Nos2 Inactivation Promotes the Development of Medulloblastoma in Ptch1+/− Mice by Deregulation of Gap43–Dependent Granule Cell Precursor Migration

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Abstract

Medulloblastoma is the most common malignant brain tumor in children. A subset of medulloblastoma originates from granule cell precursors (GCPs) of the developing cerebellum and demonstrates aberrant hedgehog signaling, typically due to inactivating mutations in the receptor PTCH1, a pathomechanism recapitulated in Ptch1+/− mice. As nitric oxide may regulate GCP proliferation and differentiation, we crossed Ptch1+/− mice with mice lacking inducible nitric oxide synthase (Nos2) to investigate a possible influence on tumorigenesis. We observed a two-fold higher medulloblastoma rate in Ptch1+/− Nos2−/− mice compared to Ptch1+/− Nos2+/− mice. To identify the molecular mechanisms underlying this finding, we performed gene expression profiling of medulloblastomas from both genotypes, as well as normal cerebellar tissue samples of different developmental stages and genotypes. Downregulation of hedgehog target genes was observed in postnatal cerebellum from Ptch1+/− Nos2−/− mice but not from Ptch1+/− Nos2+/− mice. The most consistent effect of Nos2 deficiency was downregulation of growth-associated protein 43 (Gap43). Functional studies in neuronal progenitor cells demonstrated nitric oxide dependence of Gap43 expression and impaired migration upon Gap43 knock-down. Both effects were confirmed in situ by immunofluorescence analyses on tissue sections of the developing cerebellum. Finally, the number of proliferating GCPs at the cerebellar periphery was decreased in Ptch1+/− Nos2−/− mice but increased in Ptch1+/− Nos2+/− mice relative to Ptch1+/− Nos2+/− mice. Taken together, these results indicate that Nos2 deficiency promotes medulloblastoma development in Ptch1+/− mice through retention of proliferating GCPs in the external granular layer due to reduced Gap43 expression. This study illustrates a new role of nitric oxide signaling in cerebellar development and demonstrates that the localization of pre-neoplastic cells during morphogenesis is crucial for their malignant progression.

Introduction

Medulloblastoma (MB) is a highly malignant tumor of the cerebellum that preferentially develops in children and adolescents. Although the survival rate for standard risk MB is around 70% [1] surviving patients often suffer from neurodevelopmental and cognitive side effects of the aggressive therapy [2]. Therefore, improved understanding of the molecular pathomechanisms driving MB growth is necessary to develop less toxic and more effective treatments. Recent molecular profiling studies suggested at least four MB subtypes that are associated with distinct expression profiles, genomic aberrations and clinical features [3,4]. One of these MB subtypes is characterized by aberrant activation of the hedgehog (Hh) pathway and typically corresponds to the desmoplastic (nodular) MB variant. This subtype is supposed to develop from granule cell precursors (GCPs) of the external granular layer (EGL) [5].

The EGL is a transient germinal zone at the subpial cerebellar surface consisting of rhombic lip-derived progenitor cells that have migrated tangentially to the emerging cerebellar cortex at late stages of embryonal brain development [6]. During the early postnatal period in mouse, the morphogenetic factor sonic hedgehog (Shh) is secreted by subjacent Purkinje cells and binds to patched receptors (Ptch1 and Ptch2) expressed on the GCP surface [7]. Ligand binding to Ptch1 then leads to functional de-repression of Smoh (Drosophila smooth homolog) and subsequent activation of Gli (Gloma-associated oncogene family zinc finger) transcription factors [8]. This launches a temporally concerted gene expression pattern causing a proliferation burst and massive expansion of the GCP population during the first two postnatal weeks [7].

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Author Summary

Medulloblastoma is a common pediatric brain tumor, a subtype of which is driven by aberrant hedgehog pathway activation in cerebellar granule cell precursors. Although this tumor etiology has been intensively investigated in the well-established Ptch1−/− mouse model, knowledge is still lacking about the molecular interactions between neoplastic transformation and other developmental processes. Nitric oxide (NO) has been reported to be involved in controlling proliferation and differentiation of these cells. Therefore, inactivation of the NO−producing enzyme Nos2 in combination with the mutated Ptch1 gene should provide insight into how developmental regulation influences pathogenesis. Here, we describe a new role for NO in developing neuronal precursors of the cerebellum facilitating physiologically accurate migration via regulation of Gap43. We further demonstrate that disturbance of these processes leads to retention of granule precursor cells to the cerebellar periphery. Together with the sustained proliferation of these cells in combined Ptch1−/− Nos2−/− mice, this effect results in an increased medulloblastoma incidence relative to Ptch1−/− mice and demonstrates a new disease-promoting mechanism in this tumor entity.

particular, the direct Gli-target N-myc [9,10] and D type cyclins [11] were shown to be crucial for the growth and neoplastic transformation of GCPs [12]. In addition, the set of genes targeted by activated Gli transcription factors also include components of the canonical Hh pathway for feedback-loop regulation, such as the receptors Ptch1 and Ptch2 as well as the hedgehog-interacting protein (Hhip) [10,13]. After several rounds of cell division, GCPs normally exit cell cycle and accumulate at the inner site of the EGL [14], where they start to migrate through the molecular layer (ML) and the Purkinje cell layer to form the inner granular layer (IGL) [15]. The mechanisms underlying the attenuation of the mitotic response and eventually the stop of GCP proliferation are not well understood. The most evident concepts describe extrinsic cues in gradient-based models to trigger GCP differentiation with increasing distance to the region of the outer EGL [16]. Finally, the EGL disappears at about three weeks after birth in mice.

