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EGFR-Stat3 signalling in nerve glial cells modifies neurofibroma initiation

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Abstract

Neurofibromatosis type 1 (NF1) is an inherited disease in which affected patients are predisposed to develop benign Schwann cell (SC) tumours called neurofibromas. In the mouse, loss of *Nf1* in the SC lineage causes neurofibroma formation. The tyrosine kinase receptor EGFR is expressed in Schwann cell precursors (SCP), which have been implicated in plexiform neurofibroma initiation. To test if EGFR activity affects neurofibroma initiation, size, and/or number, we studied mice expressing human EGFR in SCs and SCP in the context of mice that form neurofibromas. Neurofibroma number increased in homozygous *CNP-hEGFR* mice versus heterozygous littermates, and neurofibroma number and size increased when *CNP-hEGFR* was crossed to *Nf1^{fl/fl};DhhCre* mice. Conversely, diminished EGFR signalling in *Nf1^{fl/fl};DhhCre;Wt2/4* mice decreased neurofibroma number. *In vivo* transplantation verified the correlation between EGFR activity and neurofibroma formation. Mechanistically, expression of *CNP-hEGFR* increased SCP/neurofibroma-initiating cell self-renewal, a surrogate for tumour initiation, and activated P-Stat3. Further, IL-6 reinforced Jak2/Stat3 activation in SCPs and SCs. These gain- and loss-of function assays show that levels of tyrosine kinase expression in SCPs modify neurofibroma initiation.

Introduction

Neurofibromatosis type 1 (NF1) patients develop highly variable numbers of benign neurofibromas. Neurofibromas are Schwann cell (SC) -driven peripheral nerve tumours that may cause significant morbidity by disfigurement or by compression of adjacent organs or nerves, and cause mortality when they compress vital organs.¹ In mouse models, a modifier of plexiform neurofibroma formation is an *Nf1* mutant genetic background. When *Krox20Cre* drives *Nf1* loss in developing SCs, neurofibromas do not form, unless all mouse cells carry one *Nf1* mutant allele.² In P0Cre mice, the peripheral nerve must be injured.^{3,4} Thus, one way that modifier genes may affect neurofibroma formation is through

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Conflict of Interest

The authors declare no conflict of interest.

modulation of the microenvironment. Unlike highly penetrant mutations in tumour suppressor genes, modifier genes exert subtle effects through their normal functions by changing the efficiency of steps in tumorigenesis. We posited that neurofibroma initiation, number and/or size might also be modified by the strength of growth factor signalling.

Neurofibroma development requires SCs and/or Schwann cell precursors (SCPs) to develop biallelic *Nf1* mutations.^{2,5–9} When the SCP driver *DhhCre* elicits *Nf1* loss in developing SCs, neurofibromas form without a requirement for an *Nf1* genetic background.⁶ Overexpression of glial growth factor $\beta 3$, a neuregulin-1 isoform, which is a major SC mitogen, also results in neurofibroma development in the absence of *Nf1* mutation.^{10–12} Overexpression of human EGFR (*CNP-hEGFR*) in mouse SCs using a CNPase promoter elicited the mast cell accumulation, hyperplasia, fibrosis and disruption of Remak bundles characteristic of neurofibromas,¹³ but neurofibromas were found in only 5% of mice. These data suggest that levels of growth factor receptor activation modify neurofibroma number. In addition, it is known that several growth factor receptors, including EGFR, are frequently overexpressed in human neurofibroma, and that p75+ SCP-like cells in neurofibroma express EGFR.^{14–16}

The epidermal growth factor receptor EGFR/ErbB1 is a 170 kD transmembrane protein with intrinsic tyrosine kinase activity. EGFR activation triggers signalling processes that promote cell proliferation, migration, adhesion, angiogenesis and inhibition of apoptosis.¹⁷ S100 β +p75+ human neurofibroma SCP-like cells that respond to EGF stimulation by self-renewal are detectable in human and mouse neurofibroma.^{15,18} In the mouse, these SCP-like cells form neurofibromas after transplantation into immunocompromised mice.^{9,18,19} Downstream of EGFR activation, Stat3 phosphorylation and dimerization cause nuclear translocation and gene transcription in many systems.²⁰ Of note, deletion of *Stat3* in SCs and SCPs largely ablates neurofibroma formation in a mouse model.¹⁹

Despite these considerable data, the mechanism(s) by which EGFR expression affects neurofibroma formation remains unknown. Here we report that EGFR levels control neurofibroma number, and that EGFR provides a major input to Stat3 signalling and to II-6 in this system. Together these data suggest that levels of EGFR signalling can modify neurofibroma initiation.

