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Hedgehog/GLI1 activation leads to leukemic transformation of myelodysplastic syndrome in vivo and GLI1 inhibition results in antitumor activity

Bonnie W. Lau¹, Kyounghee Huh¹, Rafael Madero-Marroquin¹, Federico De Marchi¹, Yiting Lim^{1,2}, Qiuju Wang¹, Francisco Lobo^{1,3}, Luigi Marchionni¹, Douglas B. Smith¹, Amy DeZern¹, Mark J. Levis¹, Peter D. Aplan⁴, William Matsui⁵, and Lukasz P. Gondek¹

¹The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

²Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

³Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

⁴Genetics Branch National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

⁵LIVESTRONG Cancer Institutes, Dell Medical School, The University of Texas at Austin, Austin, TX 78712USA, USA

Abstract

Myelodysplastic syndromes (MDSs) are stem cell disorders with risk of transformation to acute myeloid leukemia (AML). Gene expression profiling reveals transcriptional expression of *GLI1*, of Hedgehog (Hh) signaling, in poor-risk MDS/AML. Using a murine model of MDS we demonstrated that constitutive Hh/Gli1 activation accelerated leukemic transformation and decreased overall survival. Hh/Gli1 activation resulted in clonal expansion of phenotypically defined granulocyte macrophage progenitors (GMPs) and acquisition of self-renewal potential in a non-self-renewing progenitor compartment. Transcriptome analysis of GMPs revealed enrichment in gene signatures of self-renewal pathways, operating via direct *Gli1* activation. Using human cell lines we demonstrated that in addition to canonical Hh signaling, *GLI1* is activated in a Smoothened-independent manner. *GLI1* knockdown or inhibition with GANT61 resulted in decreased proliferation and clonogenic potential. Our data suggest that *GLI1* activation is frequent in MDS during disease progression and inhibition of GLI1 is an attractive therapeutic target for a subset of patients.

William Matsui matsui@jhmi.edu, Lukasz P. Gondek lgondek1@jhmi.edu.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Introduction

Myelodysplastic syndromes (MDSs) are clonal hematopoietic stem cell (HSC) disorders characterized by ineffective hematopoiesis manifested by peripheral blood cytopenias and bone marrow hypocellularity [1, 2]. Although low-risk MDS generally carries a good prognosis, clinical outcomes considerably deteriorate upon progression to secondary acute myeloid leukemia (sAML) with a dismal median survival of <6 months [3, 4]. Several mechanisms, including the acquisition of structural chromosomal changes, point mutations, and the distortion of epigenetic signatures are associated with leukemic transformation of MDS [5–7]. However, the cellular pathways involved in this process have not been clearly defined. Consequently, with the exception of lenalidomide in 5q– syndrome, targeted therapies proven to be effective in other human malignancies are not currently available for patients with MDS. Approved therapeutic options are limited to hypomethylating (HMA) and immunomodulatory agents, but these are not curative and only marginally prolong survival in a subset of patients [8]. More aggressive approaches like induction chemotherapy and allogeneic bone marrow transplantation are frequently limited in this group of patients due to excessive toxicity and/or low efficacy [9, 10].

The Hedgehog (Hh) signaling pathway is active during embryogenesis and silenced in most adult tissues. Abnormal pathway activation has been demonstrated in a variety of solid and hematologic malignancies [11]. Hh signaling is initiated by binding of one of the three ligands to the cell surface receptor Patched (PTCH) that normally inhibits the protein Smoothened (SMO), and is further transmitted via a protein complex that includes Suppressor of Fused and downstream zinc-finger Gli transcription factors (TFs). GLI1 functions exclusively as an activator, GLI3 is a repressor, and GLI2 can operate as either activator or repressor [12, 13]. In addition to canonical Hh signaling, the activity of GLI1 can be modulated by other pathways frequently involved in human tumorigenesis, including Wnt, Notch, RAS-MEK, and transforming growth factor- β (TGF- β) [14–17].

