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Neuropilin-2 contributes to tumorigenicity in a mouse model of Hedgehog pathway medulloblastoma

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Abstract

The Hedgehog (Hh) signaling pathway has been implicated in the most common childhood brain tumor, medulloblastoma (MB). Given the toxicity of post-surgical treatments for MB, continued

need exists for new, targeted therapies. Based upon our finding that Neuropilin (Nrp) transmembrane proteins are required for Hh signal transduction, we investigated the role of Nrp in MB cells. Cultured cells derived from a mouse *Ptch*^{+/-}; *LacZ* MB (Med1-MB), effectively modeled the Hh pathway-related subcategory of human MBs in vitro. Med1-MB cells maintained constitutively active Hh target gene transcription, and consistently formed tumors within one month after injection into mouse cerebella. The proliferation rate of Med1-MBs in culture was dependent upon Nrp2, while reducing Nrp1 function had little effect. Knockdown of Nrp2 prior to cell implantation significantly increased mouse survival, compared to transfection with a non-targeting siRNA. Knocking down Nrp2 specifically in MB cells avoided any direct effect on tumor vascularization. Nrp2 should be further investigated as a potential target for adjuvant therapy in patients with MB.

Keywords

Neuropilin; Hedgehog pathway; Medulloblastoma; Proliferation; Brain tumor; Pediatric

Introduction

Gene expression data have distinguished four classes of MBs: Hedgehog (Hh), Wnt, Group 3, and Group 4 [1–3]. The newly recognized tumor categories require specific tools to investigate the distinct cancer biology and response to treatment for each MB class. About 30 % of MBs appear to originate from damage to Hh signal transduction [1, 2]. The *PTCH* gene encodes the Hh receptor patched (Ptc1), a negative regulator in the Hh transduction pathway (Fig. 1). Hh ligands bind Ptc1, and promote Smo activation, which in turn inhibits the cytoplasmic regulator SuFu. SuFu is a negative regulator of the Gli transcription factors, so Smo inhibition of SuFu activates Gli proteins [4–6]. Mutations of the *PTCH* gene lead to constitutive activity of the Hh pathway, resulting in MBs both in humans and in mice [7, 8].

Neuropilins (Nrps) have a positive role in Hh signal transduction in 3T3 cells, primary skin cells, and zebrafish embryos (Fig. 1, [9]). Nrp1 and Nrp2 are single-pass transmembrane proteins that act as co-receptors during axon chemotaxis in response to repellent Semaphorin signals and promote angiogenesis in concert with VEGF [10–14]. The role of Nrps as VEGF co-receptors in tumor angiogenesis and metastases is the basis for current trials of anti-neuropilin antibodies for cancer therapies [15, 16]. Inhibition of either or both Nrps strongly reduces Hh signal transduction, as measured by transcription of target genes such as *Glil* [9]. Nrps act between Smo and SuFu through an unknown mechanism [9]. Our present work explored the importance of Nrps in MB tumor cells by blocking Nrp function and Hh signal transduction specifically in tumor cells, rather than in associated vasculature. We reduced either Nrp1 or Nrp2 function in cultured tumors cells before testing their tumorigenesis potential in MB grafts into the cerebellum. Nrps positively regulated Hh signal transduction in MB cells, and inhibition of Nrp2 reduced MB tumor growth. Our work lays the foundation for further investigation into the potential for Nrp2-directed therapy for MB. Effective therapies might act both on the tumor vasculature and on individual tumor cells.

Materials and methods

Cell culture

Med1-MB cells derived from a *Ptch*^{+/-};*LacZ* mouse MB, a gift from Dr. Ervin Epstein, were cultured in 10 % fetal bovine serum (FBS) in complete DMEM. Medium conditioned with active amino-terminal ShhN ligand was produced using a HEK 293 line that stably secretes the protein [17]. For Shh, SAG, or SANT-1 treatment, cells were switched to DMEM supplemented with 0.5 % FBS for 24 hours to promote ciliation [5]. All experiments were conducted 72 h after RNAi transfection, the pre-determined optimal knockdown timepoint for reducing the level of either Nrp protein [9].

