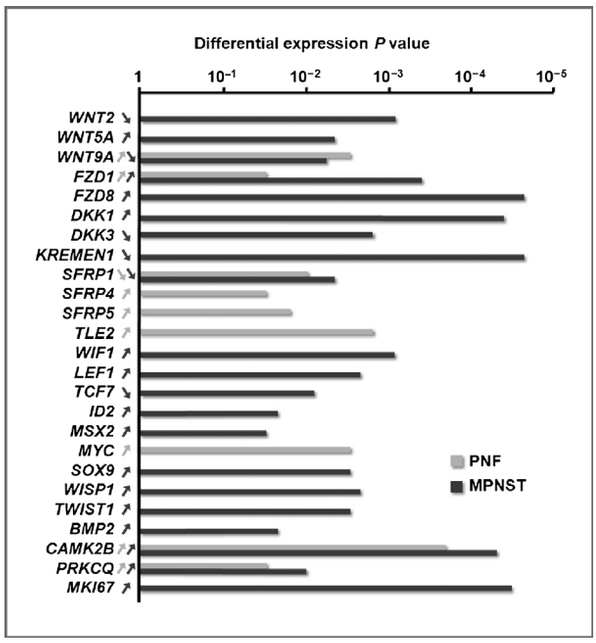

Figure 1. Diagram depicting *P* values (horizontal bars) for significant gene expression differences between MPNSTs and/or plexiform neurofibromas (PNF) and dermal neurofibromas. Arrows indicate increased (up arrow) or decreased (down arrow) differential expression.

These 20 deregulated genes in MPNSTs encode 3 Wnt ligands (WNT2, WNT5A, and WNT9A), 2 Wnt receptors (FZD1 and FZD8), 5 proteins involved in canonical Wnt signal transduction (DKK1, DKK3, KREMEN1, SFRP1, and WIF1), 2 transcription factors (LEF1 and TCF7), 6 LEF/TCF-inducible proteins (ID2, MSX2, SOX9, WISP1, TWIST1, and BMP2), and 2 proteins involved in the Wnt/calcium pathway (CAMK2B and PRKCQ).

Taken together, our results showed that 4 genes were specifically deregulated in plexiform neurofibromas, 15 were specifically dysregulated in MPNSTs, and 6 were dysregulated in both tumor types.

In the same set of 57 samples, we also examined the expression of the *NF1* gene, and the proliferation-associated gene *MKI67* that encodes the proliferation-related antigen Ki-67. *NF1* expression was similar in the 3 groups of tumors whereas *MKI67* showed significant overexpression (30.5-fold) in MPNSTs ($P = 0.00005$; Fig. 1 and Supplementary Table S4). This lack of expression difference for *NF1* is probably because of the fact that the *NF1* gene is ubiquitously expressed and therefore expressed in the different cell components of the neurofibromas and MPNSTs, and that $NF1^{-/-}$ Schwann cells represent only a fraction of the total Schwann cell population in tumor samples.

The immunohistochemical study of β-catenin in MPNSTs showed a strong diffuse and cytoplasmic staining (Fig. 2). In contrast, a weaker β-catenin staining was observed in the plexiform neurofibromas (Fig. 2).

mRNA expression of 32 identified Wnt pathway genes in paired plexiform neurofibroma and MPNST samples from 4 patients

We analyzed the mRNA levels of the 32 deregulated genes identified by "screening set" analysis in 4 patients from whom both plexiform neurofibroma and matched MPNST samples were available. We found a clear decrease in the mRNA level of *WNT9A*, *DKK3*, *SFRP4* (all patients), and *WISP1* (patients 1, 3, and 4), upon progression from plexiform neurofibroma to MPNST (Supplementary Fig. S2). We also found a clear increase in the mRNA level of *DKK1* (all patients), *TLE2*, *TCF7*, *WNT2* (patients 1, 2, and 3), and *WNT5A* (patients 3 and 4) during this transition (Supplementary Fig. S2).

mRNA expression of the 24 differentially expressed Wnt pathway genes in 4 non-NF1 (sporadic) MPNSTs

The expression levels of the 24 differentially expressed Wnt pathway genes were also determined in 4 non-NF1 (sporadic) human MPNST biopsies (Supplementary Table S5). According with the data obtained in NF1-associated MPNSTs, the majority of the 14 genes upregulated and of the 6 downregulated in MPNST biopsies showed a trend to be also up- and downregulated.