Hh signaling plays a pivotal role in the development and progression of various hematologic malignancies [18–21]. Most recently we established that *GLI2* expression is an independent predictor of poor outcome in *FLT3-ITD*-mutated AML, and Hh activation leads to accelerated progression and leukemic transformation of *FLT3-ITD*-driven indolent myeloproliferative disease in mice [18]. Only limited data exist on Hh signaling in MDS and very little is known about the role of pathway activation in disease development and progression [22]. Hh inhibitors have been shown to be effective in preclinical studies in MDS and AML, and preliminary data from ongoing clinical trials using SMO inhibitors demonstrate promising antitumor activity [23, 24]. However, optimal Hh-targeting strategies remain to be determined.

Here we examine the clinical impact of aberrant Hh signaling in MDS and further explore the mechanism of Hh-driven disease progression and leukemic transformation using the Nup98-HoxD13 (NHD13) animal model of MDS. We have found that constitutive activation of Hh signaling results in accelerated leukemic transformation via acquisition of self-renewal in phenotypically defined granulocyte macrophage progenitors (GMPs). We have

also demonstrated that the inhibition of Hh pathway at the level of *GLI1* rather than upstream SMO may be a more effective targeted therapeutic approach in MDS and sAML.

Results

Hh signaling is silenced in normal hematopoietic stem and progenitor cells and aberrantly activated in a subset of MDS patients

Hh signaling activation results in transcriptional activation of *GLI1*. To determine the activity of Hh signaling pathway in normal and malignant hematopoiesis we analyzed the expression of *GLI1* using publicly available expression datasets. *GLI1* was found to be significantly upregulated in MDS (Figure S1), poor-risk AML (Fig. 1a), and AML with complex cytogenetics (Fig. 1b). These subtypes of AML frequently arise from antecedent hematological disorders like MDS. Moreover, high *GLI1* expression correlated with shorter overall survival (median survival 24.1 vs. 16.3 months, $p = 0.03$) (Fig. 1c). Further analysis revealed that *GLI1* was overexpressed in CD34+ cells in a subset of MDS patients but not by normal CD34+ hematopoietic stem and progenitor cells (HSPCs) (Fig. 1d). To determine whether Hh pathway activation marked the disease progression and leukemic transformation, we analyzed the expression of Hh targets using serial bone marrow samples. While the expression of *GLI1* and *PTCH1* was absent in CD34+ cells from healthy controls and low-risk MDS patients, the pathway activation coincided with the progression to AML in 4/6 (67%) patients (Fig. 1e).

Hh pathway activation results in accelerated leukemic transformation of NHD13 mouse MDS

Recurrent chromosomal abnormalities have been identified in MDS and AML, including the translocation of chromosome 2 and 11 leading to fusion of the NHD13 genes. Transgenic mice expressing the NHD13 fusion protein develop highly penetrant and reproducible myelodysplasia and progression to leukemia with the median survival of 14 months [25]. To determine the role of aberrant Hh signaling in MDS progression, we crossed transgenic NHD13 mice with mice expressing the constitutively active *Smo* mutant *SmoM2* fused to yellow fluorescent protein (YFP) [25, 26]. Conditional expression of *SmoM2* within the hematopoietic system was achieved with *Mx1*Cre expression and treatment with poly(I:C) (Fig. 2a). *SmoM2*-driven expression of Hh signaling was confirmed by the presence of YFP-positive hematopoietic cells and the expression of *GLI1*, which was observed in NHD13/*SmoM2* and *SmoM2* mice and remained undetectable in NHD13 and wild-type (WT) animals (Figure S2a, b).