Results

***CNP-hEGFR/CNP-hEGFR* homozygous mice develop more neurofibromas**

When the CNPase promoter drives overexpression of human EGFR in mouse SCs, 5% of *CNP-hEGFR* heterozygous mice (*CNP-hEGFR*+) developed neurofibromas.¹³ To test if increasing EGFR signalling in mouse SCs increases neurofibroma number we interbred these heterozygous mice and obtained EGFR homozygotes (*CNP-hEGFR/CNP-hEGFR*). These mice showed similar survival compared to *CNP-hEGFR*+ mice (Figure 1a) ($P=0.22$, Gehan–Breslow–Wilcoxon test). However, on whole body dissection, we identified enlarged dorsal root ganglia (DRG) in the *CNP-hEGFR/CNP-hEGFR* mice (Figure 1b). Hematoxylin and eosin (H&E) and S100 β immunostaining confirmed that these were GEM grade I neurofibromas (Figure 1c and d). About 28% (5 out of 18) of *CNP-hEGFR/CNP-hEGFR*

mice developed neurofibromas (Figure 1e). Normal nerves contain Remak bundles with a single non-myelinating SC wrapping multiple small caliber axons, which can be visualized by electron microscopy (Figure 1f). As previously shown, *CNP-hEGFR*⁺ nerves show disrupted Remak bundles (Figure 1g). This phenotype was enhanced in age matched *CNP-hEGFR/CNP-hEGFR* mouse nerves (Figure 1h). We also performed hotplate tests to indirectly quantify small caliber axon ensheathment by non-myelin forming SCs, as these axons convey sensory information. Decreased heat sensitivity (hypoalgesia) was a feature of *CNP-hEGFR/CNP-hEGFR* and *CNP-hEGFR*⁺ mice (not shown).²¹ Thus, increasing EGFR signalling in mouse SCs increases Remak bundle disruption and promotes neurofibroma formation. However, the extent of neurofibroma formation remained much less than that in the *Nf1*^{fl/fl}; *DhhCre* model, in which the *Nf1* gene is biallelically mutated in SCs and 100% of mice develop at least four neurofibromas.⁶

Expression of *CNP-hEGFR* in an *Nf1* mutant background increases tumour burden

To test whether increased EGFR expression cooperates with *Nf1* loss in neurofibroma formation, we bred *Nf1*^{fl/+}; *DhhCre*; *CNP-hEGFR*⁺ mice with *Nf1*^{fl/fl} mice. We obtained *Nf1*^{fl/fl}; *DhhCre*; *CNP-hEGFR*⁺ or *Nf1*^{fl/fl}; *DhhCre* mice in expected Mendelian ratios. The median life span of *Nf1*^{fl/fl}; *DhhCre*; *CNP-hEGFR*⁺ mice did not differ from *Nf1*^{fl/fl}; *DhhCre* ($P = 0.26$, Gehan–Breslow–Wilcoxon test) (Figure 2a).

We performed gross dissections on the *Nf1*^{fl/fl}; *DhhCre*; *CNP-hEGFR*⁺ mice ($n = 15$) and *Nf1*^{fl/fl}; *DhhCre* mice ($n = 13$). Neurofibroma size was significantly increased ($P < 0.05$, unpaired t test, two-tailed, Figure 2b and c). Neurofibroma number also increased in the *Nf1*^{fl/fl}; *DhhCre*; *CNP-hEGFR*⁺ mice compared to the *Nf1*^{fl/fl}; *DhhCre* mice ($P < 0.05$, unpaired t test, two-tailed) (Figure 2d). Tumour locations were also altered. In *Nf1*^{fl/fl}; *DhhCre* mice most tumours are found adjacent to the cervical spinal cord, and occasionally in the thoracic region. In *Nf1*^{fl/fl}; *DhhCre*; *CNP-hEGFR* mice, tumours were present predominately at lower cervical levels. Smaller tumours were frequent at thoracic and lumbar levels, and in cauda equina (not shown). All tumours showed classic neurofibroma histology (GEM neurofibroma Grade I or GEM II) with a heterogeneous cell population of S100 β ⁺ cells and S100 β [–] cells and mast cells, typical for the *Nf1*^{fl/fl}; *DhhCre* model (Figure 2e – g). Tumours also contained entrapped neurofilament⁺ axons and myxoid matrix characteristic of neurofibroma (not shown). As reported, 25% of mice ultimately developed sarcoma (GEM 3 PNSTs).²²

Diminished EGFR signalling in the *Nf1*^{fl/fl}; *DhhCre* mice decreases neurofibroma number

To test whether EGFR activity is necessary for neurofibroma formation in *Nf1*^{fl/fl}; *DhhCre* mice, we bred *Nf1*^{fl/+}; *DhhCre*⁺; *Wa2*^{+/+} mice with *Nf1*^{fl/fl} mice. *Wa2*^{+/+} mice carry an hypomorphic *Egfr* mutation allele that reduces Egfr activity by 90%.²³ We obtained *Nf1*^{fl/fl}; *DhhCre*; *Wa2*^{+/+} or *Nf1*^{fl/fl}; *DhhCre* mice in expected Mendelian ratios. There was no significant difference in survival between *Nf1*^{fl/fl}; *DhhCre*; *Wa2*^{+/+} mice and littermate control *Nf1*^{fl/fl}; *DhhCre* mice ($P = 0.21$, Gehan–Breslow–Wilcoxon test) (Figure 3a). We dissected spinal cords with peripheral nerves to determine tumour number and size. *Nf1*^{fl/fl}; *DhhCre*; *Wa2*^{+/+} mice ($n = 10$) had decreased tumour number compared to littermate *Nf1*^{fl/fl}; *DhhCre* mice ($n = 9$, $P < 0.05$, unpaired t test, two-tailed) (Figure 3b). There was no

significant difference in tumour size measured by diameter ($P = 0.17$, unpaired t test, two-tailed) (Figure 3c). Histology showed GEM Grade I neurofibroma (not shown). Thus, decreasing EGFR activity modifies tumour number but not tumour size.