PTCH1 was identified as a frequent target of inactivating mutations or genomic loss in sporadic MBs [17–19] that belong to the molecular subtype hallmarkmed by an aberrant activity of hedgehog signaling. The monoallelic inactivation of the Ptch1 gene in mice and thus downstream activation of the Hh pathway leads to MB development at a frequency of about 10–15% [20]. This mouse model has provided substantial insights into the pathogenesis of Hh-dependent MBs and has been used in different cross-breeding experiments to investigate tumor suppressor gene functions in this particular context [21,22].

Nitric oxide (NO) is a highly reactive gaseous molecule involved in various physiological processes ranging from vasculature modulation to neurotransmission [23,24]. NO is produced by three distinct enzyme isoforms: i) neuronal nitric oxide synthase (nNOS/Nos1), ii) inducible nitric oxide synthase (iNOS/Nos2), and iii) endothelial nitric oxide synthase (eNOS/Nos3). Though being constitutively expressed in their respective tissue, nNOS and eNOS activity strongly depends on calcium [25], whereas calcium-independent iNOS is primarily regulated by transcriptional induction, e.g. by inflammatory cytokines and endotoxins [26], which permits higher quantities of NO generation. The role of NO in cancer initiation and progression is heterogeneous with opposing effects in different malignancies [27]. Considering effects of tumor stroma, increased angiogenesis was reported to be associated with elevated Nos activity [28] and some immune-related processes were found to be mediated by NO [29], including cytotoxicity of activated microglia [30]. Finally, NO release by vascular endothelial cells was reported to build a niche-like microenvironment for maintenance of glioma stem cells [31]. In the context of cerebellar development, Nos2 (inducible Nos) is initially expressed in early GCPs, whereas Nos1 (neuronal Nos) is hardly present before postnatal day 7 (Cerebellar Development Transcriprome Database [32]). Successively, Nos1 expression increases along with granule cell differentiation [33] and predominantly contributes to the common NO signaling that becomes apparent in the IGL as development proceeds [34]. Evidence has been provided that NO negatively acts on proliferation of neuronal precursors during adult neurogenesis [35]. Similarly, Ciani and colleagues demonstrated enhanced proliferation of cerebellar precursor cells upon inhibition of NO synthases [36].

Here, we report on the generation of Ptch1−/− Nos2−/− mice to investigate the impact of Nos2 on tumor development in Ptch1 hemizygous mutant mice. Interestingly, we observed an approximately two-fold increase in the incidence of spontaneous MB in Ptch1−/− Nos2−/− mice in comparison to Ptch1−/− Nos2+/+ mice. To characterize the molecular pathomechanism underlying the tumor-promoting effect of Nos2 deficiency in Ptch1−/− mice, we performed comprehensive expression and DNA copy number profiling of MB tumors (Ptch1−/− Nos2−/− versus Ptch1−/− Nos2+/+) as well as expression profiling of normal cerebellar tissue samples from different developmental stages and various genotypes (Ptch1−/− Nos2−/−, Ptch1−/− Nos2−/− mixed, and wild-type mice). Downregulation of the growth-associated protein 43 (Gap43) was the most striking feature in the cerebellum of Nos2−/− mice when compared to Ptch1−/− Nos2−/− and wild-type mice. Subsequent functional analyses and results from in situ studies of GCPs in postnatal cerebellum allowed us to formulate a model for the tumor promoting role of Nos2 deficiency in Ptch1 mutant mice via deregulation of Gap43-dependent migration of GCPs.

Results

Loss of Nos2 increases the rate of spontaneous MB in Ptch1−/− mice

Survival analyses of 315 wild-type mice, 412 Ptch1−/− Nos2−/− mice, 215 Ptch1−/− Nos2−/− mice and 221 Ptch1−/− Nos2+/+ mice demonstrated a significantly higher MB incidence in the group of Ptch1−/− Nos2−/− mice relative to the group of Ptch1−/− Nos2+/+ mice (p = 0.0007, Logrank test, Figure 1A). In total, 11% of the Ptch1−/− Nos2−/− mice (24/215) and 21% of the Ptch1−/− Nos2−/− mice (47/221) were sacrificed due to the development of cerebellar MB. None of the 315 wild-type and the 412 Ptch1−/− Nos2−/− mice developed MBs. These observations indicate a MB-promoting role of Nos2 deficiency in Ptch1−/− mice.

MBs in Ptch1−/− Nos2−/− and Ptch1−/− Nos2−/− mice show identical histological features

In humans, Hh-dependent MBs typically correspond to the desmoplasic subtype. MBs in Ptch1−/− mice, however, microscopically resemble the classic MB subtype [20]. Histological analysis of MBs in Ptch1−/− Nos2−/− and Ptch1−/− Nos2−/− mice demonstrated similar morphological features (Figure 1B–1E). The tumors were composed of densely packed sheets of cells with hyperchromatic carrot-shaped nuclei and scant cytoplasm. There
were no obvious histopathological differences between MBs of the two genotypes.

Molecular analyses of MBs in $\text{Ptch1}^{+/+} \text{Nos2}^{+/+}$ and $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ mice

For an initial assessment of the molecular tumor characteristics, gene expression of hedgehog signaling pathway components were measured in 21 MBs and 24 normal (adult) cerebellar tissue samples from both $\text{Ptch1}^{+/+} \text{Nos2}^{+/+}$ and $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ mice. Using quantitative real-time PCR (qRT-PCR), significant down-regulation of the wild-type $\text{Ptch1}$ transcript and upregulation of the Shh target genes $\text{Gli1}$ and N-myc were generally observed in the tumor samples (Figure S1), indicating all examined MBs to be of the same Hh-dependent molecular subtype. However, there were no significant differences for these genes between MBs of the two genotypes. Furthermore, targeted genetic analyses showed a loss of the wild-type $\text{Ptch1}$ allele in 10 of the 21 MBs investigated, while none of the tumors demonstrated a $\text{Tp53}$ mutation or $\text{N-myc}$ amplification. The $\text{Cdkn2a}$/p16$^{ink4a}$ locus was retained in all tumors while a single MB demonstrated a homozygous $\text{p19}{\text{Arf}}$ deletion (see Table S1 and Text S1 for details).