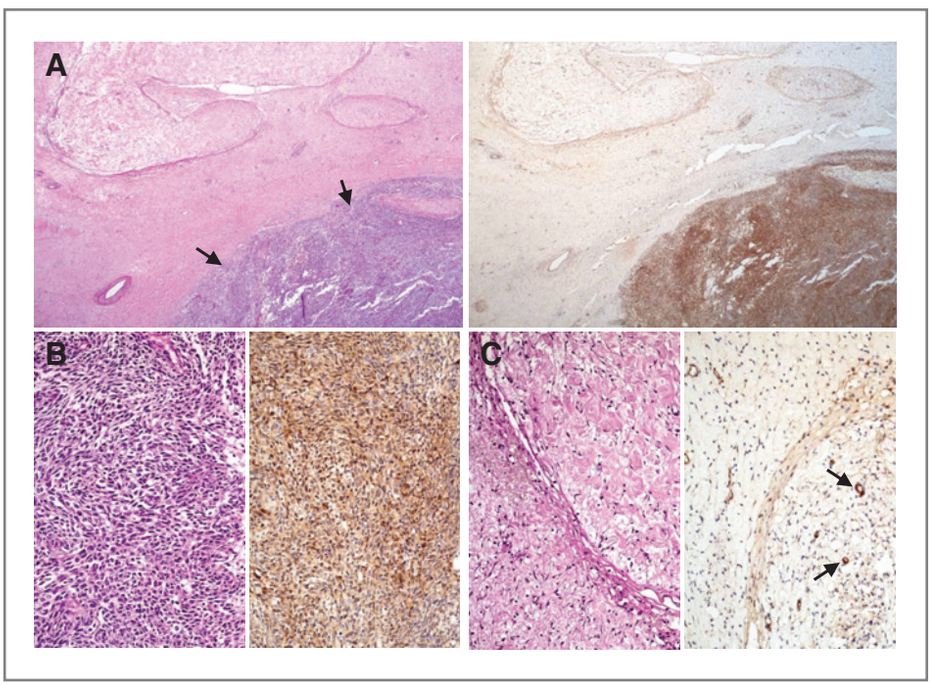

Figure 2. Representative immunohistochemical study of β-catenin in MPNST and plexiform neurofibroma. A, this tissue sample shows an area of plexiform neurofibroma surrounding the MPNST (arrows). The MPNST shows an intense β-catenin staining (left: hematoxylin, eosin, and saffron stain; right: immunohistochemistry revealed by diaminobenzidine, original magnifications ×25). B, the MPNST areas show a diffuse and cytoplasmic expression of β-catenin (left: hematoxylin, eosin, and saffron stain; right: immunohistochemistry revealed by diaminobenzidine, original magnifications ×200). C, the plexiform neurofibromas show a weak cytoplasmic expression of β-catenin in a proportion of Schwann cells, whereas intratumoral vessels are strongly stained, taken as internal positive controls (left: hematoxylin, eosin, and saffron stain; right: immunohistochemistry revealed by diaminobenzidine, original magnifications ×200).

mRNA expression of the 24 differentially Wnt pathway expressed genes in normal human Schwann cells, fibroblasts, endothelial cells, and mast cells

Neurofibromas and MPNSTs are both heterogeneous tumors mainly composed of Schwann cells (60–80%), together with fibroblasts, mast cells, and endothelial cells (3). To investigate cell type–specific expression of the 24 previously identified altered genes in NF1 plexiform neurofibromas and/or MPNSTs (Fig. 1 and Supplementary Table S4), we analyzed their mRNA levels by means of real-time RT-PCR in normal human Schwann cells, fibroblasts, endothelial cells, and mast cells (Table 1). *MYC*, *CAMK2B*, *KREMEN1*, and *BMP2* were similarly expressed in the 4 cell types, suggesting a ubiquitous expression. *TLE2*, *DKK3*, *TCF7*, *FZD1*, *FZD8*, and *DKK1* were similarly expressed in Schwann cells, fibroblasts, and endothelial cells, but were not expressed in mast cells. Six of these 24 genes (*WISP1*, *WNT5A*, *SFRP1*, *ID2*, *TWIST1*, and *SOX9*) were similarly expressed in Schwann cells and fibroblasts, but were not expressed (Ct > 32) in endothelial cells or mast cells. *WNT2*, *MSX2*, *LEF1*, *SFRP4*, and *SFRP5* were Schwann cell–specific, being expressed more than 20 times in the Schwann cells than in the other 3 cell types. *PRKCQ* was mainly expressed in endothelial cells and mast cells, and *WIF1* only in Schwann cells and endothelial cells. Finally, *WNT9A* was endothelial cells specific (being expressed 10 times more than in the other 3 cell types). It is noteworthy that the endothelial cells specificity of *WNT9A* could explain its expression profile in NF1 tumors, in particular its overexpression in plexiform neurofibromas (well-known to be more angiogenic than the dermal neurofibromas).