Increased self-renewal is driven by EGFR and *Nf1*

Accumulating evidence, including the findings that *Nf1* mutant SCP show increased self-renewal and form neurofibromas on transplantation, supports the idea that SCPs are a cell of origin for neurofibromas.^{9, 18, 24} We tested if addition of EGF or EGFR expression modifies numbers of neurofibroma-initiating cells in an *in vitro* colony-formation assay, in which loss of *Nf1* increases EGFR-dependent self-renewal of progenitor cells.^{18, 24} Addition of EGF to SCP had little effect on the percent of cells forming primary or secondary colonies in wild-type (WT) cells (Figure 4a). Colony formation increased in *Nf1*-deficient cells when EGF was added upon passage to secondary ($P < 0.01$, unpaired t test, two-tailed) and tertiary ($P < 0.01$, unpaired t test, two-tailed) colonies (Figure 4b). We generated spheres from embryonic DRG of EGFR-overexpressing or diminished EGFR-expressing transgenic mice (*Wa2/+*) crossed with *Nf1^{fl/fl};DhhCre* mice, and assessed self-renewal by serial passage at clonal density. Overexpression of EGFR in *Nf1^{fl/fl};DhhCre* mice significantly increased sphere numbers compared to *Nf1^{fl/fl};DhhCre* mice. Decreased *Egfr* activity by *Wa2* significantly decreased sphere numbers (Figure 4c) (oneway ANOVA).

To determine whether the levels of EGFR in SCP sphere-forming cells is relevant to their ability to form tumours *in vivo*, we transplanted SCPs with varying levels of EGFR activity subcutaneously into nude mice. The percent of mice developing small neurofibroma-like lesions 7 weeks after transplantation of *Nf1^{fl/fl};DhhCre* cells was 80%, as previously shown.¹⁹ The frequency of tumour formation by *Nf1^{fl/fl};DhhCre;Wa2* cells was significantly reduced as compared to *Nf1^{fl/fl};DhhCre* cells ($P < 0.05$, Fisher's exact test, two-tailed). In contrast, all mice implanted with *Nf1^{fl/fl};DhhCre;EGFR* derived sphere cells developed tumours, and there was no significant difference from *Nf1^{fl/fl};DhhCre* cells ($P = 0.47$, Fisher's exact test). We previously showed that some *Nf1^{fl/fl};DhhCre;EGFR* mice develop GEM-PNST.²² Consistent with that finding, mice implanted with *Nf1^{fl/fl};DhhCre;EGFR* cells developed tumours with the morphology of GEM-PNST, with high cell density, numerous mitoses and necrosis (not shown). Taken together, our data suggest a role for EGFR activity in maintenance of a tumorigenic progenitor population, and cell transformation.

An *EGFR/STAT3* pathway is activated in neurofibroma

The EGFR tyrosine kinase is expressed in SCPs, not in differentiated SCs.²⁵ Genetic deletion of *Stat3* significantly reduced neurofibroma formation.¹⁹ To determine if EGFR regulates *Stat3* in mouse sciatic nerve, we performed western blots. P-Stat3-Y705 was detected in nerves from *CNP-hEGFR/CNP-hEGFR* mice, but not WT nerves or nerves from *Wa2* mice (Figure 5a). The EGFR tyrosine kinase inhibitor OSI-774 reduced P-Stat3-Y705 in mouse neurofibroma-derived SCP-like cells (Figure 5b). Immunostaining of tissue sections showed P-Stat3⁺ cells in nerve and decreased staining in *Nf1^{fl/fl};DhhCre;Wa2* mouse nerve (Figure 5c – e). In neurofibroma lysates, a profound reduction in P-Stat3 was observed in *Nf1^{fl/fl};DhhCre;Wa2* versus control (Figure 5f). We also detected decreased P-Jak2 and Jak2

expression in *Nf1^{fl/fl};DhhCre; Wa2* neurofibroma lysates, suggesting that Egfr prides input, directly or indirectly, into Jak signalling. We treated *Nf1^{fl/fl};DhhCre* mice ($n = 3$) with AZD1480, a Jak2 inhibitor, for 5 days; P-Stat3 decreased in tumour lysates after this treatment (Figure 5g). These genetic gain- and loss-of-function experiments show that the EGFR/Stat3 pathway is activated, and that EGFR provides a major input into activation of Stat3 in neurofibromas.