We and others have previously shown that the expression of *SmoM2* has no effect on hematopoiesis and HSPCs [18, 27, 28]. In contrast, activation of Hh signaling in NHD13/*SmoM2* mice resulted in significantly shorter median survival compared to NHD13 mice (3 vs. months, $p < 0.0001$) (Fig. 2b). The white blood cell count and the mean spleen weight were significantly increased in NHD13/*SmoM2* compared to NHD13, *SmoM2*, or WT mice ($p < 0.05$ and < 0.0001 , respectively) (Fig. 2c). Peripheral blood at 6–8 weeks post poly(I:C) injection showed significant myeloid skewing in NHD13 mice, which was even more pronounced in NHD13/*SmoM2* animals (Figure S2c). The analysis of complete peripheral

blood count showed significant anemia and leukocytosis (Figure S2d). These findings were consistent with the observed increased penetrance of combined NHD13/SmoM2 transgenes toward myeloid leukemia compared to NHD13 alone (58% vs. 42%). Immunophenotyping of peripheral blood, bone marrow, and spleen showed the emergence of an immature myeloid population (Mac1⁺Gr1^{dim}) and significant expansion of leukemic blasts in NHD13/SmoM2 animals (Fig. 3a, b, S3c). Histologically, peripheral blood, bone marrow, and spleen were infiltrated by morphologically immature cells (Fig. 3a). Necropsy of NHD13/SmoM2 animals revealed multiple sites of extramedullary involvement by aggressive leukemia (Figure S3a, b).

Hh signaling results in expansion of immature myeloid cells and gain of aberrant self-renewal potential in GMPs

Bone marrow cellularity was significantly increased in NHD13/SmoM2 animals compared to NHD13 controls (Fig. 4a). To better characterize the HSPC compartment we utilized multiparameter flow cytometry. Since multiple groups have demonstrated no differences in HSPC frequencies between WT and SmoM2 animals [18], we studied NHD13/SmoM2 and NHD13 animals and found similar frequencies of Lin⁻Sca1⁺cKit⁺, Lin⁻CD34⁺FcγR⁻ multipotent progenitors, and Lin⁻CD34⁺FcγR⁺ common myeloid progenitors (Fig. 4b). However, Lin⁻CD34⁺FcγR⁺ GMPs were significantly expanded in NHD13/SmoM2 mice ($p < 0.05$) (Fig. 4b, S4a). Additionally, whole bone marrow cells from NHD13/SmoM2 showed significantly higher in vitro clonogenic potential; colonies showed morphologic characteristics of Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) consistent with myeloid differentiation (Fig. 4c). In order to define the long-term leukemia-initiating potential in vivo we transplanted total bone marrow from NHD13/SmoM2 mice to congenic recipients after sublethal irradiation (Figure S4b). The recipient mice developed rapidly progressing leukemia with leukocytosis, splenomegaly, expansion of Mac1⁺Gr1^{dim} leukemic blasts, and median survival of 2 months (Figure S4c, d). Furthermore, the expansion of GMPs was maintained (Figure S4e). To determine whether Hh activation resulted in aberrant self-renewal of the GMP compartment, we serially transplanted GMPs from NHD13/SmoM2 and NHD13 mice (Fig. 4d). NHD13 GMPs showed only transient engraftment ($n = 3$), characteristic of non-self-renewing progenitors. In contrast, NHD13/SmoM2 GMPs robustly engrafted and significantly expanded over time, and all recipient animals ($n = 3$) developed aggressive leukemia within 2–3 months. Engraftment potential was also maintained during secondary transplantation, and all secondary recipients succumbed to leukemia (Fig. 4d). These findings suggest that the activation of Hh signaling in NHD13 animals results in acquisition of aberrant self-renewal in normally non-self-renewing progenitors leading to the development of lethal AML.

Hh signaling activates self-renewal program in differentiated progenitors in MDS

To delineate the mechanisms leading to aberrant self-renewal in GMPs, we compared the transcriptional profile of GMPs isolated from NHD13/SmoM2 and NHD13 mice. Gene expression clustering showed a distinct transcriptional pattern between NHD13/SmoM2 and NHD13 cells (Figure S5a). We used Gene Set Enrichment Analysis (GSEA) to determine the key biological pathways involved in oncogenic transformation. As expected, we confirmed activation of the transcription signature of Hh signaling (Figure S5b).

