Published in final edited form as:

Biochem Biophys Res Commun. 2012 January 13; 417(2): 874–879. doi:10.1016/j.bbrc.2011.12.065.

SPARC expression induces cell cycle arrest via STAT3 signaling pathway in medulloblastoma cells

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Abstract

Dynamic cell interaction with ECM components has profound influence in cancer progression. SPARC is a component of the ECM, impairs the proliferation of different cell types and modulates tumor cell aggressive features. We previously reported that SPARC expression significantly impairs medulloblastoma tumor growth *in vivo*. In this study, we demonstrate that expression of SPARC inhibits medulloblastoma cell proliferation. MTT assay indicated a dose-dependent reduction in tumor cell proliferation in adenoviral mediated expression of SPARC full length cDNA (Ad-DsRed-SP) in D425 and UW228 cells. Flow cytometric analysis showed that Ad-DsRed-SP-infected cells accumulate in the G2/M phase of cell cycle. Further, immunoblot and immunoprecipitation analyses revealed that SPARC induced G2/M cell cycle arrest was mediated through inhibition of the Cyclin-B-regulated signaling pathway involving p21 and Cdc2 expression. Additionally, expression of SPARC decreased STAT3 phosphorylation at Tyr-705; constitutively active STAT3 expression reversed SPARC induced G2/M arrest. Ad-DsRed-SP significantly inhibited the pre-established orthotopic tumor growth and tumor volume in nude mice. Immunohistochemical analysis of tumor sections from mice treated with Ad-DsRed-SP showed decreased immunoreactivity for pSTAT3 and increased immunoreactivity for p21 compared to tumor section from mice treated with mock and Ad-DsRed. Taken together our studies further reveal that STAT3 plays a key role in SPARC induced G2/M arrest in medulloblastoma cells. These new findings provide a molecular basis for the mechanistic understanding of the effects of SPARC on medulloblastoma tumor cell proliferation.

Keywords

SPARC; STAT3; Cell cycle; medulloblastoma

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INTRODUCTION

Medulloblastoma is the most familiar brain tumor in children's, with a prevalence of 0.6 per 1×10^5 patient-years according to the Central Brain Tumor Registry of the USA [1]. Even though multimodal treatment including surgery, radiation, and chemotherapy, tumor reappearance is frequent, and the most of these patients eventually die from progressive tumor [2]. These treatments are also toxic and can lead to long-term disabilities [3]. Consequently, finding novel ways to suppress the tumor growth and low-toxicity therapies for children with medulloblastoma is a major goal of several cancer laboratories.

Secreted protein, acidic and rich in cysteine (SPARC) has been shown to be involved in multiple biological functions, including cell proliferation [4]. Even though the behaviors ascribed to SPARC are many, but the cellular and molecular mechanisms of SPARC that mediate these processes are largely unknown.

Constitutive activation of STAT proteins are members of a ubiquitously expressed family of transcription factors activated in response to growth factors and cytokines [5]. STAT3 has been shown to be an oncogene [6], and many types of human cancer express constitutively active STAT3 [7–9]. STAT3 correlates with cell proliferation in breast carcinoma [10] and non-small cell lung cancer [11], and also inhibits apoptosis [9]. Conversely, inhibition of JAK/STAT signaling suppresses cancer cell growth and induces apoptosis in various cancers [9;12]. Previously we have shown that SPARC induced neuronal like phenotype in medulloblastoma cells through its inhibitory effect on IL-6-regulated suppression of Notch-mediated STAT3 signaling pathway [13]. It was also demonstrated by others that siRNA mediated inhibition of STAT3-induced p16, p21 and p27 mediated cell cycle arrest and apoptosis in colorectal cancer cells [12]. Further, p21 known to induces G2/M phase arrest by inhibiting Cyclin-B/Cdc2 [14]. Cdc25 is required for removal of inhibitory phosphotyrosines on Cyclin-B/Cdc2 and Cyclin-A/Cdk2 kinase complexes that mediate entry into mitosis [15].

