**ORIGINAL ARTICLE**

**CD133⁺ brain tumor-initiating cells are dependent on STAT3 signaling to drive medulloblastoma recurrence**

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Medulloblastoma (MB), the most common malignant paediatric brain tumor, is currently treated using a combination of surgery, craniospinal radiotherapy and chemotherapy. Owing to MB stem cells (MBSCs), a subset of MB patients remains untreatable despite standard therapy. CD133 is used to identify MBSCs although its functional role in tumorigenesis has yet to be determined. In this work, we showed enrichment of CD133 in Group 3 MB is associated with increased rate of metastasis and poor clinical outcome. The signal transducers and activators of transcription-3 (STAT3) pathway are selectively activated in CD133⁺ MBSCs and promote tumorigenesis through regulation of c-MYC, a key genetic driver of Group 3 MB. We screened compound libraries for STAT3 inhibitors and treatment with the selected STAT3 inhibitors resulted in tumor size reduction in vivo. We propose that inhibition of STAT3 signaling in MBSCs may represent a potential therapeutic strategy to treat patients with recurrent MB.

**INTRODUCTION**

Brain tumors represent the leading cause of childhood cancer mortality, of which medulloblastoma (MB) is the most common malignant pediatric brain tumor. MB most often arises in the cerebellum of infants and young children, and was originally described as a primitive neuroectodermal tumor.1,2 Although tremendous improvements in therapy regimens using a combination of surgical resection, craniospinal irradiation and chemotherapy with vincristine, cisplatin and cyclophosphamide have yielded 5-year overall survival rates of 70–80%, survivors are left with substantial neurocognitive impairments and treatment-related morbidity.3–6

MB has been classified into four principal subgroups using integrative genomic platforms.1–2 Groups 1 and 2, referred to as WNT and sonic hedgehog (SHH), derive from dysregulation of these key developmental signaling pathways and Groups 3 and 4 are associated with the strong upregulation of c-MYC and N-MYC, respectively.2,5,6 This subgroup stratification has generated four genetically distinct tumor identities, each with a characteristic clinical presentation and prognosis.7 Group 3 tumors have shown the worst prognosis of all, emphasizing the need for the development of specialized therapeutics in this subgroup.8 Recent reports suggest the presence of somatic copy-number aberrations and single-nucleotide variants that may present subgroup-specific therapeutic targets2,9 but to date, druggable targets for Group 3 MB have not been identified.

As in many other solid tumors10 and hematopoietic cancers,12 MB is characterized by the presence of cancer stem cells (CSCs) or MB stem cells (MBSCs) that possess the ability to sustain tumor growth. These cells were first isolated and characterized in 2004.13 CD133 (Prom1), a pentaspan membrane glycoprotein, is a surface marker for MBSCs, although its functional role and signaling mechanisms are not well understood.13,14 Although some studies debate the role of CD133 in initiating tumors,15 CD133-positive cells continue to retain a higher self-renewal capacity and multipotency.16

The signal transducers and activators of transcription (STAT) family of transcription factors are present in the cytoplasm as inactive monomeric/dimeric forms and upon phosphorylation at key tyrosine residues (Tyr705), STATs translocate to the nucleus. This movement from cytoplasm to nucleus is due to reciprocal SH2 domain–pTyr binding interactions between two STAT molecules, leading to the formation of activated homodimers.17 Upon nuclear translocation, these activated homodimers promote expression of genes that regulate cell growth, cell survival and cell cycle regulation. In cancer cells, continual activation of STATs, more specifically STAT3, leads to modulation of several cell cycle regulators to promote a state of tumorigenesis.

Numerous cancers such as leukemia, lymphoma18 breast,19 ovarian19 and prostate20 cancer display aberrant activation of STAT3 signaling. In brain tumors, especially glioblastoma, recent signaling network profiling identified STAT3 as one of the key transcription factors modulating poor outcome in the mesenchymal subtype.21 In patient-derived glioblastoma brain tumor-initiating cells, STAT3 signaling is prognostically significant and can be targeted in orthotopic mouse models.22

Here, we describe a method for treatment of Group 3 MBSCs by targeting STAT3 transcription factor signaling. We sorted human

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Group 3 MBSCs based on CD133 expression and showed upregulation of p-STAT3 and c-MYC in these cells compared with CD133-negative subpopulations. Thereafter using structure-based drug design, we developed novel STAT3 inhibitors that have the potential to cross the blood-brain-barrier, to treat MB in vivo. Although CD133 has been used primarily as a marker to identify CSCs in multiple human cancers including brain tumors, its functional role remains obscure and cannot be currently targeted using small molecules or inhibitors. Similarly, c-MYC is difficult to target due to our poor understanding of binding ligands and inability to specify context-dependent MYC-associated transcriptional gene expression across different cancers. Our study presents a preclinical model applying STAT3 inhibitors to selectively target CD133+ MBSCs in Group 3 MB.

RESULTS
CD133 expression is upregulated in Group 3 MB cells CD133 is a marker used to enrich for human MBSCs, but its role and expression within subgroups remains unclear. To evaluate CD133 protein expression in MBSCs, we propagated in neural stem cell (NSC) media the MB Group 3 lines Med8A, D425, D458, CHLA-01R-MED, and measured CD133 expression by flow cytometry (Figure 1a). It is noteworthy that D425 was derived from a primary cerebellar MB in a 6-year-old boy, whereas D458 was established from cerebrospinal metastasis after treatment failure from the same patient; thus D425 and D458 represent a matched primary and recurrent pair. Interestingly, CD133 expression was significantly higher in recurrent MB lines (D458, CHLA-01R-MED) compared with primary (D425) lines. Phase-contrast pictures of MB cell lines are shown in Figure 1b.