II-6 reinforces EGFR activation via JAK2 in neurofibroma cells

As noted above, Jak kinases can phosphorylate Stat3, reinforcing Egfr mediated Stat3 activation. This often occurs subsequent to activation of cytokine loops initiated by Stat3 binding to cytokine promoters. Oncostatin, leukemia inhibitory factor (LIF) and II-6 have each been implicated as STAT3 target genes. II-6 mRNA was reported as elevated in *Nf1* mouse neurofibromas versus control nerves.²⁶ We detected elevated II-6 protein in three out of four mouse neurofibromas versus nerve (Figure 6a). In an ELISA assay, II-6 was present in medium produced by *Nf1^{fl/fl};DhhCre* spheres, and II-6 decreased in medium from spheres exposed to either the EGFR inhibitor OSI-774 or the Jak2/Stat3 inhibitor FLLL32 (Figure 6b). This suggested that an EGFR-Stat3 signalling might upregulate II-6 expression with or without activation. Indeed, secreted II-6 regulates P-Stat, as there was an additive effect on sphere number when a function blocking II-6 antibody was added to SCP together with OSI-774 group (Figure 6c). P-Jak2, P-Stat3 and total Jak2 protein decreased only in spheres treated with anti-II-6 antibody and OSI-774 (Figure 6d). Sphere number also decreased in mouse neurofibroma spheres treated with shII-6 (Figure 6e). Thus II-6 mediated activation of P-Jak2 depends on EGFR-mediated Stat3 activation in SCPs, and maintains viability of SCPs. We also treated sphere cells derived from neurofibroma and associated nerve/DRG with the Jak1/2 inhibitor AZD1480 and the Jak3 inhibitor CP-690550, using spheres from *Nf1^{fl/fl};DhhCre*, *Nf1^{fl/fl}; DhhCre;EGFR* and *Nf1^{fl/fl};DhhCre;Wa2* mice. To facilitate comparisons among genotypes, we normalized sphere number for each genotype to its DMSO control. AZD1480 inhibited *Nf1^{fl/fl};DhhCre* and *Nf1^{fl/fl};DhhCre;EGFR* sphere number in a dose-dependent manner, but had no effect on *Nf1^{fl/fl};DhhCre;Wa2* spheres. CP-690550 had no effect on cells from any of the three genotypes (Figure 6g). Given that FLLL32 is a Jak2/Stat3 inhibitor, the compiled results suggest that Jak2 is the major Jak upstream input to Stat3 activation.

We wondered if II-6 also signals in neurofibroma SCs. We confirmed that, as previously described,²⁷ adult mouse SCs exposed to II-6 (50ng/mL) increase P-Stat3 (Figure 7a). FLLL32 (a Jak2/Stat3 inhibitor, Figure 7b), decreased FACS-sorted EGFP⁺ mouse neurofibroma SCs viability. Anti-II-6 did not affect SC viability in this setting (not shown). Anti-II-6 antibody did, however, decrease P-Stat3 in SCs (Figure 7c) indicating that SC produce as well as respond to II-6.

DISCUSSION

We find that EGFR levels modify neurofibroma number, a surrogate of tumour initiation, based on in vivo gain- and loss-of-function assays. In vivo, the persistence of an enlarged EGFR⁺ SCP population is predicted to provide an enlarged field of cells susceptible to

additional genetic and epigenetic changes, leading to an increased number of neurofibromas. In vitro assays show that EGFR increases self-renewal of tumour initiating SCPs upstream of Stat3 activation, explaining these effects.

Increased tyrosine kinase signalling disrupts adult nerve homeostasis and predisposes to SC tumorigenesis. Remak bundle disruption is characteristic of the CNP-hEGFR and Nf1-driven models,^{2,13,18,28} and is observed when the neuregulin ligand glial growth factor $\beta 3$ is overexpressed in nerve.¹¹ While *EGFR* expression disrupted nerve structure and caused neurofibroma formation in 5% of mice,¹³ doubling levels of *CNP-hEGFR* increased nerve disruption, and increased neurofibroma formation fivefold (Figure 1). Only when *Nf1* was mutant in SCs and SCPs did all animals form neurofibromas, and numbers of tumours increased with expression of EGFR (Figure 2). In this setting, profound decreases in EGFR activity using the *Wa2* hypomorphic allele reduced tumour number by half, but did not affect tumour size, reinforcing our conclusion that EGFR's main role is in tumour initiation (Figure 3). In the *Wa2* mice, 10% residual EGFR activity remains.²³ We have not excluded the possibility that complete EGFR loss would show additional effects. Completely blocking SC ErbB2 and ErbB3 receptor signalling by biallelic dominant negative ErbB4 expression in Remak bundle SCs increased SC proliferation, and increased cell apoptosis.²⁹ Remak bundles in the transgenic mice were similar to those in mice with EGFR overexpression (Figure 1), with single axons wrapped by SCs. It is possible that when the neuregulin receptors are blocked, that EGFR is aberrantly expressed in SCs. Alternatively, appropriate levels of tyrosine kinase signalling are required for nerve homeostasis.

Our finding that neurofibroma formation increases when EGFR is overexpressed and *Nf1* is also mutant indicates that both receptor and signalling contribute to benign tumour formation. This is consistent with the fact that growth factors are required in *Nf1* mutant cells for Ras activation, and with the known role of *Nf1* in terminating Ras signals.³⁰ We favour the interpretation that EGFR signalling modifies neurofibroma initiation in the context of *Nf1* mutation and that other mitogenic pathways can contribute to post-initiation tumour growth. In lung cancer, *Nf1* somatic mutations lead to mitogen-activated protein kinases kinase 1/2 activation, correlating with resistance to EGFR inhibition.³¹ It remains possible that the limited effect of EGFR loss we find in neurofibromas would be improved by concurrent mitogen-activated protein kinases kinase 1/2 inhibition. The systems also may not be comparable: *EGFR* is mutated in lung cancer, while in our studies, and in human NF1, *EGFR* is overexpressed but not mutated.