In the present study, study, we investigated the effects of SPARC expression on cellular growth, and cell cycle distribution. We demonstrate that SPARC expression suppresses proliferation of medulloblastoma cells via the stimulation of G2/M phase arrest. We also provide experimental and mechanistic evidences that abnormalities of STAT3 signaling contribute to the SPARC induced cell cycle arrest.

MATERIALS AND METHODS

Antibodies and reagents

Primary antibodies against SPARC, Cdc2, phospho-Cdc2 (T14/Y15), Cyclin-B1, Chk1, Chk2, STAT3 and phospho-STAT3 (Y705), GAPDH, HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), 3,3'-diaminobenzidine peroxidase (DAB), propidium iodide (Sigma, St.Louis, MO), MTT reagent (R&D Systems, Minneapolis, MN), and ECL system Thermo scientific, Rockford, IL) were used in this study.

Construction of SPARC-over expressing vectors

Adenovirus vector carrying full length human SPARC cDNA (Ad-DsRed-SP) and an empty vector (Ad-DsRed) were constructed using Adeno-X ViraTrak Expression System-2 (Clontech Laboratories, Mountain View, CA) and amplified as described previously [13;16]. Constitutively active STAT3 Plasmid (pSTAT3C) obtained from Addgene (Cambridge, MA).

Cell cultures and treatment

D425 and UW228 cells were kindly provided by Dr. Darell D. Bigner and Dr. Ali-Osman (Duke University Medical Center) respectively, and cells were cultured as described previously [13]. Cells were cultured either in culture flasks (D425) or in petridishes (UW228) for 24hrs and infected with mock, Ad-DsRed or Ad-DsRed-SP as described previously [13]. For constitutively active STAT3C (pSTAT3C) and vector (pEV; empty vector) transfections, FuGENE HD transfection reagent (Roche, Indianapolis, IN) was used as per manufacturer's instructions.

Reverse transcription PCR (RT-PCR)

RT-PCR was performed as described previously [16]. The PCR primers used for this study are: SPARC sense 5'-GGAAGAACTGTGGCAGAGG-3' and antisense 5'-ATTGCTGCACACCTTCTCAA-3' and GAPDH sense 5'-AGCCACATCGCTCAGACACC-3' and antisense 5'-GTACTCAGCGGCCAGCATCG-3'. The expected PCR products were resolved on 2% agarose gels and visualized using ethidium bromide staining.

Immunoblot analysis

Cells were infected with mock, 100MOI of Ad-DsRed or 50 and 100 MOI of Ad-DsRed-SP for 24hrs, and then lysed with lysis buffer containing protease inhibitors. Equal amounts of protein aliquots were resolved over SDS-PAGE gels and transferred on to PVDF membrane. Blots were blocked with nonfat dry milk for 1hr at room temperature and incubated with primary antibodies for overnight at 4°C followed by appropriate secondary antibody conjugated with HRP. The membranes were developed by using ECL reagent protocol. The blots were further probed for GAPDH, which was used as a loading control.

Cell proliferation assays

Cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described [17]. Cells (2×10^3 /well) were plated and infected with mock, Ad-DsRed, or Ad-DsRed-SP as described above. After 12,24,36,48,60,72,84 and 96hrs MTT reagent was added to each well and absorbance of the formazan from each sample was measured at a test wavelength 550nm and reference wavelength 655nm. The % of viability was calculated as $100 \times \text{mean absorbance of sample} / \text{mean absorbance of mock}$.