When we probed multiple published MB transcriptional databases (Toronto, Boston, Amsterdam and Memphis) for CD133 we found its expression was strongly associated with Group 3 MB (Figure 1c). Data mined from the Boston database showed lower survival in CD133high tumors (P = 0.01, hazard ratio = 2.4) compared with CD133low tumors (Figure 1d). To provide a functional context for the clinical utility of CD133 expression in Group 3 MB, we compared the proliferative potential between sorted CD133-positive and CD133-negative populations. When compared with CD133-negative cells, CD133-positive cells had significantly higher proliferation capacity in both primary and matched recurrent cells lines (D425 and D458, respectively, Figures 1e and f).

To further validate the functional role of CD133 in tumor formation, we xenografted mice intracranially with sorted CD133-positive or CD133-negative Group 3 MB cells. We found that CD133-positive cells formed very large tumors, whereas tumors obtained by xenografting CD133-negative cells were much smaller (Figure 1g). Our data suggests that CD133 expression characterizes and may contribute to poor outcome in Group 3 MB through perpetuation of stem cell populations that drive tumorigenesis.

Activated STAT3 is enriched in CD133-high Group 3 MB Activated STAT3 signaling has been described in other CSC populations, including CD133-positive human colon cancer cell lines and CD133-high hepatocellular carcinoma cell lines. As high CD133 expression is a poor prognostic marker in many cancers, we hypothesized that hyperactive signaling pathways in CD133-positive cells could be ideal therapeutic targets. In order to determine transcript levels of STAT3 among MB subgroups, we probed existing MB genomic platforms for its expression levels in different subgroups, as described previously for CD133. STAT3 expression levels were enriched in poor outcome non-Shh/Wnt subgroups, namely Group 3 and Group 4 MB (Figure 2a). We then determined protein levels of CD133, STAT3 and p-STAT3 in Group 3 MB cells using Western blot, and again validated that recurrent lines, D458 and CHLA-01RMED have comparatively higher expression of CD133 than D425 line (Figure 2b). Although STAT3 expression is not significantly altered, activated STAT3 (p-STAT3) correlates with CD133 expression, and is especially elevated in recurrent Group 3 MB (Figure 2b). To further probe the relationship between CD133 and activated STAT3 signaling, we ectopically overexpressed CD133 in the CD133low Med8A Group 3 line. We validated overexpression of CD133 by RTPCR (Figure 2c) and Flow analysis (Figure 2d). Upon overexpression of CD133, Med8A cells showed a significant increase in p-STAT3 (Figure 2e). Med8A cells over-expressing CD133 also showed increased proliferation capacity (Figure 2f). Our data demonstrates that STAT3 signaling is activated in CD133+ Group 3 recurrent MB cells, and CD133 overexpression activates STAT3 to promote cell proliferation and growth of Group 3 MB.

p-STAT3 contributes to tumorigenesis in Group 3 MB To investigate the role of STAT3 signaling in MB pathogenesis, we undertook lentiviral short-hairpin RNA-mediated knockdown of STAT3 in Group 3 patient-matched MB cell lines D425 (primary) and D458 (recurrent). Out of two vectors, shSTAT3-1 showed efficient knockdown in both cell lines D425 (Figure 3a) and D458 (Figure 3b) with prominent reduction in activated p-STAT3 levels. This knockdown correlated with reduction in proliferation capacity in both primary and recurrent MB (Figures 3c and d). To further elucidate the role of STAT3 in tumor initiation/maintenance, we performed intracranial injections of STAT3-knockdown MB cells into NOD-SCID mice and found a significant reduction in tumor size when compared with control (shControl-transduced) tumors (Figures 3e–g). Moreover, mice xenografted with shSTAT3-transduced cells treated survived longer than control mice (Figures 3h and i). These data demonstrate the prominent role of STAT3 in regulating proliferation and tumorigenesis of Group 3 MBSCs.

Interplay between CD133, STAT3 and c-MYC may drive Group 3 MB c-MYC drives a regulatory network in embryonic stem cells that is also active in many cancers and drives poor outcomes. c-MYC is further implicated in regulating Group 3 murine MBs that resemble their human counterpart. CD133 and c-MYC have clearly been associated with relapse and poor prognosis in MB patients. To explore how CD133, c-MYC and STAT3 may collectively regulate MBSCs, we first probed MB transcriptome data to validate that c-MYC is highly enriched in Group 3 MB tumors (Supplementary Figure 1). Although Group 3 MB lines used (D425, D458, Med8A and CHLA-01R-MED) showed varied mRNA expression levels of c-MYC (Figure 4a), protein levels of c-MYC were higher in Group 3 recurrent lines D458 and CHLA-01R-MED (Figure 4b).