Table 1. mRNA expression of 24 identified genes in normal human cells

	Mast cells	Schwann cells	Fibroblasts	Endothelial cells
Wnt ligands ($n = 3$)				
WNT2	0.00	**0.28**[a]	0.00	0.00
WNT5A	0.00	**33.84**	**39.80**	**0.20**
WNT9A	0.01	0.04	0.03	**0.47**
Wnt receptors ($n = 2$)				
FZD1	0.03	**0.44**	**1.30**	**0.13**
FZD8	0.00	**3.70**	**0.21**	**0.87**
Wnt signal transduction ($n = 8$)				
DKK1	0.00	**486.00**	**930.00**	**396.00**
DKK3	0.01	**3.71**	**0.64**	**0.29**
KREMEN1	**0.03**	**0.13**	**0.40**	**0.10**
SFRP1	0.00	**0.55**	**0.27**	0.02
SFRP4	0.00	**0.83**	0.02	0.00
SFRP5	0.00	**0.62**	0.00	0.03
TLE2	0.00	**0.11**	**0.30**	**0.41**
WIF1	0.00	**1.05**	0.00	**0.60**
Wnt transcriptional factors ($n = 2$)				
LEF1	0.00	**0.38**	0.00	0.01
TCF7	0.00	**0.40**	**0.72**	**0.20**
Lef/Tcf-inducible proteins ($n = 7$)				
ID2	0.18	**2.84**	**17.90**	0.19
MSX2	0.00	**0.96**	0.00	0.04
MYC	**0.55**	**0.82**	**0.45**	**0.56**
SOX9	0.00	**0.93**	**0.31**	0.00
WISP1	0.00	**10.65**	**5.16**	0.02
TWIST1	0.00	**0.60**	**7.02**	0.01
BMP2	0.40	**0.30**	**5.96**	**21.38**
Noncanonical Wnt signal: Wnt/calcium pathway ($n = 2$)				
CAMK2B	**0.60**	**1.26**	**0.46**	**2.46**
PRKCQ	**2.28**	0.08	0.00	**1.11**

NOTE: The gene mRNA levels (calculated as described in Materials and Methods) were based on the amount of the target message relative to the endogenous control *TBP* message, in order to normalize the starting amount and quality of total RNA. Similar results were obtained with a second endogenous control, the *RPLP0* gene (also known as 36B4).

Genes indicated in bold were found to be highly expressed as compared with one or several other normal human cells.

[a]For each gene, mRNA levels were normalized to the 10 dermal neurofibromas.

mRNA expression of the 24 differentially expressed Wnt pathway genes in 7 NF1-associated MPNST cell lines

The expression levels of the 24 identified genes was determined in 7 well-characterized NF1-associated MPNST cell lines, namely NMS-2, NMS-2PC, 88-3, ST88-14, 90-8, S462, and T265 (Table 2). All genes (except *WT9A* in agreement with the fact that it is an endothelial cell–specific gene) were expressed (Ct < 32) in at least 1 NF1-associated MPNST cell line. According with the data obtained in MPNSTs, the majority of the 14 genes upregulated and of the 6 downregulated in MPNST biopsies was also up- and downregulated (>3-fold the median value in 10 dermal neurofibromas) in a fraction of the MPNST cell lines. For example, *WT5A*, *FZD8*, *DKK1*, *WIF1*, *MSX2*, and *CAMK2B* were upregulated in at least 5 of the 7 MPNST cell lines, whereas *WNT2*, *WNT9A*, *DKK3*, *KREMEN1*, and *SFRP1* were downregulated in at least 5 of the 7 MPNST cell lines. These findings confirm the Schwann cell expression of these genes and their dysregulation in MPNST tumorigenesis.

Relationship between mRNA levels of 24 differentially expressed Wnt pathway genes and 2 major stem cell markers (*PROM1* and *NKX2.2*)

Several studies supported the role of the canonical Wnt signaling in the formation and maintenance of stem cells and cancer stem cells (14, 20–22). To explore the possible involvement of the 24 identified Wnt genes (deregulated in NF1 tumors) in the formation of cancer stem cells, we tested the relationship between the expression of these 24 differentially expressed Wnt genes and 2 major stem cell marker genes, that is *PROM1* (also known as *CD133*) and *NKX2-2*, in our series of 57 NF1-associated tumors. *PROM1* and *NKX2-2* were overexpressed (144- and 87-fold, respectively) in MPNSTs ($P = 0.0007$ and 0.002, respectively; Mann–Whitney *U* test) suggesting an increase of the proportion of cancer stem cells in this malign tumor group. We observed

Table 2. mRNA expression of 24 identified genes in 7 NF1-associated MPNST cell lines