Mouse survival was similar in *Nf1^{fl/fl};DhhCre* mice versus *Nf1^{fl/fl}; DhhCre;CNP-hEGFR* mice, and *Nf1^{fl/fl};DhhCre* mice versus *Nf1^{fl/fl}; DhhCre;Wa2/+* mice, in spite of difference in tumour size, number and location among the models. Even small tumours that compress the spinal cord cause paralysis and failure to feed, leading to early mortality, indicating that tumour location plays a more important role than tumour size in determining mouse survival. Strain variation is known to modify mouse tumorigenesis.^{32, 33} To minimize potential strain variation, we used C57Bl/6 *CNP-hEGFR* and *Wa2* for crosses to *Nf1^{fl/fl};DhhCre* mice. Furthermore, we used littermates to serve as controls in our experiments. We nevertheless observed shifts in control Kaplan–Meier survival curves among experiments that may be attributable to additional strain-specific modifier genes or to

limited sample numbers in control cohorts. Notably, in glial growth factor $\beta 3$ overexpressing mice certain strains formed neurofibromas, while others formed mainly or exclusively malignant peripheral nerve sheath tumor.³⁴

Our studies were carried out in a mouse model of plexiform neurofibroma, and existing evidence suggests that these tumours arise stochastically in patients, as twins are mainly discordant for development of plexiform neurofibroma.³⁵ In contrast, the extreme variation in dermal neurofibroma burden among NF1 patients, even within families whose members carry the same *NF1* mutation, supports the idea that polymorphic modifiers of dermal neurofibroma number exist. The absence of cutaneous neurofibromas in a family with a 3 base pair *NF1* internal deletion indicates that *NF1* genotype itself can act as an NF1 modifier.³⁶ In addition, patients with *NF1* microdeletions have increased numbers of early-developing dermal neurofibromas,^{37,38} suggesting that a gene in proximity to *NF1* is an *NF1* modifier gene. Importantly, studies on twins with NF1 show more concordance in dermal neurofibroma number than fraternal twins.^{35,39} No modifiers of dermal neurofibroma number have yet been identified. Boundary cap cells and/or skin-derived precursors are proposed to be the cell of origin for dermal neurofibroma, while plexiform neurofibroma cells-of-origin are Dhh+, Krox20+ or GAP43+;PLP+. Both, like SCPs, can be propagated in EGF and express EGFR. Furthermore, EGFR is expressed in human neurofibroma progenitor-like cells in tissue sections.^{15,16} It is plausible, therefore, that the pathways we describe modify dermal and plexiform neurofibroma number.

Genetic and pharmacological experiments showed that Stat3 plays a key role in driving neurofibroma initiation.¹⁹ EGFR modifies hematopoietic stem cell mobilization.⁴⁰ Here, we show EGFR expression is relevant to sphere formation, a surrogate of tumour initiation (Figure 4), and that EGFR levels and activity mediate downstream activation of Stat3 *in vivo* and *in vitro* (Figure 5). On both secondary and tertiary passaging, *Nf1* mutant SCPs showed increased sphere formation in response to epidermal growth factor (Figure 4). Our study is limited in that we have not yet shown that numbers of SCP can be altered *in vivo* when EGFR expression levels change.

Downstream of EGFR/Stat in other systems, feed forward loops can include IL-6, LIF and Oncostatin-driven activation of JAK/STAT.^{41,42} We tentatively identify Jak2 as the Janus kinase activating Stat3 downstream of receptor signalling based on pharmacologic tests. We showed that in SCP, EGFR-driven P-Stat3 stimulates IL-6 secretion, and IL6 reinforced EGFR/Stat signalling (Figure 6). Furthermore, IL-6 activated SC P-Stat3 (Figure 7), likely contributing to the robust expression of P-Stat3 in neurofibroma SCs.¹⁹ Intriguingly, the absence of gp130, a co-receptor for IL-6, rescued the wound-induced plasticity in *Nf1*+/- mice.³ It remains possible that LIF, Oncostatin and/or other pathways also contribute to Stat3 activation in neurofibroma cells *in vivo*. However, IL-6, but not ciliary neurotrophic factor or LIF, activated Stat3 in SCs.²⁷ Overall, gain- and loss-of-function experiments support the relevance of EGFR levels to formation of neurofibromas, and targeting pathways downstream of EGFR, including Stat3, should identify neurofibroma therapeutic targets.