c-MYC is a known downstream target of STAT3 signaling in ovarian cancer and in glioblastoma stem cells, where STAT3 inhibitors have also reduced expression of stem cell-associated genes such as CD133. We show that p-STAT3 is upregulated in sorted CD133-positive populations from Group 3 MB lines, which also show enhanced c-MYC levels compared with CD133-negative populations (Figure 4c). Moreover, as a proof of principle we looked at the levels of STAT3 and c-MYC after knockdown of STAT3 in both D425 and D458 cell lines and found significant downregulation of c-MYC after STAT3 knockdown (Supplementary Figure 2). Expression levels of c-MYC are significantly higher in sorted CD133-positive cells compared with CD133-negative cells in D458 MB (Figure 4d). Given the high levels of c-MYC in Group 3 MB, we performed proliferation assays using a cell permeable
c-MYC inhibitor, 10058-F4, 35 to investigate whether targeting c-MYC could reduce tumor cell growth. Our results (Figure 4e) showed that Group 3 MB cell proliferation is attenuated by this c-MYC inhibitor, but only with an 50% inhibitory concentration values (IC$_{50}$; concentration at which cell proliferation is inhibited by 50%) in the high micromolar range (D425: 189.148 μM and D458: 133.277 μM). However, pharmacodynamic studies and screening of other c-Myc inhibitors are necessary to draw conclusions about the utility of c-Myc inhibitors as therapeutics for Group 3 MB. Nevertheless, c-MYC is a highly pleiotropic transcription factor that controls expression of hundreds of genes and may prove challenging to target. This prompted us to seek

Figure 1. CD133 characterizes group 3 MB and CD133$^+$ cells show increased tumorigenicity and proliferation as compared with CD133$^-$ populations: MB cell lines are grown in their respective media conditions as described in the ‘Materials and methods’ section. (a) Representative flow cytometry plots exhibiting percentage of CD133 protein levels in Med8A (4.75%), D425 (39.13%), D458 (68.62%) and CHLA-01R-MED (98.27%). (b) Phase-contrast pictures of the above cell lines showing difference in their morphology and growth patterns. (c) CD133 expression identifies Group 3 MB (indicated as Group C) that was probed using affymetrix exon array data and expression levels represented by log2–transformed intensity signal. Four independent databases were probed to measure CD133 expression levels. (d) The CD133 signature was originally identified and described by Venupogal et al. (55) and CD133 signature genes are included as Supplementary Table 2. CD133-high signature reflects a reduced overall survival of MB patients compared with CD133-low tumors (hazard ratio = 2.4, $P = 0.01$). (e and f) Group 3 MB cells D425 and D458 were flow cytometrically sorted into CD133$^+$ and CD133$^-$ populations and Presto Blue Assay assessed their proliferative potential. CD133$^+$ cells are more proliferative when compared with CD133$^-$ cells. Bar represents mean fluorescence intensity (arbitrary units (a.u.)) of three technical replicates, mean ± s.d., two-tailed t-test, $P < 0.0007$ (g) 500 000 cells of each of CD133$^+$ and CD133$^-$ populations were xenografted intracranially in mice brains. CD133$^+$ cells generated larger and infiltrative tumors than CD133$^-$ cells (scale bar = 4 mm).
Alternate means to target the CD133/STAT3/c-MYC signaling axis in Group 3 MB.

Ex vivo treatment with STAT3 inhibitors reduces MB tumor burden in vivo

We tested various STAT3 inhibitors from our library (Supplementary Figures 3 and 4) and found PG-S3-002 to have selective activity towards CD133-positive MBSCs with IC50 in the low micromolar range, in both primary and recurrent MB cells (Figures 5a and b). A reduction in p-STAT3 protein levels was observed following treatment with PG-S3-002 at IC50 (Figures 5c and d). Intracranial xenograft injections of 1.0 × 10^5 viable STAT3 inhibitor-treated cells resulted in significantly smaller tumors (Figures 5e–g) compared with control cells. When we treated normal human NSCs with PG-S3-002, we found an IC50 value also in the low micromolar range (Supplementary Figure 5). This data shows that STAT3 inhibitor treatment at doses that would be tolerable in patients can decrease tumor burden of Group 3 MB xenografts, but with equal toxicity towards NSCs; implicating neurotoxicity as a potential side effect.

Systemic treatment with STAT3 inhibitors PG-S3-009 and PG-S3-010 reduces tumor formation in vivo

As STAT3 signaling is active in MBSCs, we then investigated the role of STAT3 compounds in reducing tumor formation in our human-mouse xenograft model of Group 3 MB. We chose to...
xenograft recurrent MB lines that are refractory to conventional chemoradiotherapy, and first tested whether STAT3 inhibitors would target these cells in vitro. Both PG-S3-009 and PG-S3-010 compounds showed IC_{50} values in the low micromolar range and selectivity for recurrent MBSCs (Figure 6a, Supplementary Figures 7a and b). These two inhibitors are highly selective for recurrent (D458) MB, when compared with matched primary MB (Supplementary Figure 6). Our 2-week treatment protocol comprised PG-S3-009 and PG-S3-010 administration by intraperitoneal injections (20 mg/kg dose, twice a week), following tumor engraftment. Mice were culled and brains harvested at treatment completion for both control (DMSO) and inhibitor-treated mice (Figure 6b). Mice treated with PG-S3-009 or PG-S3-010 maintained a reduction in tumor burden (Figures 6c and d), with PG-S3-009 showing the greatest efficacy and potency. These data demonstrates selected STAT3 inhibitors to be efficacious in targeting recurrent, treatment-refractory Group 3 MBSCs.