Cell cycle analysis by fluorescence-activated cells sorting (FACS)

The allocation of cells in the cell cycle phases was determined using FACS analysis of DNA content. The cells were transfected with pSTAT3C plasmid and then incubated for 24hrs. Then cells were treated with Ad-DsRed-SP for further 24hrs. Cells were collected and fixed in 70% ethanol and stained with propidium iodide for 30 min and analyzed for DNA content by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

In vivo studies

The animal experiments were carried out as described previously [13]. D425 (1×10^5 cells/10 μ l) cells were stereotactically implanted. After 14 days of tumor cell implantation, the animals were randomized into 3 groups (10 mice/group). Each mouse received 3 intracranial injections (10 μ l) on days 15, 17, and 19 with PBS, Ad-DsRed or Ad-DsRed-SP (5×10^7 PFU). Animals were monitored for up to 90 days, which is when we arbitrarily terminated the experiment. Mice brains were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections (5 μ) were obtained from the paraffin blocks and stained with hematoxylin

and eosin (H&E) by using standard histologic techniques. Tumor volume was assessed by methods described previously [13].

Immunohistochemical analyses

We used a previously described protocol with minor changes [13]. Briefly, the tissue sections (5 μ) were deparaffinized, rehydrated, permeabilized, and incubated overnight with primary antibodies, HRP-conjugated secondary antibodies, followed by DAB substrate solution, and then counterstained with hematoxylin and mounted. The images were processed as described previously [13].

Analysis—ImageJ software (NIH) was used to quantify the mRNA and protein band intensities. Statistical comparisons were performed using analysis of variance for analysis of significance between different values using GraphPad Prism software (San Diego, CA). Values are expressed as mean of triplicate experiments $\pm$ S.D. from at least three separate experiments, and differences were considered significant at a *p* value of less than 0.05.

RESULTS

Overexpression of SPARC in medulloblastoma cells

To study the effects of SPARC overexpression, we infected UW228 and D425 cell lines with 50 and 100MOI of adenoviral vector harboring SPARC cDNA sequence (Ad-DsRed-SP) driven by a CMV promoter. PCR analysis was carried out using the primers for SPARC and GAPDH. Clear amplification of SPARC transcript up to 2.5-4 folds was observed in Ad-DsRed-SP-infected cells when compared to mock or Ad-DsRed-infected cells. The levels of GAPDH remained unchanged in all treatments, indicating uniform levels of mRNA taken in all cases (Fig. 1A). Immunoblot analysis confirmed SPARC overexpression increased up to 2–3 fold in cells infected with indicated 100MOI of Ad-DsRed-SP compared to mock and Ad-DsRed-infected cells (Fig. 1B).

Overexpression of SPARC decreases D425 and UW228 cell proliferation

To determine whether SPARC overexpression affected the growth of D425 and UW228 cells, the growth rates of SPARC-overexpressed cells were compared with those of mock and vector control. A very minimal decrease in proliferation was observed at 24hrs (5-8%). At 48hrs, there was a 15–20% decrease in proliferation in Ad-DsRed-SP-infected cells (100MOI), compared with mock and Ad-DsRed-infected controls. Finally, at 96hrs, there was a 65–75% inhibition of cell proliferation in Ad-DsRed-SP-infected cells (100MOI) compared with mock and Ad-DsRed-infected cells (Fig. 1C).

Overexpression of SPARC modulates G2/M cell cycle

To determine whether SPARC expression induces cell cycle arrest in medulloblastoma cells, we analyzed DNA content by FACS analysis. FACS analysis indicated that cells treated with Ad-DsRed-SP accumulated in G2/M phase cells. Around 60% (50MOI) and >65% (100MOI) of Ad-DsRed-SP-infected cells were arrested in G2/M, compared to mock or Ad-DsRed-infected controls (Fig. 2A). These results indicate that Ad-DsRed-SP-induced cell cycle arrest at G2/M phase in medulloblastoma cell.

We next explored potential mechanisms of the altered cell cycle arrest profile. We determined p21 protein level in the total cell lysates of SPARC overexpressed cells by immunoblotting. SPARC expression induced p21 protein levels by 2–3 folds compared to controls (Fig. 2B). G2/M arrest was shown to involve an initial inhibition of Cyclin-B1/Cdc2 activity by p21 and a subsequent reduction of Cyclin-B1 and Cdc2 [18]. To study the

disparity in G2/M cell cycle molecule expression with SPARC expression, we carried out immunoblot analysis for various cell cycle controlling proteins. We found that protein levels of Chk1, Chk2, Cdc25A, Cdc25C, Cyclin-B1 and Cdc2 were down-regulated in the Ad-DsRed-SP-infected cells compared to mock and Ad-DsRed-infected cells (Fig. 2C).