In order to identify the molecular pathomechanism contributing to the increased MB rate in Nos2-deficient $\text{Ptch1}$ mutant mice, we performed array-based gene expression profiling of three $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ versus six $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ and comparative genomic hybridization (array-CGH) analyses of five $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ versus seven $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ MB tissue samples. All specimens investigated had tumor cell contents between 70% and 90% as determined on corresponding formalin-fixed and paraffin-embedded (FFPE) reference sections. Differential expression of selected candidate genes was validated by qRT-PCR on an expanded, partially overlapping tumor set of seven $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ versus seven $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ MB samples.

The expression profiling analysis revealed a total of 87 differentially regulated genes between tumors of the two genotypes (Table S2) with the vast majority (87%) showing lower transcript levels in $\text{Ptch1}^{+/+} \text{Nos2}^{-/-}$ when compared to $\text{Ptch1}^{+/+} \text{Nos2}^{+/+}$ mice. As expected from the initial targeted qRT-PCR measurements, there was no difference detectable concerning the activation of Hh pathway genes. Due to the important role of Nos2 during angiogenesis and cancer-associated immune response, including microglia, stromal effects need to be particularly considered in a systemic Nos2 knockout model. However, neither the set of significantly deregulated genes nor selective determination of marker expression for pericytes, vascular endothelial cells or microglia suggested any differences in the tumor stroma between the two genotypes (see Table S3 and Text S1 for details).

According to the findings of Ciani and co-workers [37], reduction of NO enhances GCP proliferation through an increased activation of Hh pathway genes. Due to the important role of Nos2 during angiogenesis and cancer-associated immune response, including microglia, stromal effects need to be particularly considered in a systemic Nos2 knockout model. However, neither the set of significantly deregulated genes nor selective determination of marker expression for pericytes, vascular endothelial cells or microglia suggested any differences in the tumor stroma between the two genotypes (see Table S3 and Text S1 for details).

Analyses of genomic copy number alterations revealed a trisomy of chromosome 6 in the majority of MBs from both groups (11/12, Figure 2A and 2B). Moreover, a small region on chromosome 13, approximately 1.5 Mb upstream of the $\text{Ptch1}$ gene, showed a hemizygous deletion in healthy cerebella of $\text{Ptch1}$-mutant mice. As expected from the initial targeted qRT-PCR measurements, there was no difference detectable concerning the activation of Hh pathway genes. Due to the important role of Nos2 during angiogenesis and cancer-associated immune response, including microglia, stromal effects need to be particularly considered in a systemic Nos2 knockout model. However, neither the set of significantly deregulated genes nor selective determination of marker expression for pericytes, vascular endothelial cells or microglia suggested any differences in the tumor stroma between the two genotypes (see Table S3 and Text S1 for details).

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A direct comparison between gene expression profiles from $\text{Pch}^{1+/-} \text{Nos}^{2-/-}$ and wild-type P9 cerebellar tissue samples resulted in a total of 904 deregulated genes with 755 genes (76.7%) showing a decreased expression in $\text{Pch}^{1+/-} \text{Nos}^{2-/-}$ mice (Table S4). P9 cerebellum from $\text{Pch}^{1+/-} \text{Nos}^{2-/-}$ and $\text{Pch}^{1+/-} \text{Nos}^{2+/-}$ mice revealed only 5 and 32 deregulated genes relative to wild-type, respectively (Table S5 and Table S6). This large deviation of postnatal gene expression in the $\text{Pch}^{1+/-} \text{Nos}^{2-/-}$ genotype included a set of downregulated genes that are essential for proliferation of GCPs (e.g. cyclin D1, cyclin D2 and N-myc, Figure 3B). As hedgehog signaling constitutes the main regulatory pathway for neuronal cell proliferation in GCPs of the EGL, the 904 deregulated genes were analyzed for enrichment of Gli transcription factor targets. Matching this list to a set of recently identified Gli-targets in GCPs [10] yielded a significant overrepresentation of Gli-regulated genes ($p = 0.005$, chi-square test). Hence, the reduced transcript levels of these target genes suggests an attenuated hedgehog signaling in postnatal $\text{Pch}^{1+/-} \text{Nos}^{2-/-}$ cerebellum compared to wild-type (or any other genotype).

Notably, the decreased expression of Gli-targets and proliferation-associated genes observed in $\text{Nos}^{2-/-}$-deficient cerebellar tissue was abolished upon additional inactivation of the hedgehog receptor $\text{Pch}^{1}$ (in $\text{Pch}^{1+/-} \text{Nos}^{2-/-}$ mice). Therefore, we examined the transcript levels of patched receptors themselves in $\text{Pch}^{1+/-} \text{Ptch}^{1+/-}$ $\text{Nos}^{2-/-}$ P9 cerebellum, a significant increase of $\text{Pch}^{1}$ and a minor increase of $\text{Pch}^{2}$ expression were observed in $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2-/-}$ mice relative to wild-type mice (Figure 3C). Notably, in $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2-/-}$ cerebellar tissue samples, $\text{Ptch}^{2}$ expression was more elevated than $\text{Pch}^{1}$. However, since $\text{Ptch}^{2}$ is not capable of inhibiting $\text{smo}$ (smooth), it probably failed to take over the attenuating effect on Gli activity [38]. MB specimens from $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2-/-}$ versus $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2+/-}$ mice showed no significant difference in expression levels of either patched receptor, with $\text{Ptch}^{2}$ being substantially increased over $\text{Pch}^{1}$ in both groups (Figure 3C). These findings indicate that $\text{Nos}^{2}$ deficiency leads to an upregulation of $\text{Pch}^{1}$ in GCPs, which results in a downregulation of mitotic genes and Gli-targets only in a $\text{Pch}^{1}$-wild-type background.