	NMS-2	NMS-2PC	88-3	ST88-14	90-8	S462	T265
Wnt ligands ($n = 3$)							
WNT2	**0,08**[a]	**0.04**	**0.09**	**0.32**	**0.04**	**0.18**	0.44
WNT5A	**5.76**	**11.52**	**12.65**	**8.79**	1.42	1.13	**16.15**
WNT9A	**0.04**	**0.00**	**0.01**	**0.08**	**0.20**	**0.07**	**0.05**
Wnt receptors ($n = 2$)							
FZD1	1.21	0.96	0.44	1.33	0.56	**0.21**	0.32
FZD8	0.45	**3.99**	**3.56**	**4.17**	**6.93**	0.22	**5.38**
Wnt signal transduction ($n = 8$)							
DKK1	**868.00**	**716.00**	**796.00**	**6146.00**	**1252.00**	**806.00**	**1648.00**
DKK3	**0.12**	**0.10**	**0.23**	0.91	**0.17**	**0.03**	**0.23**
KREMEN1	**0.13**	0.36	**0.12**	0.45	**0.14**	**0.05**	**0.11**
SFRP1	**0.04**	**0.14**	0.42	**0.29**	**0.28**	**0.15**	**0.05**
SFRP4	**0.02**	**0.04**	**0.00**	**0.01**	**0.00**	**0.00**	**0.01**
SFRP5	2.71	0.70	0.57	0.63	**0.00**	1.19	0.88
TLE2	**0.11**	0.35	**0.22**	0.79	0.67	0.38	**0.12**
WIF1	**35.7**	**37.4**	**27.2**	**96.8**	**48.3**	**30.0**	**25.8**
Wnt transcriptional factors ($n = 2$)							
LEF1	0.63	0.98	**0.24**	0.61	**0.33**	0.55	**0.20**
TCF7	0.88	1.00	0.75	1.75	0.77	**0.14**	0.45
Lef/Tcf-inducible proteins ($n = 7$)							
ID2	**3.25**	0.41	1.17	0.76	0.35	1.30	0.95
MSX2	**38.20**	**14.00**	**11.80**	**8.00**	**3.28**	**4.80**	2.46
MYC	1.64	1.52	1.59	0.35	1.73	**3.96**	1.84
SOX9	1.25	**4.86**	1.80	**5.09**	2.43	**0.24**	1.12
WISP1	**0.18**	0.86	**0.11**	**0.00**	1.20	**0.12**	**0.02**
TWIST1	1.31	1.20	0.31	**3.50**	0.71	1.39	0.56
BMP2	0.92	0.96	**13.18**	**5.18**	0.86	0.20	**4.48**
Noncanonical Wnt signal: Wnt/calcium pathway ($n = 2$)							
CAMK2B	**12.18**	**18.36**	**4.08**	0.00	**3.54**	0.00	**3.48**
PRKCQ	**3.13**	0.91	**3.36**	**6.16**	1.92	1.95	2.00

NOTE: The genes indicated in bold were found to be differentially expressed.

[a]For each gene, mRNA levels were normalized to the 10 dermal neurofibromas.

Table 3. Relationship between mRNA levels of the 24 identified genes and stem cell marker and EMT marker genes

	Stem cell markers		EMT markers		
	PROM1	***NKX2-2***	***TWIST1***	***SLUG***	***CDH1***
Wnt ligands ($n = 3$)					
WNT2	**−0.355**[a] **$P = 0.007$**[b]	**−0.365** **$P = 0.005$**	+0.285 $P = 0.03$	NS	NS
WNT5A	**+0.484** **$P = 0.0002$**	**+0.353** **$P = 0.007$**	NS	NS	NS
WNT9A	−0.319 $P = 0.015$	−0.406 $P = 0.002$	NS	NS	NS
Wnt receptors ($n = 2$)					
FZD1	NS	NS	NS	NS	NS
FZD8	+0.398 $P = 0.02$	NS	**+0.468** **$P = 0.0003$**	NS	−0.278 $P = 0.03$
Wnt signal transduction ($n = 8$)					
DKK1	**+0.610** **$P = 0.000001$**	**+0.385** **$P = 0.003$**	+0.318 $P = 0.015$	NS	NS
DKK3	**−0.386** **$P = 0.003$**	**−0.413** **$P = 0.0015$**	NS	NS	NS
KREMEN1	**−0.439** **$P = 0.0008$**	**−0.327** **$P = 0.01$**	NS	NS	**+0.371** **$P = 0.0046$**
SFRP1	NS	NS	**+0.342** **$P = 0.009$**	NS	NS
SFRP4	NS	−0.296 $P = 0.024$	NS	−0.337 $P = 0.024$	NS
SFRP5	NS	NS	NS	−0.322 $P = 0.014$	NS
TLE2	NS	NS	NS	NS	NS
WIF1	**+0.527** **$P = 0.00004$**	**+0.609** **$P = 0.000001$**	+0.307 $P = 0.019$	**+0.392** **$P = 0.003$**	NS
Wnt transcriptional factors ($n = 2$)					
LEF1	NS	NS	NS	NS	NS
TCF7	**−0.520** **$P = 0.00006$**	**−0.400** **$P = 0.002$**	NS	NS	NS
Lef/Tcf-inducible proteins ($n = 7$)					
ID2	+0377 $P = 0.004$	NS	**+0.360** **$P = 0.006$**	NS	NS
MSX2	**+0.573** **$P = 0.000007$**	**+0.384** **$P = 0.003$**	**+0.433** **$P = 0.0009$**	+0.293 $P = 0.02$	NS
MYC	NS	NS	+0.305 $P = 0.02$	NS	NS
SOX9	NS	NS	**+0.344** **$P = 0.009$**	NS	NS
WISP1	**+0.630** **$P = 0.0000005$**	**+0.418** **$P = 0.001$**	+0.309 $P = 0.018$	+0.305 $P = 0.02$	NS
TWIST1	NS	NS	+1.000 $P < 10^{-7}$	+0.289 $P = 0.028$	NS
BMP2	+0.291 $P = 0.027$	+0.314 $P = 0.017$	**+0.377** **$P = 0.0039$**	**+0.505** **$P = 0.00009$**	NS
Noncanonical Wnt signal: Wnt/calcium pathway ($n = 2$)					
CAMK2B	+0.314 $P = 0.017$	NS	NS	NS	NS