MATERIALS AND METHODS

Animals

We housed mice in temperature- and humidity-controlled facilities on a 12-h dark–light cycle with free access to food and water. We complied with ethical regulation. The animal care and use committee of Cincinnati Children's Hospital Medical Center approved all animal use. We interbred C57Bl/6 *CNP-hEGFR* transgenic mice (*CNP-hEGFR*^{+/+})¹³ to obtain homo-zygous mice (*CNP-hEGFR/CNP-hEGFR*). Homozygous genotypes were confirmed by out-crossing putative homozygotes with WT mice; all offsprings of homozygotes were heterozygous for the transgene. Both male and female mice were used for experiments. The *Nf1*^{fl/fl}; *DhhCre* mouse was described⁶ and was on a 129/B1/6 mixed background. We bred *DhhCre* mice (C57Bl/6) onto the *Nf1*^{fl/fl} background to obtain the F1 generation (*Nf1*^{fl/+}; *DhhCre*^{+/+}); we bred F1 mice with *CNP-hEGFR/CNP-hEGFR* mice to obtain F2 *Nf1*^{fl/+}; *DhhCre*^{+/+}; *CNP-hEGFR*^{+/+} mice. We bred F2 mice with *Nf1*^{fl/fl} mice to obtain *Nf1*^{fl/fl}; *DhhCre*^{+/+}; *CNP-hEGFR*^{+/+} mice. We separately bred F1 (*Nf1*^{fl/+}; *DhhCre*^{+/+}) with C57Bl/6 background *Wave2/Wave2* (*Wa2/Wa2*) mice (The Jackson Laboratory, Maine, USA) to obtain F2 *Nf1*^{fl/+}; *DhhCre*^{+/+}; *Wa2*^{+/+}. We bred F2 mice with *Nf1*^{fl/fl} to obtain *Nf1*^{fl/fl}; *DhhCre*^{+/+}; *Wa2*^{+/+} mice. Littermates were used for controls. Animal genotypes were not blinded to investigator. For analysis of embryos, we established timed pregnancies with the morning of the vaginal plug defined as E0.5. For *in vivo* cell transplantation, we injected 5×10^5 single cells per mouse as described.¹⁹ Based on power analysis, we estimated that we need to use 10 mice per group to reach the statistical difference.

Cell culture

We established adherent cultures of SCPs from E12.5 DRG of WT and *Nf1*^{-/-} embryos as described.⁴³ After 7 days on uncoated tissue-culture plastic in DMEM+10% FBS, 1% Pen/Strep, 50 ng/ml recombinant nerve growth factor (Harlan Products for Bioscience, Indianapolis, IN, USA) and 1% B27, we dissociated cells with collagenase type I and 0.05% trypsin-EDTA for 40 min. We plated cells in DMEM+10% FBS+1% Pen/Strep on poly-l-lysine coated tissue culture dishes at $0.5\text{--}1.0 \times 10^6$ cells per 60-cm plate. After 24 h, we changed medium to DMEM:F12 (1:1)+N2 supplements (Invitrogen; Carlsbad, CA, USA) +gentamycin. We incubated cultures at 10% CO₂ and 37 °C. After 21–28 days, colonies emerge in *Nf1*^{-/-} cultures, which are subsequently dissociated and passaged with 0.05% Trypsin-EDTA for 5 min at 37 °C. After 24 h, we changed medium to DMEM:F12 (1:1)+N2 supplements+gentamycin. At this point, cells are adherent SCP-like cells;¹⁸ we treated some cultures with recombinant human EGF or basic FGF (R&D Systems, Minneapolis, MN, USA) at 3–10 ng/ml every 3 days.

For sphere formation assays, we dissociated E12.5 DRGs and plated 4×10^4 cells per well in 24-well low binding tissue culture plates. Nascent sphere formation was scored from 7 to 10 days for 2–4 embryos per genotype in replicates of at least 6 per embryo. Spheres were fed every 3–5 days with DMEM:F12 (3:1)+1% B27 and 20 ng/ml both EGF and bFGF.

Fluorescence-activated cell sorting

We performed cell sorting in a dual-laser (Argon 488 and dye laser 630 or HeNe 633) FACSCanto (Becton-Dickinson, Franklin Lakes, NJ, USA) and analysed on an 'alive' gate based on light scatter parameters and 7-AAD staining negativity as described previously.⁶

Measurement of tumour number and volume

We perfused mice intracardially with 4% paraformaldehyde (w/v) in PBS. Overnight incubation of mice in 300 ml 4% paraformaldehyde (w/v) preceded removal of skin and muscle. We randomly chose five mice per group and placed each mouse in 50 ml decalcification solution overnight (Cal-Rite, Richard Allan Scientific, Kalamazoo, MI, USA) then transferred to PBS. We then dissected the spinal cord with attached DRG, nerve roots and tumours using a Leica dissecting microscope. A tumour was defined as a mass >1 mm measured parallel to the spinal cord and surrounding the DRG and/or nerve roots. We measured the diameter of each tumour parallel to the spinal cord and DRG/nerve roots, not tumour length parallel to the nerve as described.¹⁹

Histology and nerve ultrastructure

We embedded tissues in paraffin, and cut 6 µm sections.^{13,21} We immunostained sections with anti-S100β (Dako, #Z0311, Carpinteria, CA, USA) overnight at 4 °C followed by incubation with labelled appropriate secondary antibody. We viewed sections with a microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a digital imaging system. We stained sections with H&E, toluidine blue. For electron microscopy, we perfused mice with 3.2% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer, and post-fixed and embedded as described.¹³ Sections were analysed on a Jeol 110CX electron microscope.