**Decreased expression of Gap43 is the most common effect of Nos2 inactivation**

So far, $\text{Nos}^{2}$ inactivation was shown to counteract proliferation and antagonize hedgehog signaling in developing cerebella. To identify those $\text{Nos}^{2}$-dependent effects promoting MB induction, we determined the features that were common to $\text{Pch}^{1+/-} \text{Nos}^{2+/-}$ and $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2-/-}$ genotypes and persisted in the tumor tissues. Accordingly, the overlap of differential gene expression from three comparisons was built: i) $\text{Pch}^{1+/-} \text{Nos}^{2+/-}$ versus wild-type P9 cerebellum, ii) $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2+/-}$ versus $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2+/-}$ P9 cerebellum; and iii) $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2+/-}$ versus $\text{Pch}^{1+/-} \text{Ptch}^{2+/-}$ MB. As a result, only 2 genes were observed to be deregulated in a $\text{Nos}^{2}$-dependent manner during cerebellar development and in MBs (Figure 4A). Although $\text{Stmn}^{1}$ (stathmin 1) appeared to be upregulated in $\text{Pch}^{1+/-} \text{Nos}^{2+/-}$ MBs relative to $\text{Pch}^{1+/-} \text{Ptch}^{2+/-}$ MBs, this could not be confirmed by qRT-PCR (Figure S3). Gene expression of Gap43 was consistently reduced in $\text{Nos}^{2}$-deficient cerebellar tissue samples and downregulation in $\text{Pch}^{1+/-} \text{Ptch}^{1+/-} \text{Nos}^{2+/-}$ tumors relative to $\text{Pch}^{1+/-} \text{Ptch}^{2+/-}$ tumors was also significant in the expanded validation set (Figure 4C and 4D). To further assess the immediacy of $\text{Nos}^{2}$ inactivation and Gap43 deregulation, Gap43 transcript levels were determined in expression profiles of healthy cerebella from all developmental stages (P9, 6W and 1Y). Groups for comparison were built according to presence or absence of...
No2, irrespective of the Pch1 status. The results clearly demonstrated a close association of altered Gap43 transcript levels and No2 status (Figure 4B), and indicated downregulation of Gap43 to be the most common effect of No2 deficiency in the cerebellum.

To investigate differences in Gap43 expression on protein level in situ we performed immunofluorescent double stainings of Gap43 and the proliferation marker Ki-67 on FFPE sections of P9 cerebella from wild-type, Pch1+/−No2−/−, Pch1+/−No2+/− and Pch1+/−Ptch1−/− mice. As illustrated in Figure 4E, Gap43 immunofluorescence was particularly prominent in the outer region of the molecular layer (ML) that is connected to and partially comprised of radial GCP process extensions. Image quantification further indicate a quantitative difference of Gap43 expression in this region with sections from wild-type and Pch1+/−Ptch1−/−No2−/− mice showing a more intense staining than sections from Pch1+/−No2−/− and Pch1+/−No2+/− mice (Figure 4F).

Impaired NO signaling reduces Gap43 transcript levels

The association of No2 inactivation and decreased Gap43 expression suggests a gene-regulatory function of NO signaling. In order to investigate this possible link in vitro we used the murine cerebellar precursors cell line c17.2 and the human MB cell line D458 (see Text S2 for details). Both cell lines were treated either with the Nos inhibitor L-NAME (N-o-nitro-L-arginine methyl ester) to reduce NO levels or solvent control (Figure S4). Relative expression of Gap43 was assessed every 24 hours by qRT-PCR. In c17.2 cells, Gap43 transcript abundance was generally low and increased with culture duration. We observed a slightly decreased expression of Gap43 upon L-NAME treatment that reached significance (p = 0.023) after 120 hours (Figure 5A). NOS inhibition in D458 human MB cells resulted in a significant reduction of Gap43 transcript levels starting already after 72 hours with further decrease after 96 hours and 120 hours (Figure 5B). FACS analyses of apoptosis and cell cycle excluded these observations to be attributed to secondary effects of changing cell conditions (Figure S5). These results suggest Gap43 downregulation as a direct consequence of reduced NO levels in murine neuronal precursors and human MB cells.

Increase of Pch1 expression and impairment of GCP migration upon knockdown of Gap43

The dependency of Gap43 expression on NO signaling suggests this gene as key mediator of the effects observed in No2-deficient P9 cerebellum and Pch1+/−No2−/− MB, in particular, the upregulation of functional Pch1 in Pch1+/−No2−/− mice. Mishra et al. recently reported a central role of Gap43 in the polarization of developing GCPs by regulating centrosome positioning and thus defining correct orientation towards the IGL [39]. Since this is a prerequisite for directed migration, reduced levels of Gap43 in P9 cerebellar tissue may lead to retention of GCPs in the EGL. To test these hypotheses, shRNA-mediated knockdown of Gap43 was performed in c17.2 cells [see Text S1 for details]. Upon knockdown of Gap43 we observed a strong inverse behavior of Pch1 and Gap43 transcript levels (Figure 5C). Changes in migration characteristics were assayed in a Boyden chamber using recombinant SDF-1α (CXCL12) as chemoattractant, which was reported to participate in guiding migration of embryonal GCPs in vivo [40]. Downregulation of Gap43 yielded a significant decrease in cell migration between 14% (p = 0.013) and 20% (p = 0.007) (Figure 5D; Figure S6C). A pseudo-effect of the knockdown due to altered proliferation of the v-myc-immortalized c17.2 cells was excluded by FACS-based cell cycle analysis (Figure S7).