Ectopic expression of constitutively active STAT3 inhibits SPARC induced growth arrest

We found that treatment of cells with Ad-DsRed-SP blocked STAT3 phosphorylation (Tyr-705) in a dose-dependent fashion [13]. Activation of STAT3 was shown to play an important role in cell cycle transition with the concomitant downregulation of p21 [19]. Constitutive activation of STAT3 directly contributes to oncogenesis by promoting proliferation and/or by suppressing apoptosis [6;20]. Further, inactivation of JAK3/STAT pathway in cancer cells was shown to increase p21 expression [21]. We next investigated whether STAT3 signaling might have a role in mediating p21 associated cell growth arrest in SPARC overexpressed cells. Constitutively active STAT3 in Ad-DsRed-SP-infected cells led to suppression p21 (Fig. 3A). FACS analysis indicated that constitutively active STAT3 suppressed SPARC induced G2/M arrest. Percent of cells in G2/M cells was >65% in the cells treated with Ad-DsRed-SP alone in cells, whereas ~40% in G2/M phase in cells transfected with pSTAT3C prior to Ad-DsRed-SP-infection (Fig. 3B). These results indicate that STAT3 plays a role Ad-DsRed-SP-induced G2/M arrest in medulloblastoma.

SPARC expression causes tumor growth inhibition in nude mice

We have previously shown that SPARC expression inhibits medulloblastoma tumor growth *in vivo* in an intracranial model [13;16]. To evaluate the effect of SPARC on tumor formation and STAT3 expression *in vivo*, we stereotactically implanted D425 cells intracranially in nude mice. The tumors that arose were challenged with intratumoral injections of Ad-DsRed-SP. There was significant decrease in the tumor volume in mice treated with Ad-DsRed-SP compared to mice treated with mock and Ad-DsRed. Histological analysis of H&E stained tumor sections showed about 75% reduction of tumor volume in the brains of mice treated with Ad-DsRed-SP as compared to mock and Ad-DsRed controls (Fig. 4A).

To determine whether SPARC altered STAT3 and p21 expression *in vivo*, tumor sections were immunoassayed for SPARC, phosphor-STAT3 (Tyr-705) and p21. Consistent with our *in vitro* observations, tumor sections from Ad-DsRed-SP-treated mice showed very minimal expression of phospho-STAT3 and increased staining for SPARC and p21 (Fig. 4B).

DISCUSSION

Even though there is a large body of information available for the SPARC in cancer research, relatively few data has been published concerning the role of SPARC in cell cycle arrest. The possible roles of SPARC in cell cycle arrest appear to have received more attention than any other tumor suppressor processes [22]. SPARC are known for their multitasked functions in cell cycle progression, cell development and differentiation [22;13]. Here, we demonstrate that SPARC expression suppress STAT3 signaling leading to cell cycle arrest in medulloblastoma cells.

In this study, we demonstrated that SPARC overexpression is able to inhibit cell proliferation in medulloblastoma cell lines. The evidence for the role of SPARC in the regulation of cell proliferation has been demonstrated before [22]. For example, Both SPARC and TGF- β were an inhibitor of cell cycle progression and proliferation in several types of cells [4;23]. SPARC negatively regulates breast cancer cell proliferation without stimulating metastasis [24], and alters the proliferation of stromal but not tumor cells in