PG-S3-009 treatment significantly decreases tumor size of recurrent Group 3 MB in vivo, but does not increase survival. D458 cells were treated with PG-S3-009 in varying doses in vitro and found that cellular levels of STAT3, p-STAT3 and c-MYC gradually decreases with increasing concentration of PG-S3-009 (Figure 7a). These data implicate the direct targeting of STAT3 by PG-S3-009 and subsequent downregulation of STAT3 leading to a reduction in tumor burden. Figure 3. STAT3 knockdown using lentiviral approach reveals its role in proliferation and tumor formation. shSTAT3 lentiviral vector-mediated transduction shows reduction in both STAT3 and phosphorylated p-STAT3 protein levels as assessed by immunoblotting with shSTAT3-1 vector compared with shControl vector in both (a) D425 and (b) D458 cell lines. GAPDH was used as a loading control. Proliferative capacity as determined by Presto Blue assay shows decreased proliferation in shSTAT3-1 (c and d) when compared with shControl-transduced cells. Bar represents mean fluorescence intensity (arbitrary units (a.u.)) of three technical replicates, mean ± s.d., two-tailed t-test. *P ≤ 0.01. (e) Xenografts were generated using 100 000 cells of shControl or shSTAT3-1 vectors in D425 (upper panel) and D458 (lower panel) into the frontal lobes of NOD-SCID mice (n = 6 in each group); shSTAT3-1-treated cells displayed significantly small and less invasive tumors in both lines (arrows indicates tumor area). Mice were killed in pairs of shControl and shSTAT3-1 when one member of the pair reached end point and H&E sections of the brains are shown. (f and g) Total area of tumor was calculated using ImageJ64 software (NIH, Bethesda, MA, USA). Bar represents tumor area (mm^2) of n = 6 mice in each group, mean ± s.d. and two-tailed t-test. *P ≤ 0.005. (h and i) shSTAT3 cells xenografted mice maintain a significant survival over shControl-treated mice (n = 3 in each group). The median survival in case of D425 shSTAT3 mice increased to 38 days compared with 20 days in shControl mice (P = 0.0072) and median survival in D458 shSTAT3 mice augmented to 33 days from 24 days in shControl mice (P = 0.04).
the reduction of the downstream target c-MYC in MBSCs (Supplementary Figure 7). In order to evaluate the effect of the drug in vivo, NODSCID mice were intracranially xenografted with D458 cells and treated intraperitoneal with escalating doses of PG-S3-009 (DMSO, 5, 10 or 20 mg/kg) (Figure 7b). Cohorts treated at 20 mg/kg with PG-S3-009 showed significant reduction in tumor burden compared with DMSO or lower-dose treated mice (Figures 7c and d). However, no survival benefit was observed in treated mice compared with DMSO controls (Figure 7e). Treated mice became anorexic and withdrew from feeding and activity, suggestive of treatment-induced neurotoxicity, although no toxicity was seen in liver, kidneys and lungs (data not shown). These data implicate the direct targeting of STAT3 by PG-S3-009 and subsequent downregulation of STAT3 leading to the reduction of the downstream target c-MYC in MBSCs (Supplementary Figure 8).

**DISCUSSION**

The recent molecular classification of MB into four distinct subgroups implies that different subgroup-specific therapeutic strategies need to be devised for individualized patient therapy. This therapeutic roadmap may only be realized by elucidating the biological and genetic drivers of tumorigenesis in each subgroup, in order to discover tractable and druggable therapeutic targets. In this study, we link the high expression of CD133 with Group 3 MB and further correlate its expression with c-MYC, via STAT3. High c-Myc expression often reflects a classic Group 3 MB with metastasis at diagnosis, large cell histology and overall poor prognosis. Novel mouse models have been developed using genetics to recapitulate human Group 3 MB that have the potential to be used for preclinical testing. Our observation of enrichment of CD133 in Group 3 MB is in accordance with the expression of this cell-surface marker in multiple CSC populations, which are thought to evade current chemoradiotherapy only to seed recurrent disease and drive patient relapse and poor outcome. In light of the fact that Group 3 MB is the most aggressive among all subgroups, our understanding of the functional role of CD133 in Group 3 MB can provide a strategic platform to identify novel signaling inhibitors in CD133-positive cells for the effective management of these tumors. c-MYC expression can be regulated by numerous signaling pathways including the JAK/STAT, rendering STAT3 inhibitors attractive therapeutic options. As the Wnt subtype of...
MB (Group 1) also demonstrates elevated levels of c-Myc, STAT3 inhibitors could be considered as a therapeutic option for patients with this positive prognostic MB subtype. Activated STAT3 signaling has been shown to be essential for cell survival and tumorsphere-forming capacity in human colon cancer cell lines sorted for both ALDH (aldehyde dehydrogenase) and CD133 (ALDH+/CD133+).29 Previous studies have shown that downregulation and inhibition of STAT3 signaling suppresses proliferation and induces apoptosis in human glioma cells37-39 as well as various cancers.40,41 Xiao et al. have already shown that disruption of STAT3 signaling leads to inhibition of cell growth and apoptosis of medulloblastoma cells.42 Using bioinformatics and microarray platforms, another study revealed STAT3/p-STAT3 signaling upregulated in CD133-positive MB cells compared with CD133-negative cells.33 STAT3 has a key role in maintaining both tumor cells and its microenvironment, although little is known about both upstream and downstream regulators of JAK–STAT3 signaling. Despite advances in studying the role of STAT3 as an ideal target for CSC-driven tumors,30,33,44 challenges have arisen in developing these drugs for clinical use. In general STAT3 inhibitors targeting the SH2 domain have suffered from limited clinical utility due to inclusion of polar groups designed to mimic SH2 domain binding Tyr705 residue that has resulted in metabolic instability and poor cell permeability.42,45 Non-phosphorylated small molecule inhibitors such as STA-201,46 BP-1-102,47 BP-5-87,48 SH-4-5444 and LY542 have, to a certain extent circumvented this problem.