GCPs of the external granular layer show Gap43-associated phenotypes

Transcriptome and functional analyses suggest that a decreased Gap43 expression accounts for Pch1 upregulation and impairment of directed neuronal precursor migration in vitro. Accordingly, Pch1+/−No2−/− P9 cerebella are supposed to increasingly retain GCPs with reduced mitotic activity in the EGL compared to wild-type and Pch1+/−Ptch1−/−No2−/− mice. Moreover, the Pch1+/−Ptch1−/−No2−/− genotype is also expected to exhibit retention of GCPs, but not to show any cell cycle arrest. To further verify this hypothesis in situ we performed immunofluorescent double staining of proliferating (Ki-67+) and post-mitotic GCPs on FFPE sections of postnatal cerebellum (Figure 6A). Here, post-mitotic cells were delineated by the neuronal marker NeuN (neuronal nuclear antigen) [41]. At least three different regions of each mouse cerebellum were analyzed in three to four animals per genotype using confocal laser scanning microscopy. In accordance with the microarray data, averaged cell counts of wild-type and Pch1+/−No2−/− mice did not show significant difference. In contrast, an increase of post-mitotic GCPs (NeuN+, Ki-67−) was detectable in the EGL of Pch1+/−No2−/− and Pch1+/−Ptch1−/−No2−/− mice (Figure 6C). Concurrently, the ratio of dividing to non-dividing GCPs was similar in Pch1+/−No2−/−, wild-type and Pch1+/−Ptch1−/−No2−/− P9 cerebella but markedly decreased in Pch1+/−No2−/− mice. This recapitulated the downregulation of mitotic genes observed in the expression profiles. However, the total amount of proliferating GCPs per EGL section was significantly higher in Pch1+/−No2−/− mice compared to any other genotype (Figure 6C). These results demonstrate a tissue phenotype that corresponds to the effects of reduced Gap43 in developing cerebellar neuronal precursors (in vitro). The increased accumulation of proliferating GCPs in the EGL observed in the Pch1+/−No2−/− genotype supposedly leads to a larger pool of cells susceptible to neoplastic transformation and is therefore likely to promote medulloblastoma development.

Discussion

The Pch1+/− MB mouse model has been intensively studied and has greatly contributed to our understanding of HH-dependent MB tumorigenesis in the context of cerebellar development. The data presented here indicate a role of Nos2 and hence NO signaling in HH-dependent MB by demonstrating a significantly increased MB rate in Pch1+/−No2−/− mice compared to Pch1+/−No2−/− mice. The global genome-wide...
Figure 4. Identification of Nos2-regulated candidate genes. (A) The overlap between three group comparisons of expression profiles revealed Gap43 and Stmn1 as commonly deregulated by Nos2 inactivation according to the microarray data. (B) Gap43 gene expression was Nos2-dependent during different developmental stages of cerebellar development. Nos2-sufficient: wild-type and Ptc1+/c Nos2+/c, Nos2-deficient: Ptc1+/c Nos2−/− and Ptc1+/c Nos2−/−. (C) Gap43 was differentially expressed between Ptc1+/c Nos2+/c and Ptc1+/c Nos2−/− MB samples. Values in (B) and (C) were obtained from the microarray data and indicate log2 ratios of sample against Universal Reference RNA (Stratagene). (D) Differential Gap43 gene expression was confirmed by qRT-PCR in an expanded tumor sample set (n = 7 per genotype). Linear expression values are normalized to housekeeping genes. Significant expression differences between groups are indicated by asterisks (**p<0.01). Error bars reflect SEM. (E) Immunofluorescent co-staining of Gap43 (green) and Ki-67 (red) on FFPE sections from P9 cerebella. Blue: DAPI-stained nuclei. Overview sections (upper left corner) were acquired by wide-field microscopy and detail sections represent confocal laser scanning microscopy images. Intense Gap43 signal was observed in wild-type and Ptc1+/c Nos2−/− cerebella, in particular at the outer ML, scale bar = 50 μm. (F) Quantification of Gap43 staining.
screens performed in the present study did not reveal obvious molecular differences between MBs in \( \text{Ptch1}^{++} \text{Nos2}^{++} \) versus \( \text{Ptch1}^{++} \text{Nos2}^{--} \) animals. Assessment of genomic alterations using array-CGH identified trisomy of chromosome 6 as a recurrent feature in tumors of both genotypes. This corresponds to a recent report on MBs of the same molecular subtype with inactivated double-strand break repair proteins targeted to neuronal progenitors of \( p53^{++} \) mice [42]. The most common loss identified in our analyses affected two small regions on chromosome 13 encompassing the \( \text{Ptch1} \) gene and possibly indicate acquired homozygosity for the mutant allele or somatic rearrangements rather than a broad deletion of the locus. Targeted duplex PCR further confirmed loss of the functional wild-type allele to be a frequent event in these MBs. Notably, tumors of the \( \text{Ptch1}^{++} \text{Nos2}^{--} \) genotype showed a higher frequency of a small gain on chromosome 14. The affected \( \text{Entpd4} \) gene encodes for an apyrase located at the internal membrane of lysosomal vacuoles and the Golgi apparatus. It preferentially catalyzes the hydrolysis of UDP to UMP [43] and thereby facilitates the inverse directed import of UDP-GlcNAc [44]. This in turn was reported to increase glycosylation of surface receptors (e.g. EGFR and PDGFR) and foster cell growth [45]. According to the microarray and qRT-PCR expression data, \( \text{Entpd4} \) transcript levels were indeed increased in tumors with this chromosomal gain. However, this effect did not turn out to be \( \text{Nos2} \)-dependent in an expanded sample set. Consequently, \( \text{Entpd4} \) likely plays a role in MB pathogenesis but is not directly linked to loss of \( \text{Nos2} \).