Additionally, the transcriptional programs characteristic of other developmental pathways such as Wnt and TGF- β were enriched in NHD13/SmoM2 GMPs (Fig. 5a). Both pathways are well known to drive cancer stem cells self-renewal [14, 17]. Interestingly, also transcription signature of EWS/FLI1-driven model of myeloid leukemia was significantly enriched for in NHD13/SmoM2 GMPs (Fig. 5b). Analogous to our model, EWS/FLI1 expression in murine HSPC resulted in expansion of immature myeloid progenitors with aberrantly activated self-renewal potential [29]. Interestingly, *GLI1* has been reported as a direct transcriptional target of EWS/FLI1 [30–32]. Our data suggest that aberrant Hh signaling in NHD13 GMPs leads to activation of a self-renewal program via transcriptional activation of *GLI1*. Moreover, the transcriptional program activated by GLI1 resembles the transcriptional characteristics of other developmental pathways suggesting its key role as an oncogenic TF.

***GLI1* can be activated in a SMO-independent manner and GLI1 inhibition diminishes the proliferation and clonogenic potential of human AML and MDS cell lines**

To determine the role of GLI1 in human MDS and AML, we examined the expression of pathway components in several AML and MDS cell lines. We observed that *GLI1* activation may occur in both: SMO-dependent and SMO-independent fashion. In TF1 leukemia cell line for instance, *GLI1* expression was coupled with the Hh pathway signaling components *SMO* and *PTCH1* (Figure S6a) suggesting canonical Hh signal transduction. In contrast, GLI1 expression was observed in the absence of SMO in these secondary AML cell line arising from MDS (MDS-L) suggesting SMO-independent *GLI1* activation (Figure S6a). These findings suggest the key role of GLI1 that may be activated in a SMO-independent manner. To further delineate the role of SMO and GLI1 in oncogenic process we focused on TF1 (*SMO* and *GLI1* expresser) and MDS-L (*GLI1* expresser). To investigate the role of SMO we carried out short hairpin (shRNA) gene silencing and protein inhibition using PF04449913 in TF1 (Figure S6b). *SMO* knockdown and protein inhibition negatively impacted TF1 cell growth (Figure S6c) but had no effect on clonogenic potential (Figure S6d). However, both *GLI1* knockdown and treatment with GANT61 resulted in both diminished cell growth and clonogenic potential (Figure S6c, d). To examine the role of SMO-independent GLI1 activation we carried out GLI1 loss-of-function experiment in MDS-L secondary leukemia cell lines. GLI1 knockdown and inhibition with GANT61 resulted in decreased cell proliferation (Fig. 6a) and colony formation (Fig. 6b). As expected, the SMO inhibition with PF04449931 had no effect on cell proliferation (Fig. 6a) or colony formation (Fig. 6b). We next sought to elucidate the mechanism of impaired cell growth and clonogenic potential. The treatment with GANT61 but not with PF04449931 induced apoptosis in MDS-L cells. While increased apoptosis may explain impaired cell growth, it may not affect clonogenic potential. Differentiation, however, is nearly uniformly associated with decreased self-renewal. The treatment of MDS-L blasts with GANT61 resulted in acquisition of myeloid differentiation marker CD11b as well as morphological changes indicative of increased differentiation. As expected, PF04449931 showed no differentiation potential (Fig. 6c– e). We further sought to determine the relationship between GLI1 expression level and response to GLI1 and SMO inhibition using primary human secondary AML and healthy, age-matched CD34+ cells. We identified two patients: AML1 with complex cytogenetics and high GLI1, and AML2 with normal cytogenetics and

low GLI1 mRNA level (Figure S6e). SMO inhibition with PF04449931 had no impact on colony formation of either AML1 or AML2 compared to normal CD34+ cells. However, GLI1 inhibition significantly decreased clonogenic potential of AML1 (*GLI1* high), but not AML2 (*GLI1* low) human leukemia (Fig. 6f). Our data imply that GLI1 can be transcriptionally activated in a SMO-independent fashion and inhibition of the pathway at the level of GLI1, rather than upstream SMO may result in a superior anti-leukemic effect.