To enhance in vivo efficacy, SH-4-54 was optimized specifically to yield inhibitors with improved bioavailability. PG-S3-009 and PG-S3-010 were two of the most potent anti-cancer compounds to emerge from this program. These inhibitors have shown great promise in treating other treatment-refractory and aggressive cancers such as brain metastases.49

Figure 5. STAT3 inhibitors targeting MBSCs. (a) D425 and (b) D458 cells were flow cytometry sorted into CD133+/− populations and treated with PG-S3-002 STAT3 inhibitor. IC50 curves were generated using inhibition of cell proliferation using PrestoBlue assay with four technical replicates. For D425, IC50 values are: CD133+: 2917 nM and CD133−: 13 794 nM. IC50 values for D458 are CD133+: 7807 nM and CD133−: 11 424 nM. (c and d) Western blot showing protein levels of STAT3 and phosphorylated STAT3 (p-STAT3) in DMSO-treated and PG-S3-002-treated (IC80) D425 and D458, relative to GAPDH control. (e) D425 (upper panel) and D458 (lower panel) cells were treated ex vivo with their respective IC80 of PG-S3-002 for 4 days and 100 000 viable cells were injected into frontal lobes of NOD-SCID mice (n = 3 in each group). Cells treated with DMSO were used as control. Mice were killed as pairs as they fell sick. H&E sections of the brains are shown. Average tumor size reduction is 85% in (f) D425 and 70% in (g) D458. Tumor area was calculated using ImageJ software (NIH). Bar represents tumor area (mm2) of n = 3 mice in each group, mean ± s.d. and two-tailed t-test. *P < 0.005.
STAT3 in MB and its translocation to the nucleus permits activation of oncogenes and cell cycle regulatory genes such as c-MYC, cyclin-D1 and Cox-2, leading to cancer progression. In another study, DNA microarray analysis in CD133-positive MB cells showed an upregulation of c-MYC. Although both CD133 and c-MYC upregulation have clear implications in Group 3 MB pathogenesis, both are difficult to target owing to insufficient understanding of molecular mechanisms and their role in normal development and cancer. Currently there are no available drugs targeting CD133, and the c-MYC targeting compound 10058-F4 has a very high IC₅₀ value in MBSCs that may preclude clinical use. There are currently no clinically approved therapies for directly targeting c-MYC, as MYC is a pleiotropic transcription factor that controls expression of hundreds of genes. Moreover, 10058-F4 inhibits c-MYC/Max dimerization, implying multiple off-target cellular activities through modulation of various transcription factors in other tissues and organs. Within the CD133/STAT3/c-MYC signaling axis, the only attractive target for drug development is STAT3. This concept prompted us to search for novel STAT3 inhibitors to treat Group 3 MB with the potential to translate into clinical settings.

In this study, we demonstrate the enrichment of CD133 in Group 3 MBSCs and discover potential signaling mechanisms involving activated STAT3 (p-STAT3) and c-MYC in CD133-positive cells. Treatment with STAT3 dimerization inhibitor PG-S3-002 ex vivo significantly reduces tumor burden in mice. Our preclinical trial of another STAT3 inhibitor, PG-S3-009, showed reduced tumor burden in mice, but no significant survival benefit due to presumed neurotoxicity of the compound. However, if neurotoxicity could be mitigated with chemical modifications to the compound, these inhibitors show great promise in effectively treating recurrent MB (D458), an important result as Group 3 recurrence is currently treated with palliation alone. Our study describes the first preclinical evidence that novel STAT3 inhibitors cross the blood-brain-barrier and effectively treat recurrent Group 3 MB.

**MATERIALS AND METHODS**

CD133/STAT3/c-MYC gene profiling in subgroups of MB

MB microarray data for 103 MBs were downloaded from GEO (GSE21140). These data consisted of processed Affymetrix CEL files (Affymetrix Human Exon 1.0 ST Array (transcript (gene) version)) that had undergone gene-level analysis (CORE content), quantile normalization (sketch) and summarization using PLIER with PM-GCBG background correction for 103 MBs. Clinical annotations for each MB tumor, including...
subgroup (Wnt, Shh, Group 3, Group 4) were also downloaded from this location. The expression of each gene was plotted as the normalized fluorescence intensity of the corresponding affymetrix probes. Similarly, raw Affymetrix datasets for 62 primary human MBs, 40 primary human MBs, 15 Daoy MB cell line samples and 46 primary human MBs were respectively downloaded and processed from GEO (GSE10327, GSE12992 and GSE7578) or http://www.stjuderesearch.org/site/data/medulloblastoma and normalized using RMA. When available, clinical annotations for each tumor were also downloaded from these locations.