Tumour grading

We classified and graded tumours using the GEM-neurofibroma and GEM-peripheral nerve sheath tumour classification.⁴⁴

Statistics

For mouse survival analyses we used Kaplan–Meier analysis followed by a Gehan–Breslow–Wilcoxon log-rank test. We used unpaired two-tailed Student's *t*-tests to analyse significance of tumour number, tumour size, percentage of cell colonies, shII-6 or control infected sphere numbers and P-Stat3 percentage in tissue sections. We used ordinary one-way ANOVA on other experiments, and reported them as mean ± s.e.m. For *in vivo* cell transplantation experiments, we used Fisher's exact test for power analysis and *P* value. *P* < 0.05 was considered significant.

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Figure 1. *CNP-hEGFR/CNP-hEGFR* homozygous mice develop neurofibromas

(a) Kaplan–Meier survival curve. Black, *CNP-hEGFR/hEGFR* mice ($n = 18$); grey, *CNP-hEGFR/+* mice ($n = 12$). (b) Gross dissections of tumours (white arrow) surrounding several dorsal root ganglia in the cervical area in *CNP-hEGFR/CNP-hEGFR* mice on one side. Nerve roots are also enlarged. A ruler with 1 mm markings is shown. (c) H&E staining of GEM neurofibroma. (d) Anti-S100 β staining marks SCs, visualized with DAB [brown]. (e) The percentage of mice showing neurofibromas is shown. E/+ = *CNP-hEGFR* heterozygotes; E/E = *CNP-hEGFR* homozygotes. (f–h) A representative electron micrograph of 3 months mouse saphenous nerve on WT (f), E/+ (g) and E/E (h). Normal nerves contain Remak bundles, with single SCs wrapping multiple small caliber axons (black arrow head). Non-myelinating SCs fail to wrap multiple axons and are dissociated from them (black arrows). Bar = 10 μ m.

Figure 2. Overexpression of CNP-hEGFR in an *Nf1* mutant background increases neurofibroma burden

(a) Kaplan–Meier survival curve. Red, $Nf1^{fl/fl};DhhCre$ mice ($n = 9$); green $Nf1^{fl/fl};DhhCre;CNP-hEGFR$ mice ($n = 15$). The median life span of $Nf1^{fl/fl};DhhCre;CNP-hEGFR$ mice is not significantly reduced compared to $Nf1^{fl/fl};DhhCre$ (13 months) mice ($P = 0.26$). (b) Gross dissections of tumours in the cervical area in $Nf1^{fl/fl};DhhCre$ mice (upper) and $Nf1^{fl/fl};DhhCre;CNP-hEGFR$ mice (lower). A ruler showing 1 mm markings is included. (c) Quantification of tumour size. There is a significant difference between

Nf1^{fl/fl};DhhCre mice (●, $n = 38$) and *Nf1^{fl/fl};DhhCre;CNP-hEGFR* mice (■, $n = 56$, $P < 0.05$, unpaired t test, two-tailed). (d) Quantification of average tumour number per mouse ($P < 0.05$, unpaired t test, two-tailed). The variance between these two groups are being statistically compared. (e–g) Histology and immunostaining of the tumours. Paraffin embedded tissue sections from plexiform GEM-neurofibromas were stained with H&E highlighting increased cellularity (e), bar = 20 μm . An adjacent section stained with anti-S100 β antibody to mark SCs, visualized with DAB [brown] (f). Toluidine blue staining showed infiltration of metachromatic mast cells in *Nf1^{fl/fl};DhhCre;CNP-hEGFR* mice (white arrows) (g). * $P < 0.05$.

Figure 3. Diminished EGFR signalling in the *Nf1^{fl/fl}; DhhCre* mice decreases neurofibroma number

(a) Kaplan–Meier survival curve. Grey, *Nf1^{fl/fl}; DhhCre* mice ($n = 9$); black, *Nf1^{fl/fl}; DhhCre; Wa2/+* mice ($n = 10$). There is no significant difference ($P = 0.21$) in survival time between these two genotypes. (b) Quantification of average tumour number per mouse. There are more visible plexiform tumours at gross dissection in the *Nf1^{fl/fl}; DhhCre* mice (white bar, $n = 9$) as compared to the *Nf1^{fl/fl}; DhhCre; Wa2/+* mice (black bar, $n = 10$, $P < 0.05$, unpaired t test, two-tailed). (c) Quantification of tumour size. There is no significant difference between *Nf1^{fl/fl}; DhhCre* mice (●, $n = 27$) and *Nf1^{fl/fl}; DhhCre; Wa2/+* mice (■, $n = 22$, $P = 0.09$, unpaired t test, two-tailed) on tumour size. * $P < 0.05$.

Figure 4. EGF increases SC proliferation and EGFR dose modulates SCP self-renewal
(a) Cells from WT or EGFR/+ E12.5 dorsal root ganglia (DRG) plated at clonal density and colonies measured 10 days later, for three passages. (b) Cells from *Nf1^{fl/fl};DhhCre*, or *Nf1^{fl/fl};DhhCre;CNP-hEGFR* from E12.5 DRG plated at clonal density and colonies measured 10 days later, for three passages. Loss of the *Nf1* gene increases EGFR-dependent self-renewal of progenitor cells in the developing PNS. Data are shown as mean $\pm$ standard deviation. (c) Mouse neurofibroma sphere culture show that overexpression of EGFR in *Nf1^{fl/fl};DhhCre* mice (black bar) significantly increased sphere numbers compared to *Nf1^{fl/fl};DhhCre* mice (white bar), while decreased Egfr activity by Wa2 (grey bar) significantly decreased the sphere numbers. (d) Numbers of neurofibroma-like lesions after subcutaneous injection of *Nf1^{fl/fl};DhhCre*, *Nf1^{fl/fl};DhhCre;EGFR* or *Nf1^{fl/fl};DhhCre;Wa2* neurofibroma sphere cells into nude mice. * P <0.05, n.s. = no significant difference. ** P <0.01, *** P <0.001.