Discussion

We report that Hh pathway activation marks disease progression in a subset of patients with MDS. GLI1, a pure transcriptional activator, is not present in healthy HSPC but becomes aberrantly activated in a subset of patients with MDS upon leukemic transformation. Using a murine model, we demonstrate that GLI1 activation via canonical Hh signaling accelerates the leukemic transformation of NHD13 mice through pathologic activation of a self-renewal program in differentiated progenitors. Moreover, in human disease, GLI1 can be activated in a SMO-independent fashion and the inhibition of GLI1 at the mRNA or protein level reduces proliferation and clonogenic potential of human AML and MDS cells. Therefore, Hh inhibition appears to be an attractive therapeutic approach in patients with MDS and AML.

We have previously demonstrated the cooperative role of Hh signaling with *FLT3*-ITD in leukemic transformation of myeloproliferative neoplasm and the advantage of combination therapy with Hh and FLT3 inhibitors [18]. While Hh signaling has already emerged as an important pathway in certain myeloid and lymphoid malignancies, its role in MDS has not been extensively studied. Several groups have shown the role of Hh ligands and Hh interacting protein in the maintenance of MDS clones [33, 34]. Hh activation and *GLI1* expression was recently found to be associated with high-risk disease and GLI1 inhibitors enhanced the effects of HMA agents [35]. Based on these findings, SMO inhibitors were tested as a single agent in phase 1 and in combination with low-dose chemotherapy in randomized phase 2 clinical trials and showed promising activity with improved complete remission rate and overall survival [23, 24, 36].

In our current studies, we used a constitutively active *Smo* mutant to activate *GLI1* via canonical Hh signaling. Even though we found the evidence of transcriptional activation of *GLI1* in cell lines and primary MDS samples, the genetic mutations and abnormal expression of upstream pathway components like SMO and PTCH are infrequent in human MDS and AML (The Cancer Genome Atlas (TCGA) (<http://cancergenome.nih.gov>). In addition to canonical Hh signaling, the activity of GLI TFs can be modulated by other pathways frequently involved in human tumorigenesis like Wnt, Notch, TGF- β , MAPK, and PI3K/AKT [14–17, 37, 38]. Our data suggest that *GLI1* expression is not always coupled with the presence of upstream pathway components, indicating that its activation may be modulated in a SMO-independent manner (Fig. 7). Similar findings were recently published in AML [39]. Thus, GLI1 TFs seem to be a convergence point of multiple cancer-associated pathways and may confer an attractive target with broader therapeutic effects than currently used SMO inhibitors.

We previously found that Hh pathway drives aberrant self-renewal within putative cancer stem cells in multiple myeloma, pancreatic carcinoma, and glioblastoma [40–42]. The precise role of Hh signaling in myeloid disorders is unclear as mouse models have revealed that it is required for the maintenance of chronic myeloid leukemia stem cells but not for the development of mixed-lineage leukemia (MLL)-driven AML [28, 43]. Our data suggest that pathologic activation of Hh signaling accelerates progression of MDS via aberrant activation of self-renewal in a normally non-self-renewing compartment. Acquisition of abnormal self-renewal in GMP compartment via activation of Wnt signaling was also observed in HoxA9-Meis1 and MLL-AF9 mouse models of AML [44]. Similar activation of self-renewal in myeloid progenitors was found during leukemic transformation of chronic myeloid leukemia [45]. Consistent with the aforementioned findings, the gene expression analysis of GMPs from NHD13/SmoM2 and NHD13 controls revealed relative enrichment in transcriptional targets of not only Hh but also other developmental pathways like Wnt and TGF- β . Interestingly, transcriptome of NHD13/SmoM2 GMPs resembled the profile of EWS/FLI1-driven leukemia. Notably, EWS/FLI1 was shown to exert its effect via abnormal activation of self-renewal and expansion of hematopoietic progenitors [29]. We have previously shown that *GLI1* is a direct target of EWS/FLI1 oncoprotein suggesting *GLI1* as a key mediator of oncogenic transformation [30].