The examination of tumor-relevant changes in developing cerebellum as a consequence of impaired \( \text{Nos2} \) activity and hence NO signaling surprisingly revealed a decreased proliferation of GCPs in the cerebellum of \( \text{Ptch1}^{++} \text{Nos2}^{--} \) mice. The concurrent upregulation of \( \text{Ptch1} \) and the significant enrichment of downregulated Gli1-target genes strongly suggest that this effect is a consequence of reduced hedgehog signaling. Moreover, this phenotype was completely abrogated by a concomitant \( \text{Ptch1} \) mutation. The slight increase of \( \text{Ptch2} \) in \( \text{Ptch1}^{++} \text{Nos2}^{--} \) cells points to a compensatory effect and further supports the notion of an inhibitory function of \( \text{Nos2} \) loss on the hedgehog pathway in postnatal cerebellum. Since neither a Smoh-regulating domain [38] nor a function for cell cycle arrest through seizing cyclin B1 [46] were reported for \( \text{Ptch2} \), its upregulation may be insufficient for preventing MB induction. In contrast to these observations, Ciani et al. demonstrated that proliferation of cultured GCPs increased upon withdrawal of NO and that this effect was...
mediated by augmented N-myc levels [37]. However, N-myc was not differentially expressed between Ptch1+/− Nos2−/− and Ptch1+/− Nos2+/− MBs of our series. A possible explanation for this discrepancy might be an unrecognized heterogeneity in the isolated cerebellar cell population used in the Giani study. Since eNos and nNos are known to attenuate the mitotic activity of subventricular neuronal stem cells [47,48] Nos inhibitor treatment possibly resulted in a selective growth advantage over GCPs. Downregulation of Gap43 was the only feature observed in a selective growth advantage over GCPs. Downregulation of Gap43-mediated NO signaling [51]. Finally, the present study demonstrated that indeed ELAV-like family member HuD was binds to 3’UTR regions of GAP43 mRNA levels are post-transcriptionally regulated during neuronal differentiation and that elements of the 3’UTR confer transcript instability, which is abolished upon TPA treatment (inter alia inducing NOS2) [54]. At the same time, Chung et al. demonstrated that indeed ELAV-like family member HuD was binding to 3’UTR regions of GAP43 [55]. Taken together, NO accumulation possibly decreases cellular levels of mRNA-stabilizing AUFI protein and thus might contribute to a high transcript abundance of Gap43.

Gap43 is a membrane-anchored protein at the cytoplasmic side of neuronal cell projections and found to be highly expressed during development of the CNS [56]. It is particularly localized in axonal growth cones and participates in the coordination of extrinsic stimuli and intrinsic cell remodeling [57] by regulating cytoskeleton dynamics [58]. Granule cell (GC) migration follows a sequence of tangential and radial movements controlled by successive formation of leading projections [59]. As maturating GCPs exit cell cycle, positioning of the centrosome determines the site of axon growth cone emergence and thus neuronal polarity including localization of such projections [60]. This defines the structural orientation of GCPs in terms of directing its dendrite to the IGL. However, centrosome positioning and therefore accurate polarization of GCPs require phosphorylated Gap43 to bind to the centrosome-associated microtubule-organizing center [61]. Hence,
inaccurate GCP migration was observed in Gap43\(^{-/-}\) animals [39], a finding that is in full agreement with our data from the functional Gap43 knockdown assays. Downregulation of Gap43 in Nos2-deficient P9 cerebellum therefore likely mediates the retention of GCPs observed in FFPE sections. Accordingly, NO/ cGMP signaling was demonstrated to be crucial for accurate migration of the neuronal precursor cell line NT2 [62]. Furthermore, slice culture experiments of neonatal cerebella (P9) exhibited a substantial reduction of proliferation and migration of maturing granule cells to the EGL upon application of NO synthase inhibitors [63]. The elevation of Ptch1 levels upon Gap43 reduction in vitro fits to the data by Shen et al. who reported an upregulation of Ptch1 gene expression in inner EGL regions of Gap43\(^{-/-}\) mice compared to wild-type animals. Moreover, cultured Gap43-deficient GCPs show decreased proliferation in response to administered recombinant Shh protein [64]. A possible regulatory link was recently provided as the activation of the hedgehog signaling component Smoh was found to depend on PI4P (phosphatidylinositol 4-phosphate) levels that immediately increase when Shh binds to Ptch1 or when functional Ptch1 is absent [65]. The authors further showed that imbalanced conversion of the precursor molecule PI into PI4P influences hedgehog pathway activity. Alternatively, the production of PI4P can also result from a specific dephosphorylation of PI(4,5)P2 [66]. In this context, Gap43 protein was recently demonstrated to build oligomeric structures in the plasma membrane which sequester specifically PI(4,5)P2 [67]. A similar finding has been reported earlier showing that GAP43 participates in the accumulation of plasmalemma rafts, which promoted retention of PI(4,5)P2 [68]. The amount of Gap43 associated with the plasma membrane therefore possibly modulates the utilization of PI(4,5)P2, including its conversion into PI4P, which in turn directly affects hedgehog signaling through Smoh activation. However, the effective impact on downstream Gli-targets would still be difficult to conclude regarding the multitude of responses to Shh, including negative feedback regulation [13]. Further studies applying depletion and enrichment of specific phosphatidylinositol derivatives and selective silencing of hedgehog pathway elements will be necessary to elucidate the molecular nature of this proposed signaling axis.