Transient transfections

Mouse Nrp1 siRNA (#1, 5'-GCACAAUCUCUGAA-ACUA-3', Dharmacon), mouse Nrp2 siRNA (#1, 5'-GAC-AAUGGCUGGACACCCA-3', Sigma), mouse Smo siRNA (SASI_Mm01_00346929, Sigma), and non-targeting (NT) siRNAs (Dharmacon) were dissolved in nuclease-free water and stored as 5 μ M stocks. RNAi molecules were introduced into Med1-MB cells via a "wet" reverse transfection procedure using Lipofectamine 2000 (Invitrogen).

qPCR

Total RNA was isolated from cells and tissue using Trizol (Invitrogen). One microgram of RNA was reverse-transcribed with random hexamer primers using Superscript III reverse transcriptase (Invitrogen). A fraction of the resultant cDNA was used as template for interrogation with TaqMan qPCR probes (Applied Biosystems) on a Applied Biosystems 7500 Fast thermocycler: Gapdh (Mm99999915_g1), Gli1 (Mm00494645_m1), Ptc1 (Mm00436026_m1), Nrp1 (Mm00435371_m1), and Nrp2 (Mm00803099_m1). RNA levels were normalized to GAPDH RNA.

Western blots

Cultured Med1-MB cells were scraped into cold PBS, sedimented at 1,000 $\times g$ for 5 min, and lysed in modified RIPA buffer (25 mM Na-Tris pH7.4, 150 mM NaCl, 2 % v/v NP- 40, 0.25 % w/v sodium deoxycholate, 1 mM DTT, 1 mM PMSF, and Roche complete protease inhibitor cocktail with EDTA) for 60 min at 4°. Tissues were homogenized for 3 min in modified RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 % v/v NP-40, 0.25 % sodium deoxycholate, 1 mM DTT, 0.1 % SDS, 5 mM EDTA pH 8.0, 5 mM EGTA pH 8.0, 2 mM sodium pyrophosphate, 5 mM sodium fluoride, 2 mM sodium orthovanadate, Roche Complete protease inhibitor cocktail). Lysates were clarified by centrifugation for 30 min at 20,000 $\times g$. Protein concentrations were determined using the detergent-insensitive BCA kit (Pierce). Samples were mixed with SDS sample buffer, incubated at room temperature (RT) for 15 min, resolved by SDS-PAGE, and processed for immunoblotting. Anti-p38 (1:50,000, Sigma), Anti-gapdh (1:20,000, cell signalling technology), anti-Gli1 (1:500, cell signalling technology), anti-Nrp1 (1:1,000, Abcam), and anti-Nrp2 (1:1,000, cell signalling technology) were purchased. Anti-Smo (1:500) antibody was made [5]. Primary antibody incubations were carried out overnight at 4° in 5 % non-fat dry milk, tris-buffered saline, pH

7.4, containing 0.05 % Tween-20. Secondary antibody incubation was performed in the same block buffer at RT for 1 h.

Imaging

Med1-MB cells were harvested 48 hours after RNAi treatment and re-plated on 8-well chamber slides. For imaging primary ciliation, cells were brought to confluence and serum-starved for 24 hours prior to fixation. Seventy-two hours after transfection, cells were fixed with 4 % paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and washed three times with PBS. Fixed cells were placed in block solution (PBS with 1 % v/v Normal Donkey Serum and 0.1 % v/v Triton X-100) for 30 min. Primary antibodies [1:500 anti-Nrp1 (R&D Systems), 1:500 anti-Smo [5]; 1:2,000 anti-acetylated tubulin (Sigma)] were diluted in block and used to stain cells overnight at 4°. After washing three times in PBS, Alexa dye-coupled secondary antibodies were added in block solution at 1:250 for 1 hour at RT. Hoechst dye (Invitrogen) at 1:1,000 was included in final washes with PBS. Samples were mounted in Fluoromount G (Southern Biotech). Microscopy was on a Leica DMIRE2 laser-scanning confocal microscope.

Migration

For a wound-healing assay Med1-MB cells were plated in a 96-well plate. Once they formed a confluent monolayer, cells were stained with Hoechst dye, uniformly scratched, and washed with PBS. Cells were imaged in an ImageXpress 5000 robotic epifluorescence microscope (Axon Instruments) for 12 hours at 37 °C, with photos taken every 15 min. Analysis was completed using MatLab (MathWorks).