melanoma [25]. They also observed that SPARC inhibited endothelial cell proliferation, spreading, and migration in five different sources of cells. In fact, low expression of integrins following exposure to SPARC considerably reduced cell proliferation and adhesion, in part by down-regulating the activation of Akt, FAK, MAPK 44/42 and Src [26]. Over-expression of SPARC protein and mRNA were frequently seen in non-proliferating, but actively secreting, Leydig, Sertoli cells and migrating cells [27], it showed that this SPARC might direct a metabolic pathway additional to the cell cycle. Even though no study has claimed SPARC to be stimulatory to cellular development, this statement showed that SPARC might suppress proliferation. SPARC is stimulated after cells have primarily proliferated and might function to withdraw cells temporarily from the cell cycle in preparation for migration [28]. In the present study, we demonstrate that expression of genes controlling cell-cycle p21 and Cyclin-B1 are affected by SPARC expression. We show that SPARC expression suppressed Cyclin-B1 levels in medulloblastoma cells. Cyclin-B1, the regulatory subunit of Cdk1, is essential for the transition from G2 phase to mitosis. [29]. Cyclin-B1 is highly expressed in various primary tumors and associated with poor prognosis [30;31]. Overexpression of Cyclin-B1 could contribute to the chromosomal instability in cancer cells through an alteration of spindle checkpoint [32]. Previous studies demonstrated that downregulation of Cyclin-B1 is an effective and specific approach to inhibit proliferation in variety of tumor cells [33].

The STAT3 gene is known for its proliferation activities in several types of cancers and is involved in different cellular processes, including regulation of cell-cycle [34;19]. Blocking the pathway by either antisense oligonucleotides or dominant-negative proteins induced growth retardation and apoptosis in DU145 cells [35]. Since STAT3 plays an important role in cell cycle transition with the concomitant downregulation of p21 [19], we analyzed the expression and biologic significance of STAT3 in SPARC mediated suppression of tumor cell proliferation. We demonstrated that treatment of cells with Ad-DsRed-SP resulted in downregulation of STAT3 and its phosphorylation form. By utilizing constitutively active mutant of STAT3, we demonstrate that STAT3 plays a critical in SPARC induced G2/M arrest. Inactivation of JAK3/STAT pathway in cancer cells was shown to increase p21 expression [21]. Thus, it is likely that observed induction of p21 by AdDsRed-SP in medulloblastoma cells is a consequence of SPARC-induced STAT3 inactivation. Our findings are in good agreement with the recent publication reporting that the functional inactivation of STAT3 resulted in significant inhibition of anchorage-dependent and -independent proliferation *in vitro* and reduced tumor growth *in vivo* due to inhibition of cyclin-dependent kinase 2 activity based on increased expression of p21^{WAF1} *in vitro* and *in vivo* [36].

In summary, signal transduction pathways are likely essential therapeutic targets that are aberrantly operated in various cancers. Intervention of activated STAT3 is a promising therapy to treat malignant cancers [37;38]. Our findings clearly indicate that SPARC targets STAT3 signaling network induce growth arrest and that manipulating its tumor-suppressive activity may be a useful for rational cancer therapy.

Acknowledgments

FUNDING

This research was supported by National Cancer Institute Grant CA132853 (to S.S.L.).

We also thankful to Dr. Darell D. Bigner and Dr. Ali-Osman, (Duke University Medical Center for providing D425 and UW228 cells, respectively. The authors also thankful to Prof. J.E. Darnell, Jr., for providing constitutively active STAT3 (Addgene plasmid: #8722).