Initial reports revealed that Hh signaling is dispensable in normal definitive hematopoiesis [27, 28]. Subsequent data showed only minor impact of Hh perturbations on hematopoietic stem cell frequencies and function [46]. These findings indicate that Hh inhibition may selectively target malignant cells while sparing healthy counterparts. This is further supported by the lack of hematologic toxicities with the use of SMO inhibitors in clinical trials [47]. Our data using MDS and AML cell lines showed that inhibition of GLI1 either with shRNA or GANT61 reduced cell proliferation and clonogenic potential more effectively than SMO inhibition. The lack of SMO expression in MDS-L cells suggests that GLI1 activation may also occur in a SMO-independent fashion. Consistently with our data, *GLI1* knockdown was shown to inhibit cell proliferation and induce cell cycle arrest and apoptosis in MDS bone marrow cells [48]. The use of GANT61 resulted in growth arrest, apoptosis, and cell differentiation in AML cells [49, 50]. In a recent study, T-cell acute lymphoblastic leukemia cells with high *GLI1* expression were sensitive to GANT61 in ex vivo cultures and in vivo xenograft models [19]. In summary, our data suggest that both SMO-dependent and -independent activation of GLI1 is frequent in MDS/sAML and therapeutic strategies targeting Gli TFs may be more effective than currently used SMO inhibitors.

Materials and methods

Clinical specimens, cell lines, and drug treatment

The study was approved by the Institutional Review Board at the Johns Hopkins School of Medicine. Bone marrow specimens were obtained from patients with MDS at the time of diagnosis and upon progression to AML. CD34+ cells were selected using MicroBeads (Miltenyi Biotec, San Diego, CA) and the expression of effectors of Hh signaling was determined by quantitative PCR. Human whole bone marrow with >80% AML blasts were

cultured in stem cell media H4435 (Stem Cell Technologies) supplemented with FLT3-ligand, stem cell factor, and interleukin (IL)-6. Human cell lines TF1 (American Type Culture Collection) and MDS-L (kindly provided by Dr. Daniel Starczynowski) were cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, and granulocyte macrophage colony-stimulating factor (2 ng/ml) or recombinant IL-3 (10 ng/ml), respectively. Drug treatments were done with PF04449913 (Sigma-Aldrich), GANT61 (Stem Cell Technologies), or dimethyl sulfoxide vehicle control for 72–96 h. Cell proliferation was assayed by Trypan blue dye exclusion or MTT assay (Abcam). Apoptosis was determined by flow cytometry after Annexin V-PE (BD Pharmingen™) staining. Cell differentiation was determined by increase in CD11b expression and histologically after cytospin and modified Wright-Giemsa staining. Colony formation assay was performed by growing cells (100–500 cells/ml) in methylcellulose for 11 days, and counting colonies using an inverted Nikon microscope.

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee. SmoM2 and *Mx1Cre* C57Bl6 mice were obtained from Jackson Laboratories and crossed with NHD13 C57Bl6 mice. Genotyping was determined by PCR. NHD13 mice and mice carrying the *Mx1Cre* allele alone (WT) were used as littermate controls. All mice were treated with five doses of poly(I:C) (300 µg, Sigma) intraperitoneally every other day. Successful transgene excision in NHD13/SmoM2 and SmoM2 animals was confirmed by PCR and YFP expression by flow cytometry (FACSCalibur, BD Biosciences) of peripheral blood cells (Figure S1a). Development of AML was assessed by the presence of Mac1⁺Gr1^{dim} cells in the peripheral blood and/ or bone marrow. Complete blood counts were quantified using a Procyte Dx Hematology analyzer (Idexx). Formalin-fixed tissues were stained with hematoxylin and eosin, and blood smears with Wright-Giemsa stain. At least three mice per experimental or control group were used for each experiment as a sample size estimate limited by the number of mice per litter for littermate controls. Animals who were found dead for >24 h were excluded from obtaining tissue and therefore not analyzed. The criteria were pre-established. No randomization or blinding was used for these studies on observations based on individual mouse genotype.