(Continued on the following page)

Table 3. Relationship between mRNA levels of the 24 identified genes and stem cell marker and EMT marker genes (Cont'd)

	Stem cell markers		EMT markers		
	PROM1	***NKX2-2***	***TWIST1***	***SLUG***	***CDH1***
PRKCQ	NS	NS	+0.260 $P = 0.048$	+0.312 $P = 0.017$	NS

Abbreviation: NS, not significant.

[a]Spearman correlation coefficient.

[b]*P* value, Spearman rank correlation test.

The genes indicated in bold were found to be marked significantly differentially expressed ($P < 0.01$).

high statistical significant positive associations between *PROM1* and *NKX2-2*, and *WNT5A*, *DKK1*, *WIF1*, *MSX2*, and *WISP1* ($P < 0.01$ with both *PROM1* and *NKX2-2*), and high statistical significant negative associations between *PROM1* and *NKX2-2*, and *WNT2*, *DKK3*, *KREMEN1*, and *TCF7* ($P < 0.01$ with both *PROM1* and *NKX2-2*; Table 3).

Relationship between mRNA levels of 24 differentially Wnt pathway expressed genes and 5 major EMT markers (*TWIST1*, *SLUG*, *SNAIL*, *VIM*, and *CDH1*)

Regulation of the EMT is a crucial step in cancer development. *TWIST1*, an important transcription factor involved in EMT and upregulated in NF1-associated MPNST (19), is also a major LEF/TCF–inducible gene (http://www.stanford.edu/~rnusse/pathways/targets.html). In consequence, to explore the involvement of the Wnt pathway in a possible Schwann–mesenchymal transition during NF1 tumorigenesis, we first tested the expression of 5 major EMT markers (*TWIST1*, *SLUG*, *SNAIL*, *VIM*, and *CDH1*) in our series of 57 NF1-associated tumors. *TWIST1* and *SLUG* were overexpressed (1.6- and 2.1-fold, respectively) in the MPNSTs ($P = 0.005$ and 0.001, respectively), whereas *CDH1* was underexpressed (11-fold; $P = 0.0006$). *SNAIL* and *VIM* showed similar mRNA levels in dermal neurofibromas, plexiform neurofibromas, and MPNSTs. We next tested the relationship between the expression of the 24 identified Wnt genes and of the 3 differentially expressed EMT marker genes (i.e., *TWIST1*, *SLUG*, and *CDH1*). We observed high statistical significant positive associations ($P < 0.01$) between *TWIST1* and *FZD8*, *SFRP1*, *ID2*, *MSX2*, *SOX9*, and *BMP2*, between *SLUG* and *WIF1* and *BMP2*, and between *CDH1* and *KREMEN1*. *CDH1* and *KREMEN1* showed a correlated decreased expression in MPNSTs (Table 3). It is noteworthy that we observed a high statistical significant positive association between *SLUG* and both *PROM1* and *NKX2-2* ($P = 0.0033$ and 0.00068, respectively).

In vitro consequences of *NF1* inactivation on Wnt pathway activation state

To analyze the effects of the *NF1* gene inactivation on the Wnt pathway, we knocked down the expression of *NF1* in 2 human cell lines (an embryonic kidney cell line: HEK293 and a breast adenocarcinoma cell line: MCF-7) and 1 mouse Schwann cell line (MSC80) by siRNAs strategy. Cells were transfected with either a nontargeting siRNA (NT) or a siRNA directed against *NF1*. The efficacy of the knockdown of the *NF1* expression was tested by real-time RT-PCR and Western blot. In MSC80 cell line, we found a decreased *Nf1* expression at both mRNA and protein (neurofibromin) levels (Fig. 3A). In human cell lines (HEK293 and MCF-7), a decreased *NF1* expression was confirmed at mRNA level (data not shown).

In the 2 human cell lines (HEK293 and MCF-7), mRNA expression of 24 Wnt pathway genes (cf. supra) was analyzed after the knockdown of *NF1*. Those 2 non-Schwann cell lines showed no clear Wnt gene expression difference for the majority of the 14 upregulated and the 6 downregulated genes in MPNST biopsies and no difference was found in the amount of the active as well as the total β-catenin quantified by Western blot (data not shown).