Proliferation

Med1-MB cells were plated at equal concentrations in 96-well imaging plates for 48 h following RNAi transfection. After overnight incubation to ensure cell adherence, EdU (Invitrogen) was incubated with the Med1-MB cells for 4 h at 37° with CO₂. Cells were fixed, permeablized, and stained with 1:200 anti-PH3 (Millipore) at RT for 1 hour.

After a PBS wash, Hoechst dye was added prior to the final wash. Proliferation was analyzed using the Image Express and quantified with MatLab. Individual nuclei were detected by a watershed analysis of the intensity-threshold and Gaussian-filter fluorescence image of Hoechst 33342. Nuclei were gated by area in order to eliminate false nuclei. Median fluorescence intensities were determined for each nucleus from EdU or PH3 images. Nuclei were scored EdU- or PH3-positive if their readout exceeded a fixed threshold above the population mode. Graph error bars denote standard deviations. All significance tests were two-tailed Student's *t* test; *p* < 0.05 was considered significant.

Orthotopic transplantation of Med1-MB cells

Animal work was supervised under an approved Stanford University protocol. After male 6-week old nude mice (Charles River Labs) were appropriately anesthetized, a skin incision and craniotomy were performed. Each mouse received 4.8×10^5 Med1-MB cells in 4 µL of DPBS, injected with stereotactic guidance into the cerebellum. Cells were injected 72 hours following their transfection with NT siRNA (*n* = 7), Smo siRNA (*n* = 3), Nrp1 siRNA (*n* =

3), or Nrp2 siRNA ($n = 5$), where n is the number of animals injected with the designated siRNA treated cells. Each condition was tested at least three times. The survival curve had mortality, or severe morbidity requiring sacrifice, as endpoints.

Results and discussion

Med1-MB cells mirror the Hedgehog subcategory of medulloblastoma

The standard cell type for studying Hh signal transduction in vitro is the NIH 3T3 fibroblasts. To turn on Hh target genes, these cells require serum starvation, cell culture confluence, and treatment with an agonist such as Sonic Hedgehog (Shh) or SAG. Shh inhibits the Hh receptor patched (Ptc1), which otherwise prevents target gene expression by inhibiting the membrane protein smoothened (Smo). SAG acts by directly stimulating the activity of the Smo transducer, overcoming the inhibition of Smo by Ptc1. The Gli1 gene, which encodes a transcription factor in the Hh pathway, is itself a target gene and commonly used as a reporter of the state of the pathway.

A significant percentage of MBs in children originate from damaged Hh signal transduction [1,2]. MB cells where Ptc1 has been inactivated typically have high Gli1 transcription without adding agonist. In MB tissue from *Ptch*^{+/-} mice, the Gli1 transcript level was significantly elevated compared to normal surrounding cerebellum ($p < 0.003$; Fig. 2a). It is difficult to study the Hh MB subtype with cultured cells, because after establishment in culture, the cells often lose constitutive Hh target gene expression, measured by elevated Gli1 RNA levels [18]. Med1-MB cells derived from *Ptch*^{+/-}; *LacZ* mouse MB [8] had constitutively active Hh signal transduction. These cells were responsive to pathway antagonists (SANT-1, an inhibitor of Smo), and were insensitive to further pathway activation with agonists (Fig. 2d). Thus, Med1-MB cells mimicked the human Hh subtype in their maintenance of constitutively active Hh target gene expression and their responses to Hh antagonists.

Stereotactic injection of a small number of Med1-MB cells into the cerebella of nude mice (Fig. 2e) led to nearly universal death from large brain tumors within 4–6 weeks (Fig. 2f). Isolated tumor samples had elevated levels of Gli1 transcript compared to surrounding normal cerebellum (Fig. 2c), so the Med1-MB cells maintained Hh pathway activity in vivo. The ability of Med1-MB cells to maintain characteristics of Hh-associated MB and reliably form cerebellar tumors in mice made them an important tool for investigating the role of Nrps in the Hh MB subtype.