**Figure 7.** Dose-dependence study of PG-S3-009. (a) Western blot showing c-MYC, STAT3 and p-STAT3 levels compared to GAPDH after treatment of D458 with serially increasing concentration of PG-S3-009. (b) Timeline of the in vivo protocol starting with 48 mice intracranial injections (frontal lobe) of D458 at day 0, day of tumor cell implantation. After a week, (n = 12 mice in each set) were given 5, 10 or 20 mg/kg body weight of either DMSO or PG-S3-009 for 2 weeks, twice a week. At the end of third week, mice were killed (n = 6 mice in each set) for H&E staining and remaining 6 mice per set were monitored for their survival. (c) Representative H&E sections of the brains are shown after each treatment regimen (n = 6). (d) There is a significant reduction in tumor area (calculated using ImageJ software). Bar represents tumor area (mm²) of n = 6 mice in each group, mean ± s.d. and two-tailed t-test. *P = 0.03. (e) Percentage survival for mice treated with 5 mg (upper panel), 10 mg (middle panel) and 20 mg/kg body weight of PG-S3-009 (lower panel).
Targeting STAT3 in medulloblastoma stem cells
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Wilmington, DE, USA. Using 1 μg RNA, complementary DNA was synthesized using qScript cDNA Super Mix (Quanta Biosciences, Beverly, MA, USA) and a C1000 Thermal Cycler (Bio-Rad, Mississauga, ON, Canada) with the following cycle parameters: 4 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, hold at 4 °C. qRT-PCR was performed using Performa SybrGreen (Quanta Biosciences) and an ACFX96 instrument (Bio-Rad) as described previously.20 Gene expression was quantified by using CFX Manager 3.0 (Bio Rad) software and expression levels were normalized to GAPDH/Actin expression. Primers are listed in Supplementary Table 1.

Screening of MBSCs with STAT3 inhibitors
Dr Patrick Gunning provided a library of proprietary direct-binding STAT3 inhibitors. Cell proliferation in MBSCs (grown in DMEM supplemented with 20% fetal bovine serum) was determined by plotting percentage cell viability versus log dilutions of the inhibitors, giving IC₅₀. To assess tumor inhibition in mice, compound PG-S3-002 was chosen for in vivo treatment and modification of compound PG-S3-002 resulted in synthesis of compound PG-S3-009, which has increased bioavailability and was used for in vivo treatment. See Supplementary Figure 8 for details about PG-S3-009.

In vivo intracranial injections of MB stem cells and H&E staining of xenograft tumors
The McMaster University Animal Research Ethics Board (AREB) approved all experimental procedures and intracranial injections were performed as described previously.13 Briefly, live cells (numbers mentioned in figure legends) re-suspended in 10 μl of phosphate-buffered saline were injected into the frontal lobe of NOD-SCID mice (both males and females of 8-10 weeks of age). Mice injected with CD133 sorted cells (positive or negative) were killed when the CD133-positive group reached end point. Treated mice (with either shSTAT3 or ex vivo STAT3 inhibitor or in vivo STAT3 inhibitor) were killed when control Group/unreated mice reached end point as shown by signs of illness, for tumor size quantification. Mice were randomized into control or treatment groups. Group allocation was not blinded. For survival studies, treated or control mice were killed when they reached end point. The treatment did not induce adverse weight loss that would be contraindicated by our animal utilization protocol (AUP) endpoints policy.

Mouse brains were harvested, formalin-fixed and thereafter brain tissue sections were paraffin-embedded and also stained for hematoxylin and eosin (Cell Signaling). Aperio Slide Scanner (Leica Biosystems, Concord, ON, Canada) was used to scan images and images were analyzed using ImageScope v1.1.2.760 software (Aperio, Concord, ON, Canada). Survival curves were plotted using GraphPad Prism 5 (SanDiego, CA, USA).

Statistical analysis
Technical/experimental replicates from at least three samples were compiled for each experiment, unless otherwise stated in figure legends. Respective data represent mean ± s.d. with n values listed in figure legends. Student’s t-test analyses and two-way analyses of variance were performed using GraphPad Prism 5. P < 0.05 was considered significant. For in silico analyses, statistical tests were compiled and completed in R.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
NG and SKS: conception and design, development of methodology, acquisition of data, analysis and interpretation of data, writing, review and/or revision of the manuscript, and study supervision. DB: acquisition of data, writing, review and/or revision of the manuscript. CV: conception and design, analysis and interpretation of data, writing, review and/or revision of the manuscript. SM and NM: acquisition of data, analysis and interpretation of data. DAR, CCA, PSL, RFG8 AMA and DE: technical or material support. TV: acquisition of data. BM: writing, review and/or revision of the manuscript. PH: acquisition of data, analysis and interpretation of data. KHD, JMK: analysis and interpretation of data. OAA, JAH: administrative, technical or material support. PTG: conception and design, development of methodology, analysis and interpretation of data, writing, review and/or revision of the manuscript.