The increased accumulation of mitotic granule cells at the EGL seen in the combined Ptch1\(^{+/+}\) Nos2\(^{-/-}\) genotype supposedly gives a special clue to MB induction. In contrast to the classical view of neonatal EGL organization, which describes radial migration of neonatal EGL organization, which describes radial migration of maturating GCPs from outer EGL regions. Therefore, the accumulation of GCPs in the EGL in combination with the insensitivity to Ptch1-mediated cell cycle arrest in Ptch1\(^{+/+}\) Nos2\(^{-/-}\) mice provide a growth advantage and increase the number of putative transformation targets over Ptch1\(^{+/+}\) Nos2\(^{+/+}\) mice (Figure 7C).

In conclusion, the following picture emerged from our data: Homozygous deletion of Nos2 leads to a reduction of basic NO levels in immature GCPs of the EGL during postnatal development of the cerebellum. This reduction causes a downregulation of Gap43 expression, which results in an increased expression of Ptch1 and impaired directed migration of maturing GCPs. As a consequence, undifferentiated granule cell precursors exit cell cycle and are retained at the EGL (Figure 7B). In case of an additional heterozygous Ptch1 mutation, upregulation of this receptor does not suffice to exert the anti-proliferative stimulus following Gap43 decrease, which results in an increased fraction of continuously dividing cells in the EGL (Figure 7C). As reduced migration towards the IGL further leads to a withdrawal of growth-limiting signals, expansion of the GCP population is additionally supported. Finally, this advances medulloblastoma development in Ptch1\(^{+/+}\) Nos2\(^{-/-}\) mice compared to Ptch1\(^{+/+}\) Nos2\(^{+/+}\) mice. The mechanism described here illustrates a new tumor-promoting concept in MB showing that the localization of pre-neoplastic cells within the developing cerebellum is important for pathogenesis.

Materials and Methods

Generation of Ptch1\(^{+/+}\) Nos2\(^{+/+}\) mice

Ptch1\(^{+/+}\) mice (B6;129P2-Ptch1\(^{tm1Lau}\)/Ptch1\(^{+/-}\) [20]) and Nos2\(^{+/+}\) mice (B6;129P2-Nos2\(^{tm1Lau}\); [72]) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and crossbred to generate double heterozygous mice (Ptch1\(^{+/+}\) Nos2\(^{+/+}\) mice; [20]). The F1 hybrids were backcrossed with Ptch1\(^{+/+}\) Nos2\(^{-/-}\) mice to generate Ptch1\(^{+/+}\) Nos2\(^{-/-}\) mice. Later on, Ptch1\(^{+/+}\) Nos2\(^{-/-}\) mice were directly mated. For details on housing and genotyping see Text S2 and Table S9. All animal experiments were approved by the responsible federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany, Az. 50.05-230.17-06).

Microarray-based genomic and expression profiling

Total RNA of tumor specimens and normal cerebellar tissue samples was isolated via CsCl density gradient centrifugation [73] and assessed for integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). For gene expression microarrays, linear amplification of mRNA and labeling of cDNA were conducted on samples and Mouse Universal Reference RNA (Stratagene, La Jolla, USA) according to the TAgKLE protocol [74]. Both were combined for two-color hybridizations with each sample being performed as two replicates of inverse dye orientation. Global gene expression profiling was performed on self-printed oligonucleotide microarrays. Further details of microarray production and hybridization are described in Text S2. Genomic DNA of tumor specimens was isolated from the interphase of the CsCl gradient by ethanol precipitation, proteinase K digest, and phenol/chloroform extraction. DNA samples were monitored for purity and adequate fragment size using spectrophotometric measurements and gel electrophoresis. Array-based comparative genomic hybridization ([array-CGH, matrix-CGH, [75]]) was performed on Mouse Genome CGH 244 k Microarrays (Agilent). Cy3-labeled tumor DNA was combined with corresponding Cy3-labeled reference (wild-type genomic DNA) to receive either sex-matched sample pairs or pairs of different gender for internal negative or positive control. Sample preparation, microarray hybridization, and washing procedures were carried out as described in the manufacturer’s protocol. Microarray data are available in GEO (http://www.ncbi.nlm.nih.gov/geo), under accession number GSE29201.
qRT–PCR analyses

Total RNA isolated from cell culture samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) or RNA from tissue specimens was subjected to oligo(dT)-primed reverse transcription. qRT-PCR measurements were conducted in an ABI PRISM 7900HT thermal cycler (Applied Biosystems, Foster City, USA) using the SYBR green reaction and detection system (ABgene, Epsom, UK). For relative quantification mean ratios were calculated between genes of interest and a set of five housekeeping genes (Table S7) according to the Pfaffl method [76].

The expression levels of Ptch1, Gli1, N-myc, and Nos2 were determined by real-time reverse transcription PCR analysis using the ABI PRISM 5700 system (Applied Biosystems) as reported before [73]. For these experiments, the mRNA expression level of mitochondrial ribosomal protein L32 (Mrpl32) served as housekeeping reference. All primer sequences are depicted in Table S8.

Cell culture experiments

Inhibition of NO synthases was performed in c17.2 and D458 cells which were seeded at densities of 2 × 10^5 and 4 × 10^5 cells per well in 12-well plates, respectively. Cells were daily treated with either 1 mM of the inhibitor L-NNAME or 1× PBS as solvent control. For knockdown experiments of Gap43, c17.2 cells were grown in a 12-well plate to 80% confluency and transfected with 2 μg of pLKO.1-puro vector that contained either shRNA constructs targeting Gap43, shRNA against GFP, or non-target shRNA as a control (Sigma-Aldrich, St. Louis, USA) using 9 μl FuGene HD reagent (Roche, Basel, Switzerland). Transfection was repeated 2 times each after 8 hours and subjected to selection conditions (1 μg/ml puromycin) for 24 hours. Subsequently, cells were trypsinized, adjusted to 4 × 10^5 cells/ml and seeded into the inserts of a Costar Polycarbonate Membrane Transwell plate (8 μm pores, Corning, USA). After 24 hours cells were either harvested for gene expression and protein analyses or 0.1 μg/ml recombinant SDF-1α was applied to the lower compartment for migration assays. Following 12 hours of incubation, cells at the bottom of the insert membrane were methanol-fixed, hematoxylin-stained, and counted.