Decreased Hedgehog signal transduction after neuropilin knockdown

We next tested the importance of Nrp proteins within the cultured tumor cells. We first confirmed that Nrps were essential for Hh signal transduction in the Med1-MB cells (Fig. 3). siRNA molecules that targeted Nrp1 or 2 reduced protein levels at 72 h post-transfection (Fig. 3b). The siRNA sequences that had been extensively and carefully tested in our previous work [9] were used for the present study. Despite 44 % sequence similarity between Nrps, the siRNA treatments were selective; neither one inhibited the other Nrp (Fig. 3b). Using Gli1 transcript levels as a metric for Hh signal transduction, siRNA knockdown

of Nrp1 or Nrp2 in MB cells reduced the Gli1 mRNA level as potently as siRNA knockdown of Smo, the essential positive regulator of Hh transduction (Fig. 3a). Smo protein accumulates in primary cilia after cells are treated with a Hh agonist [4]. Med1-MB cells also produce primary cilia (Fig. 3c), and their loss of *Ptch* function led to constitutive localization of Smo in cilia as expected (Fig. 3c, d). Inhibition of Nrp production with siRNA did not change the frequency of ciliation or the level of Smo in cilia (Fig. 3d), in agreement with previous work with fibroblasts [9].

Knockdown of neuropilin-2 reduces tumorigenicity

Excessive Hh target gene activity is implicated in MB and other cancers [19]. Here we show that Med1-MB cells are highly tumorigenic and require Nrps for successful Hh transduction. We blocked Nrp function in Med1-MB cells and measured changes in their tumorigenicity. By reducing Nrp function with transient siRNA transfection specifically in Med1-MB tumor cells, we were able to distinguish direct effects on Hh transduction and tumor cell growth from indirect effects on the tumors due to reduced vascularization.

Nrp2 knockdown had a dramatic effect on tumors formed by engrafted Med1-MB cells and the consequent mortality (Fig. 4a). These effects were consistent with the inhibition of Med1-MB cell proliferation in vitro. Despite the transient nature of RNAi effects, mice engrafted with cells that had Nrp2 knocked down survived longer than those engrafted with cells treated with NT RNAi (Fig. 4a).

Nrp1 has a known role in cell migration in central nervous system (CNS) tumors [20–22], but no equivalent has been described for MB. We found that Nrp1 knockdown reduced Med1-MB cell migration in culture, expanding the list of CNS tumors in which Nrp1 affects cell motility (Fig. 4c). Nrp2 knockdown had a reproducibly greater effect than Nrp1 knockdown on slowing Med1-MB cell proliferation in culture (Fig. 4b). This effect was likely independent from the effect on cell motility, given Nrp2's less profound effect on migration compared to Nrp1 (Fig. 4c). Recent studies in other cancer types [23, 24] are consistent with our findings that Nrp2 may affect cell survival independently of angiogenic interactions with VEGF. Smo knockdown did not show the same effect on Med1-MB migration and proliferation, suggesting either that the effect of Nrp knockdown was independent of Hh signal transduction, or that the kinetics of the effects of Smo and Nrp knockdown are distinct (Fig. 4b).

Potential therapeutic importance of Nrp2 in medulloblastoma

Nrp2 could be a potent target for therapeutic treatment of residual, disseminated, or recurrent MB. Due to the marked propensity of MB to disseminate throughout the CNS, the current standard of care involves surgical resection followed by chemotherapy and radiation. Studies suggest Nrp2 blocking antibodies may reduce metastases by delaying primary tumor cell shedding [16], so Nrp2 may be an attractive target for therapeutic intervention. An adjuvant therapy targeting Nrp2 would have the potential to inhibit not only tumor vascularity, but also proliferation and the potential to metastasize. Nrp2 has been identified in other tumor types as an important potential therapeutic target, due to its roles in angiogenesis and tumor cell proliferation [23–25]. Animal studies have already shown that

Nrp1-blocking antibodies can inhibit vascular remodeling, enhancing susceptibility to treatment with anti-VEGF therapy [15]. Our results suggest that Nrp2-targeting agents could be useful for inhibiting tumor growth, if efficient penetration of the tumor were accomplished. This might require developing drugs that target Nrp2, since the existing trials for Nrps make use of anti-Nrp1 antibodies. In our experiments the effect of Nrp2 knockdown was more potent than inhibition of Hh signal transduction alone, so Nrp2-targeting therapies could be investigated for other CNS and peripheral tumors.