REFERENCES
1. Northcott PA, Jones DT, Kool M, Robinson GW, Gilbertson RJ, Cho YJ et al. Medulloblastomas: the end of the beginning. Nat Rev Cancer 2012; 12: 818–834.
2. Taylor MD, Northcott PA, Korshunov A, Remke M, Cho YJ, Clifford SC et al. Molecular subgroups of medulloblastoma: the current consensus. Acta Neuropatholog 2012; 123: 465–472.
3. Gajjar A, Chintagumpala M, Ashley D, Kellei S, Kun LE, Merchant TE et al. Risk-adapted craniospinal radiotherapy followed by high-dose chemotherapy and stem-cell rescue in children with newly diagnosed medulloblastoma (St Jude Medulloblastoma-96): long-term results from a prospective, multicentre trial. Lancet Oncol 2006; 7: 813–820.
4. Mabbott DJ, Spiegler BJ, Greenberg ML, Rutka JT, Hyder DJ, Bouffet E. Serial evaluation of academic and behavioral outcome after treatment with cranial radiation in childhood. J Clin Oncol 2005; 23: 2256–2263.
5. Northcott PA, Korshunov A, Witt H, Hielscher T, Eberhart CG, Mack S et al. Medulloblastoma comprises four distinct molecular variants. J Clin Oncol 2011; 29: 1408–1414.
6. Pei Y, Moore CE, Wang J, Tewari AK, Hormigo A, Kushner J, Milde T et al. Sonic hedgehog regulates Bmi1 in human medulloblastoma brain tumor-initiating cells. J Neuro-oncology 2010; 98: 355–367.
7. Robinson G, Parker M, Kranenburg TA, Lu C, Chen X, Ding L et al. Novel mutations target distinct subgroups of medulloblastoma. Nature 2012; 488: 43–48.
8. Kool M, Korshunov A, Remke M, Jones DT, Schlanstein M, Northcott PA et al. Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH Group 3, and Group 4 medulloblastomas. Acta Neuropatholog 2012; 123: 473–484.
9. Northcott PA, Shih DJ, Peacock J, Garzia L, Morrissy AS, Zichner T et al. Subgroup-specific structural variation across 1,000 medulloblastoma genomes. Nature 2012; 488: 49–56.
10. Parsons DW, Li M, Zhang X, Jones S, Leary RJ, Lin JC et al. The genetic landscape of the childhood cancer medulloblastoma. Science 2011; 331: 435–439.
11. Grosse-Gehling P, Fargeas CA, Dittfeld C, Garbe Y, Alison MR, Corbeil D et al. Expression of CD133 on leukemia- and lymphoma-derived cancer stem cells. Oncogene (2017) 606–617.
12. Cox CV, Diamanti P, Evely RS, Kearns PR, Blair A. Expression of CD133 on leukemia- and lymphoma-derived cancer stem cells. Oncogene (2017) 606–617.
13. Burke WM, Jin X, Lin HJ, Huang M, Liu R, Reynolds RK et al. Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells. Oncogene 2001; 20: 7925–7934.
14. Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonaccelli G et al. Stat3 promotes metastatic progression of prostate cancer. Am J Pathol 2008; 172: 1717–1728.
15. Carro MS, Lim WK, Alvarez MJ, Bollro RJ, Zhao X, Snyder EY et al. The transcriptional network for mesenchymal transformation of brain tumours. Nature 2010; 463: 318–325.
16. Stechishin OD, Luchman HA, Ruan Y, Blough MD, Nguyen SA, Kelly JJ et al. On-target Jak2/Stat3 inhibition slows disease progression in orthotopic xenografts of human glioblastoma brain tumor stem cells. Neuro-oncology 2015; 17: 198–207.
17. Bandopadhayay P, Bergthold G, Nguyen B, Schubert S, Gholamin S, Tang Y et al. BET bromodomain inhibition of MYC-amplified medulloblastoma. Clin Cancer Res 2014; 20: 912–925.
18. Wang X, Venugopal C, Manoranjan B, McFarlane N, O’Farrell E, Nolte S et al. Sonic hedgehog regulates Bmi1 in human medulloblastoma brain tumor-initiating cells. Oncogene 2012; 31: 187–199.
19. Friedman HS, Colvin OM, Kaufmann SH, Ludeman SM, Bullock N, Bigner DD et al. Cyclophosphamide resistance in medulloblastoma. Cancer Res 1992; 52: 5373–5378.
20. Cho YJ, Tishkarnia A, Tamayo P, Santagata S, Ligon A, Greulich H et al. Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. J Clin Oncol 2011; 29: 1424–1430.
21. Kool M, Koster J, Bunt J, Hasselt NE, Lakeman A, van Sluis P et al. Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. PLoS One 2008; 3: e3008.
22. Thompson MC, Fuller C, Hogg TL, Dalton J, Finkelstein D, Lau CC et al. Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. J Clin Oncol 2006; 24: 1924–1931.
23. Lin L, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J. Stat3 signaling pathway is necessary for cell survival and tumor sphere forming capacity in ALDH(+)/CD133(+) stem-cell-like human colon cancer cells. Biochem Biophys Res Commun 2011; 416: 246–251.
24. Won C, Kim BH, Hee YIE, Choi KJ, Kim EK, Jeong JM et al. STAT3-mediated CD133 upregulation contributes to promotion of hepatocellular carcinoma. Hepatology 2015; 62: 1160–1173.
25. Zeppenmiek F, Ahmedi R, Campos B, Dictus C, Hmelke BM, Becker N et al. Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 2008; 14: 123–129.
26. Kim J, Woo AJ, Chu J, Snow JW, Fujwara Y, Kim CG et al. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell 2010; 143: 313–324.
27. Ashizawa T, Miyata H, Iizuka A, Komiyama M, Oshita C, Kume A et al. Effect of the STAT3 inhibitor STX-0119 on the proliferation of cancer stem-like cells derived from recurrent glioblastoma. Int J Oncol 2013; 43: 219–227.
28. Sai K, Wang S, Balasubramanijay V, Conrad C, Lang FF, Aldale K et al. Induction of cell-cycle arrest and apoptosis in glioblastoma stem-like cells by WP1193, a novel small molecule inhibitor of the JAK2/STAT3 pathway. J Neuro-oncology 2012; 107: 487–501.
29. Huang MJ, Cheng YC, Liu CR, Lin S, Liu HE. A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia. Exp Hematol 2006; 34: 1480–1489.
30. Kawauchi D, Robinson G, Uziel T, Gibson P, Rej H, Gao C et al. A mouse model of the most aggressive subgroup of human medulloblastoma. Cancer Cell 2012; 21: 168–180.
31. Chen F, Yang Y, Luo Z, Deng S, Song Y, Yu K et al. Down-regulation of Stat3 decreases invasion activity and induces apoptosis of human glioma cells via modulation of molecular neuroscience. MN; 2010; 40: 353–359.
32. Li GH, Wei H, Lv SQ, Ji H, Wang DL. Knockdown of Stat3 expression by RNAi suppresses growth and induces apoptosis and differentiation in glioblastoma stem cells. International journal of oncology 2010; 37: 103–110.
33. Rahman SO, Harbor PC, Chernova O, Barnett GH, Vogelbaum MA, Haque SJ. Inhibition of constitutively active Stat3 suppresses proliferation and induces apoptosis in glioblastoma multiforme cells. Oncogene 2002; 21: 8404–8413.
34. Aoki Y, Feldman GM, Tosato G. Inhibition of Stat3 signaling induces apoptosis and decreases survival expression in primary effusion lymphoma. Blood 2003; 101: 1535–1542.
35. Ding BB, Yu JJ, Yu RJ, Mendez LM, Shakhnovich R, Zhang Y et al. Constitutively activated STAT3 promotes cell proliferation and survival in the activated B-cell subtype of diffuse large B-cell lymphomas. Blood 2008; 111: 1515–1523.
36. Xiao H, Bid HK, Jou D, Wu X, Yu W, Li C et al. A novel small molecular STAT3 inhibitor, LYS, inhibits cell viability, cell migration, and angiogenesis in medulloblastoma cells. The Journal of biological chemistry 2015; 290: 3418–3429.
37. Chang CJ, Chang CH, Song WS, Tsai SK, Wong LC, Chang CH et al. Inhibition of phosphorylated STAT3 by curcubitacin I enhances chemoradiosensitivity in medulloblastoma-derived cancer stem cells. Child Nerv Syst 2012; 28: 363–373.
44 Haftchenary S, Luchman HA, Jouk AO, Veloso AJ, Page BD, Cheng XR et al. Potent targeting of the STAT3 protein in brain cancer stem cells: a promising route for treating glioblastoma. ACS Med Chem Lett 2013; 4: 1102–1107.