Antibodies and fluorescence-activated cell sorting

Monoclonal antibodies against Mac1, Gr1, and B220 (eBioscience) were used for peripheral blood analysis using FACSCalibur flow cytometers (BD Biosciences). Biotinylated antibodies against Gr1, Ter119, B220, and CD3 (eBioscience) were used for lineage staining of whole bone marrow cells. Bone marrow cells were also stained with antibodies against cKit, Sca1, CD16/32, CD34, FLT3, and IL7R (eBioscience), and analyzed on a LSRII cytometer (BD Biosciences) (Table S1). Cells were sorted using a MoFlo Instrument (Beckman Coulter). Flow cytometry data were analyzed with FlowJo v.8.7 software, with gating strategy shown in Supplemental Fig. 4

Quantitative real-time PCR analysis

Total RNA was extracted using RNeasy Plus Mini kit (Qiagen) and reverse transcribed with Superscript III reverse transcriptase (Invitrogen) according to the manufacturers' protocol.

Quantitative real-time PCR (qRT-PCR) was carried out with Taqman and/or SYBR Green assays (Applied Biosystems) and analyzed using StepOne Plus instrument (Applied Biosystems). Primer and probe sequences are listed in Table S2.

Microarray and GSEA analysis

A published dataset available from TCGA and the NCBI Gene Expression Omnibus database (GSE 58831) [51] were analyzed for expression levels of the Hh signaling pathway and correlated with risk stratification and overall survival. The results published here are in whole or part based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>. RNA was extracted as described above from GMPs obtained from bone marrow of age-matched NHD13 and NHD13/SmoM2 mice, and gene expression profiling was carried out using Mouse Gene Expression Microarray Kit v2 (Agilent). Microarray data were analyzed using R and GSEA software (see supplementary methods).

Short hairpin RNA

TF1 and MDS-L cell lines were transfected with pLKO Tet-On lentiviral shRNA vectors targeting *GLII*, *SMO*, and luciferase (Table S3). Successful knockdown of genes was determined by qRT-PCR 72 h after doxycycline treatment.

Statistics

The data were normally distributed. *p* Values were determined using an unpaired two-tailed Student's *t* test. *p* Values < 0.05 were considered statistically significant. Survival was assessed using Kaplan-Meier analysis. GraphPad Prism v.6 was used for statistical analysis. Estimate of variance was determined for each group and were similar between groups. The detailed description of gene expression data analysis is described in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The Hedgehog signaling pathway is activated in human disease. Analysis of *GLI1* expression using publicly available expression datasets in **a** AML based on risk category and **b** AML based on cytogenetics (TCGA). **c** Overall survival based on *GLI1* expression in TCGA AML cohort (*GLI1* high: *GLI1* > mean expression; *GLI1* low: *GLI1* < mean expression), p value calculated using log-rank test. **d** The expression of *GLI1* in CD34+ HSPC from a subset of MDS patients compared to healthy controls (GSE 58831). **e** *GLI1* and *PTCH1* expression in longitudinal MDS samples as the time of diagnosis and upon progression to AML. Dot plots represent individual values; mean and standard deviation are shown as whisker plots mean and standard deviation

Fig. 2.

Constitutive Hh activation in NHD13 mice results in rapidly progressing acute leukemia. **a** Schematic of transgenic mouse model with constitutive activation of mutant Smoothened (SmoM2) in NHD13 mice. **b** Overall survival of NHD13/SmoM2 mice compared to NHD13, SmoM2, and WT controls. **c** WBC and spleen size of NHD13/SmoM2, NHD13, SmoM2, and WT controls. Dot plots represent individual values; mean and standard deviation are shown as whisker plots

Fig. 3.