Similarly, no clear expression difference was found between the MEFs derived from wild-type mice and the MEFs derived from $Nf1^{-/-}$ mice, in the majority of the 14 upregulated and of the 6 downregulated genes in MPNST biopsies (data not shown).

In MSC80 Schwann cells, we used a Wnt pathway reporter assay to determine whether the canonical Wnt signaling pathway was modulated after the knockdown of *Nf1*. The silencing of *Nf1* increased by 3-folds ($P < 0.01$) the promoter activity of SuperTOP-Flash-Luc in MSC80 (Fig. 3B). The knockdown of *Nf1* did not alter the promoter activity of SuperFOP-Flash-Luc, a construct that lacks any binding sites for TCF/LEF transcription factors (Fig. 3B). Furthermore, the transcript of *Axin2*, a target gene of Wnt pathway was increased by 2-folds after the silencing of *Nf1* in MSC80 cells (Fig. 3C). To assess whether the knockdown of *Nf1* altered the expression of β-catenin at the protein level, we have quantified by Western blot the amount of the active as well as the total β-catenin. In MSC80 transfected with the siRNA against *Nf1*, we observed a 2-folds increase of the ratio of active-β-catenin/total-β-catenin compared with the control cells (Fig. 3D).

Altogether, those data show that the knockdown of *NF1* in Schwann cells enhances the activity of Wnt/β-catenin pathway. Those data suggest cell type–specific

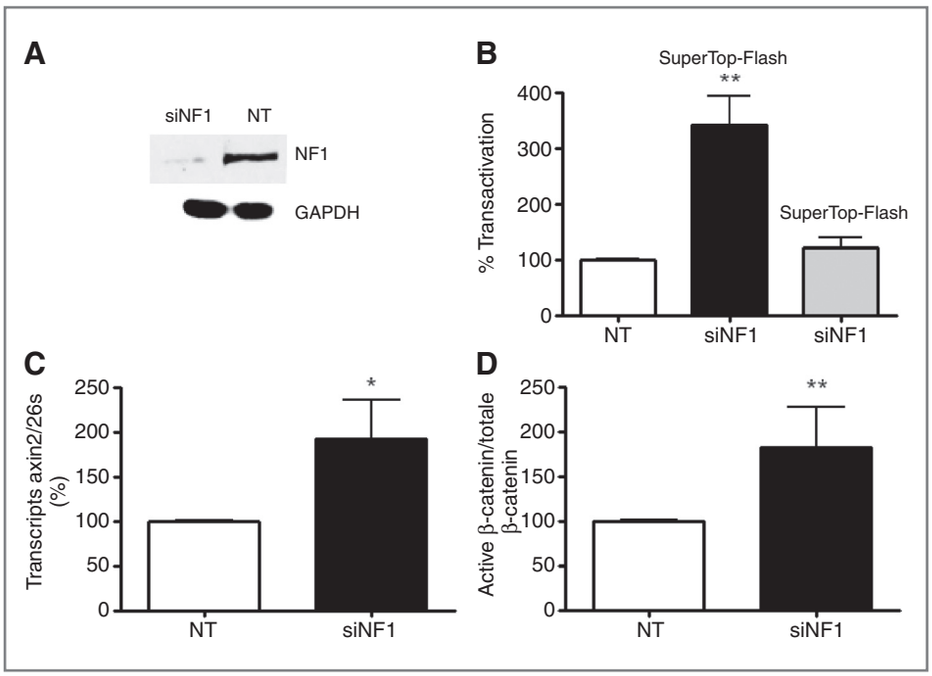

Figure 3. Effect of *Nf1* knockdown on Wnt pathway in MSC80 cells. A, MSC80 cells were transfected with either siRNA-targeting *NF1* or nontargeting siRNA (NT). Western blots using anti-neurofibromin antibodies were perfomed to verify Nf1 inactivation at protein level in MSC80 cells. GAPDH was used to normalize the Western blots. Results were reproduced in 3 independent experiments, and figures represent a typical experiment. B, MSC80 cells were transiently cotransfected by the SuperTOP-Flash Luciferase or SuperFOP-Flash Luciferase plasmids with either nontargeting (NT) siRNA or siRNA against *NF1*. Luciferase activity was then analyzed. *, $P < 0.05$, and **, $P < 0.01$ by Tukey *post hoc* tests after one-way ANOVA when compared with control. C, MSC80 cells were transfected with either NT or a siRNA against *Nf1*. Total RNA was extracted and RT-qPCR was performed using specific primer recognizing *Axin2*. D, Western blots were performed using anti-active (dephosphorylated β-catenin) and anti-total β-catenin antibodies. GAPDH was used to normalize the Western blots. The Western blots were quantified using Image J software (rsb.info.nih.gov).

consequences of *NF1* knockdown as Wnt pathway activation was not observed in epithelial (MCF-7) or kidney (HEK293) cell lines, or in MEFs cells but only in Schwann (MSC80) cell lines.