Immunofluorescence analyses

FFPE sections of postnatal cerebella were pre-processed as described in Text S2. For immunofluorescence co-staining, Gap43 (Sigma-Aldrich, clone GAP-7b10) or NeuN (Millipore, clone A60) first primary antibodies were diluted 1:1000 or 1:200, respectively.
and applied using the Dako REAL Detection System (Dako, Glostrup, Denmark). Following over night incubation at 4°C, washing in TBS, and blocking of residual biotin/streptavidin, sections were subsequently incubated with biotinylated anti-mouse secondary antibody (Dako) and stained with 20 ng/μl FITC-conjugated streptavidin (Invitrogen, Carlsbad, USA). The second primary antibody against Ki-67 (Novocastro, Wetzlar, Germany) was diluted 1:1000 and accordingly applied using biotinylated anti-rabbit secondary antibody (Dako) and 20 ng/μl Cy3-conjugated streptavidin (Invitrogen). Co-stained sections were then covered with DAPI-containing VECTASHEILD Mounting Medium (Vector, Burlingame, USA) and subjected to confocal laser scanning microscopy.

Quantification of Gap43 staining was performed for areas of interest using Image J software (NIH). Numbers of dividing and non-dividing cells in the EGL of postnatal cerebellar tissue sections were counted manually and normalized to the corresponding non-dividing cells in the EGL of postnatal cerebellar tissue sections of interest using Image J software (NIH). The EG2 distance in z-axis, and distance in y-axis, and distance in x-axis were obtained from qRT-PCR measurements on the expanded sample set and indicate mRNA expression against a pool of housekeeping genes normalized to mouse universal reference RNA (Stratagene). (A) Expression of Otx1, p = 0.071, Δmean = −2.736±1.354. (B) Expression of Pdgfra, p = 0.201, Δmean = −2.890±0.849. (C) Expression of Sin3a, p = 0.710, Δmean = 0.202±0.331.

Figure S4 Nitric oxide (NO) assay on neuronal progenitor cells (c17.2) and medulloblastoma cells (D458) upon inhibition of NO synthases. Treatment samples were supplemented with 1 mM L-NAMe and control samples were supplemented with solvent (PBS). After 24 hours control sample exceeded NO levels in treatment samples indicating successful impairment of NO production. (TIF)

Figure S5 Apoptosis and cell cycle analyses of the cell lines c17.2 and D458 by FACS, after inhibition of NO synthases (L-NAMe treatment). (A, C) PI (propidium iodide) signals of fixed cells representing different cell cycle phases. (B, D) Dot plot showing apoptosis in freshly harvested cells stained with Annexin V and 7-AAD. (E) Plotted fractions of cells in G0/G1, G2/M-phase, or S-phase. (F) Plotted fractions of dead cells and viable cells. Inhibition of NO synthases by L-NAMe application shows no prominent changes in cell physiology. I: cell debris, II: G0/G1, III: S-phase, IV: G2/M-phase, V: doubles. (TIF)

Figure S6 Reduction of c17.2 migration depends on Gap43 knockdown efficiency. (A) Western blot stained for Gap43 and α-tubulin (housekeeping protein). (B) Quantification of Gap43 protein bands normalized to α-tubulin. (C) Proportion of migrated cells relative to non-target (NT) control. The constructs sh39 and sh42 showed the highest knockdown efficiency and the strongest effect on migration. (TIF)

Figure S7 Cell cycle analysis of neuronal progenitor cells (c17.2) by FACS, 72 h after knockdown of Gap43. (A) PI (propidium iodide) signals of fixed cells representing different cell cycle phases. I: cell debris, II: G0/G1, III: S-phase, IV: G2/M-phase, V: doubles. (B) Plotted fractions of cells in G0/G1, G2/M-phase, or S-phase. (TIF)

Table S1 Targeted molecular analyses of selected genes. (DOC)

Table S2 Differentially expressed genes in medulloblastomas of Pch1+/− Nos2−/− against Pch1+/− Nos2+/+ mice. (DOC)

Table S3 Gene expression of markers for stromal cells in medulloblastomas of Pch1+/− Nos2−/− against Pch1+/− Nos2+/+ mice. (DOC)

Table S4 Differentially expressed genes in P9 cerebella of Pch1+/+ Nos2−/− against wild-type mice. (DOC)

Table S5 Differentially expressed genes in P9 cerebella of Pch1+/+ Nos2−/− against wild-type mice. (DOC)

Table S6 Differentially expressed genes in P9 cerebella of Pch1+/+ Nos2−/− against wild-type mice. (DOC)
**Table S7**  Housekeeping genes used for qRT-PCR analyses.

**Table S8**  Primer sequences used for qRT-PCR analyses.

**Table S9**  Primer sequences used for duplex-PCR analyses and genotyping.

**Text S1**  Supporting Results.

**Text S2**  Supporting Methods.

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**Author Contributions**

Conceived and designed the experiments: DH PZ JR GR PL. Performed the experiments: DH PZ VW DK KP. Analyzed the data: DH PZ GT. Contributed reagents/materials/analysis tools: GT FB ND. Wrote the paper: DH PZ GR PL. Critical revision of the manuscript: DK MH.

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