References

1. Guessous F, Li Y, Abounader R. Signaling pathways in medulloblastoma. *J Cell Physiol.* 2008; 217:577–583. [PubMed: 18651559]
2. Garzia L, Andolfo I, Cusanelli E, Marino N, Petrosino G, De Martino D, Esposito V, Galeone A, Navas L, Esposito S, Gargiulo S, Fattet S, Donofrio V, Cinalli G, Brunetti A, Vecchio LD, Northcott PA, Delattre O, Taylor MD, Iolascon A, Zollo M. MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. *PLoS One.* 2009; 4:e4998. [PubMed: 19308264]
3. Kombogiorgas D, Sgouros S, Walsh AR, Hockley AD, Stevens M, Grundy R, Peet A, English M, Spooner D. Outcome of children with posterior fossa medulloblastoma: a single institution experience over the decade 1994–2003. *Childs Nerv Syst.* 2007; 23:399–405. [PubMed: 17119978]
4. Podhajcer OL, Benedetti L, Girotti MR, Prada F, Salvatierra E, Llera AS. The role of the matricellular protein SPARC in the dynamic interaction between the tumor and the host. *Cancer Metastasis Rev.* 2008; 27:523–537. [PubMed: 18459035]
5. Darnell JE Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer.* 2002; 2:740–749. [PubMed: 12360277]
6. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. *Cell.* 1999; 98:295–303. [PubMed: 10458605]
7. Garcia R, Yu CL, Hudnall A, Catlett R, Nelson KL, Smithgall T, Fujita DJ, Ethier SP, Jove R. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ.* 1997; 8:1267–1276. [PubMed: 9419415]
8. Gouilleux-Gruart V, Gouilleux F, Desaint C, Claisse JF, Capiod JC, Delobel J, Weber-Nordt R, Dusanter-Fourt I, Dreyfus F, Groner B, Prin L. STAT-related transcription factors are constitutively activated in peripheral blood cells from acute leukemia patients. *Blood.* 1996; 87:1692–1697. [PubMed: 8634413]
9. Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S, Johnson DE, Huang L, He Y, Kim JD. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. *Proc Natl Acad Sci USA.* 2000; 97:4227–4232. [PubMed: 10760290]
10. Zhang F, Li C, Halfter H, Liu J. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. *Oncogene.* 2003; 22:894–905. [PubMed: 12584569]
11. Alvarez JV, Greulich H, Sellers WR, Meyerson M, Frank DA. Signal transducer and activator of transcription 3 is required for the oncogenic effects of non-small-cell lung cancer-associated mutations of the epidermal growth factor receptor. *Cancer Res.* 2006; 66:3162–3168. [PubMed: 16540667]
12. Xiong H, Zhang ZG, Tian XQ, Sun DF, Liang QC, Zhang YJ, Lu R, Chen YX, Fang JY. Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. *Neoplasia.* 2008; 10:287–297. [PubMed: 18320073]
13. Bhoopathi P, Chetty C, Dontula R, Gujrati M, Dinh DH, Rao JS, Lakka SS. SPARC Stimulates Neuronal Differentiation of Medulloblastoma Cells via the Notch1/STAT3 Pathway. *Cancer Res.* 2011; 71:4908–4919. [PubMed: 21613407]
14. Weinberg, RA. Garland Science. Taylor & Francis Group, LLC; New York: 2007. Moving out: invasion and metastasis, *The biology of cancer*; p. 587–654.
15. Boutros R, Lobjois V, Ducommun B. CDC25 phosphatases in cancer cells: key players? Good targets? *Nat Rev Cancer.* 2007; 7:495–507. [PubMed: 17568790]
16. Bhoopathi P, Chetty C, Gujrati M, Dinh DH, Rao JS, Lakka SS. Cathepsin B facilitates autophagy mediated apoptosis in SPARC overexpressed primitive neuroectodermal tumor cells. *Cell Death Differ.* 2010; 17:1529–1539. [PubMed: 20339379]
17. Rao JS, Bhoopathi P, Chetty C, Gujrati M, Lakka SS. Matrix metalloproteinase-9 short interfering RNA induced senescence resulting in inhibition of medulloblastoma growth via p16INK4 and mitogen-activated protein kinase pathway. *Cancer Res.* 2007; 67:4956–4964. [PubMed: 17510426]