Figure 5. The Egfr/Stat3 pathway is activated in neurofibroma

(a) Western blot of mouse sciatic nerves that are WT, EGFR-overexpressing (*EGFR/EGFR*), or with diminished levels of Egfr activity (*Wa2*), showing that Stat3 activity is increased in *EGFR/EGFR* nerves. (b) Western blot of mouse neurofibroma-derived EGFR⁺ progenitor-like sphere cells OSI-774. Inhibition of EGFR function inhibits Stat3-Y705 phosphorylation (P-Stat3). Total Stat3 (Stat3) was used as loading control. Statistics B, C, E: unpaired *t* test, two-tailed. (c–d) Immunofluorescent staining showing that P-Stat3 is detected in *Nf1^{fl/fl};DhhCre* mouse neurofibromas (C, CTRL) and decreased in *Nf1^{fl/fl};DhhCre, Wa2*

neurofibroma (D, Wa2). Bar = 20 μ m. (e) Quantification of P-Stat3⁺ cells in *Nf1^{fl/fl};DhhCre* (white bar) and *Nf1^{fl/fl};DhhCre, Wa2* (black bar). (f) Western blot of neurofibroma lysates from *Nf1^{fl/fl};DhhCre* and *Nf1^{fl/fl};DhhCre;Wa2* mice. (g) Western blot of lysates from *Nf1^{fl/fl};DhhCre* mouse neurofibromas treated with AZD1480 or vehicle for 5 days. (f and g) Stat3 and anti- β -actin serve as loading controls. Antibodies: P-Stat3, Cell Signaling, #9145; Stat3, Cell Signaling, #4904; EGFR, Santa Cruz, #SC-03; P-EGFR, Santa Cruz, #SC-12351; P-Jak2, Cell Signaling, # 3776; Jak2, Cell Signaling, #3230; β -actin, Cell Signaling, #5125. *** $P < 0.001$.

Figure 6. II-6 regulates Stat3 activities in SCPs

(a) A representative western blot of WT mouse sciatic nerve and *Nf1^{fl/fl};DhhCre* mouse neurofibroma lysates showing expression of II6 protein (II-6, Abcam, Cambridge, MA, USA; #AB6672). Anti- β -actin served as a loading control. (b) II-6 quantification on OSI-774, FLLL32, and DMSO treated medium conditioned by mouse neurofibroma spheres. Sphere medium without treatment was also used as additional control ($n = 3$ for each group). Unpaired t test, two-tailed was used. (c) Mouse neurofibroma sphere counts on II-6 antibody (II-6 Ab), OSI-774, II-6 Ab+OSI-774, IgG+DMSO treated mouse neurofibroma spheres. (d) Western blot of P-Jak2, Jak2, P-Stat3 on II-6 antibody (II-6 Ab), OSI-774, II-6 Ab+OSI-774, IgG+DMSO treated mouse neurofibroma spheres. Anti-Stat3 and anti- β -actin serve as controls. (e) Sphere counts show that two shII-6 shRNAs (#1 and #2) each significantly decrease mouse neurofibroma sphere formation, compared to non-target lentivirus yellow fluorescent protein control. (f) Western blot showing knockdown of II-6 in *Nf1^{fl/fl};DhhCre* mouse neurofibroma spheres, 4 days after sh II-6 infection using two different shRNA clones (#1, #2). Numbers below indicate the relative band intensity (50% and 40%), normalized to β -actin for each sample. (g) Inhibitory effects of the JAK1/2 inhibitor AZD1480 on *Nf1^{fl/fl};DhhCre*, and *Nf1^{fl/fl};DhhCre;EGFR* but not *Nf1^{fl/fl};DhhCre;Wa2* mouse neurofibroma spheres. DMSO was used as control. The JAK3 inhibitor CP 690550 did not affect sphere number at concentrations below 1 μ M. Three independent experiments were performed, and data are represented as mean $\pm$ s.e.m. Statistics: B, one-way ANOVA; E, unpaired t test, two-tailed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 7. II-6 and EGFR regulate Stat3 activities in mouse neurofibroma SCs
 (a) Western blot of P-Stat3-Tyr705 and Stat3 in mouse WT SCs treated with II-6 (50 ng/mL) for 0, 5, 15, or 20 min. Total Stat3 serves as control. (b) MTS assay shows FLLL32 inhibits growth of FACS-sorted EGFP⁺ mouse neurofibroma SCs. (c) Western blot of P-Stat3 and Stat3 on II-6 antibody (II-6 Ab), OSI-774, or FLLL32 treated FACS-sorted adherent cells. Anti- β -actin serves as control.