NHD13/SmoM2 mice showed significant expansion of immature myeloid blasts in blood, bone marrow, and spleen. **a** Wright-Giemsa stain of peripheral blood, and hematoxylin and eosin staining of the bone marrow and spleen sections showed expansion of immature hematopoietic progenitors. **b** Flow cytometry of peripheral blood, bone marrow, and spleen showed significant increase in the myeloid progenitor cell population (Mac1⁺/Gr1^{dim}) in NHD13/SmoM2 mice. The flow cytometry plots depict representative animals. Dot plots demonstrate individual animals; mean and standard deviation are shown as whisker plots

Fig. 4.

Granulocyte macrophage progenitors (GMPs) are expanded in NHD13/SmoM2 mice and acquire self-renewal potential. **a** Bone marrow cellularity presented as number of nucleated cells per femur, vertical lines represent mean values. **b** Absolute GMP number per femur in NHD13/SmoM2 mice and NHD13 littermate controls. **c** Colony formation assay using total nucleated bone marrow cells from NHD13/ SmoM2 ($n = 4$), NHD13 ($n = 3$), and WT animals ($n = 3$). Box plots represent mean and standard deviation. **d** Self-renewal potential of GMP was assessed in vivo. A total of 0.5×10^6 FACS-sorted GMPs from NHD13/ SmoM2 and NHD13 controls were transplanted into congenic recipients (3 mice per group). Percent engraftment in peripheral blood over time after primary and secondary transplantation; mean and standard deviation are shown

Fig. 5. Self-renewal gene signature is enhanced in NHD13/SmoM2 GMPs. **a** Gene set enrichment analysis (GSEA) of the GMPs revealed the enrichment in expression signature consistent with activation of Wnt and TGF- β signaling—developmental pathways frequently dysregulated in cancer stem cells. **b** GSEA demonstrated enrichment in genes regulated by EWS/FLI1 oncogene. Corresponding heat-maps are shown

Fig. 6.

GLII inhibition at the mRNA and protein level impedes MDS and leukemic cell growth and clonogenic potential in vitro. **a** MDS-L cell proliferation with *GLII* knockdown and inhibition with GANT61 as well as SMO inhibition with PF04449913 is shown along with shLuc- and DMSO-treated controls, respectively. Mean and standard deviation from three replicates are shown. **b** MDS-L colony formation in *GLII* knockdown cells relative to shLuc control as well as PF04449913 and GANT61-treated cells relative to DMSO-treated controls. Box plot represents mean and standard deviation from three replicates. **c** Flow cytometric apoptosis assay utilizing Annexin V antibody and 7AAD. Representative flow cytometry plots are shown. Bar graphs represent percentage of Annexin V-positive cells, mean and standard deviation calculated from experimental replicates ($n = 3$). **d** Flow cytometric analysis of CD11b acquisition by MDS-L blasts in response to SMO and *GLII* inhibition as well as DMSO-treated controls. **e** Morphology of MDS-L cells after cytospin

and modified Wright-Giemsa. Black arrowheads represent immature cells (blasts) with scant cytoplasm, open chromatin, and prominent nucleoli. Blue arrowheads represent differentiating myeloid cells with increased chromatin condensation, prominent cytoplasm, and the absence of nucleoli. **f** Clonogenic potential of bone marrow cells obtained from sAML patients: AML1 (*GLI1* high) and AML2 (*GLI1* low) treated with PF004449931 and GANT61. Presented values are relative to CD34⁺ cells obtained from age-matched healthy donors. Mean and standard deviation calculated from experimental replicates ($n = 3$)

Fig. 7. Schematic of GLI activation in human leukemia. **a** Post-translational modifications of GLI to activator forms mediated by SMO-dependent Hh signaling and SMO-independent mechanisms. Green arrows depict activation and red lines depict inhibition of GLI activation. Stars mark genes with loss-of-function mutations and/or haploinsufficiency described in human MDS or AML. **b** Direct translational activation of *GLI1*