45 Turkson J, Ryan D, Kim JS, Zhang Y, Chen Z, Haura E et al. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J Biol Chem 2001; 276: 45443–45455.

46 Song H, Wang R, Wang S, Lin J. A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. Proceedings Natl Acad Sci USA 2005; 102: 4700–4705.

47 Zhang X, Yue P, Page BD, Li T, Zhao W, Namanja AT et al. Orally bioavailable small-molecule inhibitor of transcription factor Stat3 regresses human breast and lung cancer xenografts. Proceedings Natl Acad Sci USA 2012; 109: 9623–9628.

48 Eiring AM, Page BD, Kraft IL, Mason CC, Vellore NA, Resetca D et al. Combined STAT3 and BCR-ABL1 inhibition induces synthetic lethality in therapy-resistant chronic myeloid leukemia. Leukemia 2015; 29: 586–597.

49 Singh M, Garg N, Venugopal C, Hallett R, Tokar T, McFarlane N et al. STAT3 pathway regulates lung-derived brain metastasis initiating cell capacity through miR-21 activation. Oncotarget 2015; 6: 27461–27477.

50 Schaefer LK, Ren Z, Fuller GN, Schaefer TS. Constitutive activation of Stat3alpha in brain tumors: localization to tumor endothelial cells and activation by the endothelial tyrosine kinase receptor (VEGFR-2). Oncogene 2002; 21: 2058–2065.

51 Zhao D, Pan C, Sun J, Gilbert C, Drews-Elger K, Azzam DJ et al. VEGF drives cancer-initiating stem cells through VEGFR-2/Stat3 signaling to upregulate Myc and Sox2. Oncogene 2015; 34: 3107–3119.

52 Gu C, Yokota N, Gao Y, Yamamoto J, Tokuyama T, Namba H. Gene expression of growth signaling pathways is up-regulated in CD133-positive medulloblastoma cells. Oncology Lett 2011; 2: 357–361.

53 Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003; 4: 249–264.

54 Venugopal C, McFarlane NM, Nolte S, Manoranjan B, Singh SK. Processing of primary brain tumor tissue for stem cell assays and flow sorting. J Vis Exp 2012.

55 Singh SK, Venugopal C, Hallett R, Vora P, Manoranjan B, Mahendram S et al. Pyrinium targets CD133 in human glioblastoma brain-tumor initiating cells. Clin Cancer Res 2015; 21: 5324–5337.

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