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Fig. 1.

The mammalian Hedgehog (Hh) pathway requires the primary cilium for its function. In the absence of Hh ligand (OFF), patched receptor (Ptc1) inhibits the transmembrane transducer smoothed (Smo) from accumulating in the primary cilium 1. Without inhibition from active ciliary Smo, the negative regulator suppressor of fused (SuFu) promotes processing of Gli transcription factors into its repressor forms 2, 3 and transcription is suppressed 4. When Hh ligand is present (ON), Hh binds to Ptc1 1 and relieves its inhibition of Smo 2, thereby allowing its accumulation in the primary cilium and activation 3. Active Smo then inhibits SuFu 4. This allows Gli to accumulate preferentially at the tip of the cilium where it gets fully activated before translocating to the nucleus to promote transcription 5. When Ptc1 is mutated as in *Ptc1*^{+/-} mice, the loss of Smo inhibition leads to constitutively active Hh signal transduction. Nrps, which are most abundant in the plasma membrane, positively regulate Hh transduction, acting in some way at the level between Smo and SuFu

Fig. 2.

Med1-MB cells maintain constitutive Hh target gene expression. **a** Medulloblastoma (MB) from *Ptch*^{+/-} mice have elevated levels of Gli1 transcript levels compared to normal surrounding cerebellum (CRB) ($p < 0.003$). **b** MB tumors from *Ptch*^{+/-} mice have elevated levels of Gli1 protein (160 kDa) compared to normal surrounding CRB, in keeping with Gli1 transcriptional elevation. Tumors formed by implantation of Med1-MB cells into the cerebella of nude mice also have elevated levels of Gli1 protein. **c** These MBs from Med1-MB cells had elevated Gli1 transcript levels ($p = 0.004$) in vivo compared to normal cerebellum. **d** Med1-MB cells maintained constitutive Hedgehog (Hh) Gli1 target gene transcription in vitro, which could be inhibited with SANT-1 ($p = 0.006$). Addition of Hh pathway agonists Shh or SAG gave little to no increase in Gli1 mRNA compared to no treatment (NT), indicating that Gli1 transcription in Med1 cells is already at a near-maximal level. **e** The Med1-MB cell line was derived from spontaneous tumors obtained from *Ptch*^{+/-}; *LacZ* mice. A small number of Med1-MB cells (blue *LacZ* stain) were stereotactically implanted into the normal cerebella of nude mice. **f** Within 4 weeks, nearly 100 % of the mice formed deadly MBs (blue)

Fig. 3. Knockdown of neuropilins (Nrps) in Med1-MB cells reduced Hedgehog (Hh) signal transduction. **a** In Med1-MB cells, knockdown with siRNA against either Nrp, compared to non-targeting (NT) siRNA, led to decreased Gli1 transcript level ($p < 0.007$), an effect comparable to inhibiting smoothened (Smo; $p = 0.004$). **b** Knockdown of Nrp 1 or 2 with siRNA led to specific decreases in Nrp 1 or 2 protein levels in Med1-MB cells. **c** Hh signal transduction requires primary cilia. Primary cilia in Med1-MB with neuropilin (Nrp) knockdown were of normal appearance, with Smo localized in the primary cilia, despite the lack of downstream target gene transcription. **d** Quantification of primary cilia and smoothened (Smo) accumulation in cilia in Nrp versus non-targeting (NT) RNAi treatment showed a significant decrease only in control cells Smo knockdown cells ($p = 0.02$)

Fig. 4. Neuropilin-2 (Nrp2) knockdown decreased Med1-MB cell tumorigenicity. **a** Even transient knockdown of Nrp 2 in Med1-MB cells implanted in the cerebella of nude mice increased mouse survival compared to non-targeting RNAi (NT) (Kaplan–Meier curve; $p = 0.014$). Average time to death of mice injected with the Nrp2 knockdown cells was twice that of NT. **b** Proliferation of Med1-MB depends on Nrp2, assayed by EdU ($p = 0.014$) or phospho-histone 3 (PH3; $p = 0.025$). This effect was independent of the effect on Hh signal transduction (Smo). **c** The effect on proliferation was also independent of migration, as Nrp1 KD had a greater effect on cell motility