Discussion

WNT signaling pathway orchestrates highly complex molecular events. Aberrant activation of Wnt pathway could trigger cell malignant transformation. In the last 20 years, involvement of the Wnt pathway has mainly been revealed in human epithelial malignancies (31). However, the Wnt pathway may also play a role in tumors of mesenchymal origin (32). Mesenchymal stem cells could differentiate into Schwann cell–like cells (33). In this work, we therefore apprehended the role of Wnt pathway in NF1 tumorigenesis and more particularly its link with cancer stem cells and putative Schwann–mesenchymal transition.

Wnt pathway is tightly implicated in the myelination process elicited by Schwann cells. We have shown that the selective inhibition of Wnt components by siRNA or dominant negative forms inhibits the expression of myelin genes in mouse Schwann cells (34, 35). Moreover, the activation of Wnt signaling by recombinant Wnt ligands increases the transcription of myelin genes. Importantly, loss-of-function analyses in zebrafish embryos show, *in vivo*, a key role for Wnt/β-catenin signaling in the initiation of myelination and in myelin sheath compaction in the central and peripheral nervous systems. Inhibition of Wnt/β-catenin signaling resulted in hypomyelination, without affecting Schwann cells and oligodendrocytes generation or axonal integrity (21). Thus, the dysregulation of this pathway could lead to either developmental defects or tumorigenesis.

Nine genes were significantly deregulated in plexiform neurofibromas (as compared with dermal neurofibromas): 8 genes were upregulated and 1 was downregulated (Table 4). All these genes (except *WNT9A*, which seems endothelial cell specific) were expressed in normal and tumoral Schwann cells (Table 2). Our results show that among the 3 different Wnt pathways, the canonical Wnt pathway is mainly involved in plexiform neurofibroma development with deregulation of genes encoding major components of this Wnt pathway (Table 4). Little is known about the relevance of these genes (except *MYC*) to cancer biology. A few observations have however described implications of these genes in several cancer types (36–40). Taken together, these findings suggest an alteration of the canonical Wnt pathway FZD1/SFRP1/TLE2/MYC in plexiform neurofibroma development.

Our results also showed a major activation of the Wnt canonical pathway during the malignant transformation of plexiform neurofibromas in MPNSTs from NF1 patients.

Table 4. Major deregulation of the Wnt pathway expression in plexiform neurofibromas and MPNSTs

Plexiform neurofibromas	MPNSTs	Human tumor types with previous described alterations in WNT pathway components
Wnt canonical pathway:	• Upregulated genes	
FZD1, *SFRP4*, *SFRP5*, *TLE2*, and *MYC*	***FZD1***, *FZD8*, *DKK1*, *WIF1*, *LEF1*, *MSX2*, *ID2*, *WISP1*, *BMP2*, *TWIST1*, and *SOX9*	Neuroblastoma (36), colorectal cancer (37), and astrocytoma (39), sarcoma (31)
	• Downregulated genes	
SFRP1	*WNT2*, ***SFRP1***, *DKK3*, *KREMEN1*, and *TCF7*	Breast carcinoma (38), Melanoma (40)
β-Catenin-independant Wnt pathway:	• Wnt/calcium pathway: upregulated genes	
WNT9A, ***CAMK2B*** and ***PRKCQ***	*WNT5A*, ***CAMK2B*** and ***PRKCQ***	Melanoma (42–44)
	• Wnt/calcium pathway: downregulated genes *WNT9A*	

The genes indicated in bold were found altered both in plexiform neurofibromas and in MPNSTs.
The canonical Wnt pathway is mainly involved in plexiform neurofibromas and MPNSTs. Eight genes were significantly upregulated (*WNT9A*, *FZD1*, *SFRP4*, *SFRP5*, *TLE2*, *MYC*, *CAMK2B*, and *PRKCQ*) and one was downregulated (*SFRP1*) in plexiform neurofibromas (as compared with dermal neurofibromas). Fourteen genes were significantly upregulated (*WT5A*, *FZD1*, *FZD8*, *DKK1*, *WIF1*, *LEF1*, *MSX2*, *ID2*, *WISP1*, *BMP2*, *TWIST1*, *SOX9*, *CAMK2B*, and *PRKCQ*), and 6 were downregulated (*WNT2*, *WNT9A*, *SFRP1*, *DKK3*, *KREMEN1*, and *TCF7*) in MPNSTs.

Indeed, 20 genes were significantly deregulated in the 16 NF1-related MPNSTs and the 7 MPNST cell lines (Fig. 1 and Supplementary Table S4). These deregulated genes encode molecules mainly involved in the canonical Wnt pathway (Table 4). Among them, *TWIST1* and more recently *SOX9* have already been identified as major genes involved in NF1 tumorigenesis (19, 41). By using immunohistochemical analysis, we confirmed an activation of the Wnt canonical pathway during the malignant transformation of plexiform neurofibromas in MPNSTs from NF1 patients (Fig. 3).