18. Choi YH, Lee WH, Park KY, Zhang L. p53-independent induction of p21 (WAF1/CIP1), reduction of Cyclin-B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. *Jpn J Cancer Res.* 2000 Feb; 91(2):164–73. [PubMed: 10761703]
19. Fukada T, Ohtani T, Yoshida Y, Shirogane T, Nishida K, Nakajima K, Hibi M, Hirano T. STAT3 orchestrates contradictory signals in cytokine-induced G1 to S cell-cycle transition. *EMBO J.* 1998; 17:6670–6677. [PubMed: 9822610]
20. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, Jove R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 yeloma cells. *Immunity.* 1999; 10:105–115. [PubMed: 10023775]
21. Lin Q, Lai R, Chirieac LR, Li C, Thomazy VA, Grammatikakis I, Rassidakis GZ, Zhang W, Fujio Y, Kunisada K, Hamilton SR, Amin HM. Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines: inhibition of JAK3/STAT3 signaling induces apoptosis and cell cycle arrest of colon carcinoma cells. *Am J Pathol.* 2005; 167:969–980. [PubMed: 16192633]
22. Tai IT, Tang MJ. SPARC in cancer biology: its role in cancer progression and potential for therapy. *Drug Resist Updat.* 2008; 11:231–246. [PubMed: 18849185]
23. Massague J. TGF-beta signal transduction. *Annu Rev Biochem.* 1998; 67:753–791. [PubMed: 9759503]
24. Dhanesuan N, Sharp JA, Blick T, Price JT, Thompson EW. Doxycycline-inducible expression of SPARC/Osteonectin/BM40 in MDA-MB-231 human breast cancer cells results in growth inhibition. *Breast Cancer Res Treat.* 2002; 75:73–85. [PubMed: 12500936]
25. Haber CL, Gottifredi V, Llera AS, Salvatierra E, Prada F, Alonso L, Sage EH, Podhajcer OL. SPARC modulates the proliferation of stromal but not melanoma cells unless endogenous SPARC expression is downregulated. *Int J Cancer.* 2008; 122:1465–1475. [PubMed: 18059024]
26. Said N, Socha MJ, Olearczyk JJ, Elmarakby AA, Imig JD, Motamed K. Normalization of the ovarian cancer microenvironment by SPARC. *Mol Cancer Res.* 2007; 5:1015–1030. [PubMed: 17951402]
27. Sage H, Vernon RB, Funk SE, Everitt EA, Angello J. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca²⁺-dependent binding to the extracellular matrix. *J Cell Biol.* 1989; 109:341–356. [PubMed: 2745554]
28. Funk SE, Sage EH. The Ca²⁺-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. *Proc Natl Acad Sci USA.* 1991; 88:2648–2652. [PubMed: 2011576]
29. Hoffmann I, Clarke PR, Marcote MJ, Karsenti E, Draetta G. Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J.* 1993; 12:53–63. [PubMed: 8428594]
30. Hassan KA, Ang KK, El-Naggar AK, Story MD, Lee JI, Liu D, Hong WK, Mao L. Cyclin-B1 overexpression and resistance to radiotherapy in head and neck squamous cell carcinoma. *Cancer Res.* 2002; 62:6414–6417. [PubMed: 12438226]
31. Li JQ, Kubo A, Wu F, Usuki H, Fujita J, Bandoh S, Masaki T, Sano K, Takeuchi H, Kobayashi S, Imaida K, Maeta H, Ishida T, Kuriyama S. Cyclin-B1, unlike cyclin G1, increases significantly during colorectal carcinogenesis and during later metastasis to lymph nodes. *Int J Oncol.* 2003; 22:1101–1110. [PubMed: 12684677]
32. Sarafan-Vasseur N, Lamy A, Bourguignon J, Le PF, Hieter P, Sesboue R, Bastard C, Frebourg T, Flaman JM. Overexpression of B-type cyclins alters chromosomal segregation. *Oncogene.* 2002; 21:2051–2057. [PubMed: 11960377]
33. Yuan J, Yan R, Kramer A, Eckerdt F, Roller M, Kaufmann M, Strebhardt K. Cyclin-B1 depletion inhibits proliferation and induces apoptosis in human tumor cells. *Oncogene.* 2004; 23:5843–5852. [PubMed: 15208674]
34. Pan X, Wang X, Lei W, Min L, Yang Y, Wang X, Song J. Nitric oxide suppresses transforming growth factor-beta1-induced epithelial-to-mesenchymal transition and apoptosis in mouse hepatocytes. *Hepatology.* 2009; 50:1577–1587. [PubMed: 19821529]