Wnt pathway expression analysis in a small set of non-NF1 (sporadic) MPNSTs also suggested an activation of the canonical Wnt pathway, as in the NF1-related MPNSTs. Our observation confirms previous results showing no difference in whole transcriptome profiling between sporadic and NF1-related MPNSTs that both present inactivation of the 2 *NF1* alleles (42).

An activation of the noncanonical Wnt/calcium pathway was also shown during the malignant transformation of plexiform neurofibromas in MPNSTs. Finally, our results showed a total absence of involvement of the second noncanonical Wnt pathway-planar cell polarity pathway—both in plexiform neurofibroma genesis, as well as in MPNSTs.

Very little is known about the link between Wnt pathway and receptor tyrosine kinase (RTK) RAS/MAPK and PI3K/AKT signaling pathways that are activated in NF1 tumorigenesis *via* the inactivation of the neurofibromin (encoded by *NF1*), which functions as a negative regulator of the RAS proteins. However, several studies with culture cells showed that activation of various tyrosine kinases (ERBB2, MET, and RON) can increase β-catenin signaling (43–45). Moreover, STI-571 (Glivec), a tyrosine kinase inhibitor, downregulates the β-catenin–signaling activity and suppresses cell proliferation (46). Finally, additional studies showed that RAS pathway activation can strongly cooperate with Wnt signaling to drive development of several type of carcinoma *in vivo* (47, 48), and that PI3K/AKT pathway can regulate Wnt signaling in various cancers (49). Our study emphasizes the potential cooperative link between Wnt pathway and RAS/MAPK and PI3K/AKT signaling pathways in NF1-associated tumorigenesis. To translate our observations into potential therapies, further studies will crucial to know which pathway(s) are critical for this link.

We confirmed the NF1/WNT pathway crosstalk at the functional level. We showed that the silencing of the *Nf1* gene stimulated Wnt pathway activation in Schwann cell lines, as in an *in vitro* model. *Nf1* knockdown induced (i) a Wnt pathway luciferase reporter assay activation, and (ii) an increased active-β-catenin/total-β-catenin ratio (Fig. 3). Our data also suggest Schwann cell–specific consequences of *NF1* knockdown as no significant Wnt gene expression variation was found in 3 non-Schwann cell types (HEK293, MCF-7, and MEFs).

We observed a strong association between Wnt pathway activation and both cancer stem cell reservoir (as judged on the expression level of stem cell markers *PROM1* and *NKX2.2*) and Schwann–mesenchymal transition (as judged on the expression level of EMT markers *TWIST1*, *SLUG*, and *CDH1*; Table 3). It is noteworthy that *NKX2.2* and *TWIST1* are major Wnt targets (http://www.stanford.edu/~rnusse/pathways/targets.html; ref. 50). Several studies suggested that mesenchymal stem cells could differentiate into Schwann cell–like cells in physiological conditions (32). In consequence, activation of Wnt signaling in NF1 tumorigenesis could promote the dedifferentiation of the tumoral

Schwann cells into tumoral mesenchyme cell-like. During the process of Schwann cell–mesenchyme transition, cell–cell adhesion may be downregulated and a mesenchymal phenotype acquired, associated with increased interaction with the extracellular matrix and an enhanced migratory capacity. These mesenchyme cell-like could be characterized by a higher ability to self-renew and to induce tumorigenesis, both characteristics of cancer stem cells. In this regard, we previously showed a downexpression of several key genes in the Schwann cell lineage (*ERBB3*, *ITGB4*, and *SOX1*) as well as that the deregulation of the Hedgehog pathway (19). Wnt, Hedgehog, and Notch pathways are the 3 major signaling pathways that regulate the proliferation of both endogenous normal stem cells and cancer stem cells (22). For example, Clement and colleagues suggest the Hedgehog pathway is involved in the regulation of the glioma cancer stem cells (51).

Finally, we assume that activation of the Wnt signaling pathway in NF1 tumorigenesis could explain the numerous skeletal manifestations in NF1 patients. Indeed, Wnt signaling is suggested to be the major common pathway leading to bone formation, with bone morphogenetic proteins (BMP) as only factors capable of initiating osteoblastogenesis from mesenchymal progenitors. Double inactivation of the *NF1* gene was observed in bone dysplasia found in NF1 (52). In this study, we showed upregulation of *BMP2* in MPNST samples. We can hypothesize that activation of Wnt pathway impaired $NF1^{-/-}$ osteoblasts differentiation, and has a major role in osseous abnormalities observed in the patients with NF1.

In conclusion, our study reveals that activation of the Wnt signaling pathway is consistently seen in NF1 tumorigenesis. Full confirmation of the role of this pathway in NF1 needs further *in vivo* (animal model) studies. Our results also suggest that Wnt inhibitors may represent a new therapeutic strategy for NF1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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