35. Barton BE, Karras JG, Murphy TF, Barton A, Huang HF. Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines. *Mol Cancer Ther.* 2004; 3:11–20. [PubMed: 14749471]
36. Scholz A, Heinze S, Detjen KM, Peters M, Welzel M, Hauff P, Schirner M, Wiedenmann B, Rosewicz S. Activated signal transducer and activator of transcription 3 (STAT3) supports the malignant phenotype of human pancreatic cancer. *Gastroenterology.* 2003; 125:891–905. [PubMed: 12949733]
37. Klampfer L. Signal transducers and activators of transcription (STATs): Novel targets of chemopreventive and chemotherapeutic drugs. *Curr Cancer Drug Targets.* 2006; 6:107–121. [PubMed: 16529541]
38. Yu H, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer.* 2004; 4:97–105. [PubMed: 14964307]

Highlights

- Ectopic expression of SPARC impaired cell proliferation in medulloblastoma cells
- SPARC expression induces STAT3 mediated cell cycle arrest in medulloblastoma cells
- SPARC expression significantly inhibited pre-established tumor growth in nude-mice

Figure 1. Ad-DsRed-SP inhibits proliferation of D425 and UW228 cells

(A) D425 and UW228 cells were infected with Mock, Ad-DsRed or Ad-DsRed-SP. After 24hrs of infection, total RNA was extracted using TRIzol reagent, and RT-PCR was performed for assessment of SPARC levels. GAPDH served as a control. (B) After 24hrs of Ad-DsRed-SP infection, SPARC levels were determined by immunoblot analyses using SPARC-specific antibodies. GAPDH served as a loading control. (C) Cell proliferation was determined by MTT assay. Cells were infected with Mock, Ad-DsRed or Ad-DsRed-SP, and incubated for different time intervals. MTT reagent was added and absorbance of the formazan from each sample was measured at a test wavelength 550nm. Results are representative of three independent experiments. Points, means; bars: S.D.

Figure 2. SPARC expression induces G2/M cell cycle arrest in D425 and UW228 cells
(A) D425 and UW228 cells were infected with Mock, Ad-DsRed or Ad-DsRed-SP and incubated for 24hrs. **(A)** Cells were stained with propidium iodide and DNA contents of cells were measured by FACS analysis. **(B)** Cellular extracts were subjected to immunoblotting with antibodies specific for p21. GAPDH served as a loading control. **(C)** Cellular extracts were subjected to immunoblotting with antibodies specific for Cdc2, phospho-Cdc2 (T14/Y15), Cdc25A, Cdc25C, Chk1, Chk2, and Cyclin-B1. GAPDH served as a loading control.

Figure 3. Expression of constitutively active STAT3 reverses Ad-DsRed-SP induced G2/M arrest in D425 and Uw228 cells

D425 and Uw228 cells were transfected with constitutively active STAT3 plasmid (pSTAT3C) for 24hrs. Cells were then infected with Mock, Ad-DsRed or Ad-DsRed-SP and cultured for another 24hrs. **(A)** Total cell lysates were subjected to immunoblot analyses for STAT3, Phospho-STAT3 (Y-705), SPARC and p21 using specific antibodies. GAPDH served as a loading control. **(B)** DNA contents of cells were measured by FACS analysis to determine cell cycle arrest.

Figure 4. SPARC expression inhibits tumor growth *in vivo*.

D425 cells were injected intracerebrally into athymic mice. Mice were treated with mock, AdDsRed, AdDsRed-SP as described in Materials and Methods. **(A)** Representative H&E staining of xenograft sections from mice following four weeks of treatment are shown. **(B)** immunohistochemical analysis of SPARC, phospho-STAT3 (Y-705), p21 expression are shown (60X). Inset: Negative control.