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Award Number: W81XWH-12-1-0527

TITLE: Function of Brg1 Chromatin Remodeling Factor in Sonic Hedgehog-Dependent Medulloblastoma Initiation and Maintenance

PRINCIPAL INVESTIGATOR:
Xuanming Shi

CONTRACTING ORGANIZATION:
Texas, University of, Southwestern Medical Center at Dallas

REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2014		2. REPORT TYPE Annual		3. DATES COVERED 15 SEP 2013 – 14 SEP 2014	
4. TITLE AND SUBTITLE Function of Brg1 Chromatin Remodeling Factor in Sonic Hedgehog-Dependent Medulloblastoma Initiation and Maintenance				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-12-1-0527	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Xuanming Shi, Jiang Wu E-Mail: Xuanming.Shi@UTsouthwestern.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Southwestern Medical Center, Dallas, Texas 75390-9133				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Medulloblastoma is the most common malignant pediatric brain tumor. Overactive Shh signaling in cerebellum granule neuron precursors (CGNPs) is the leading cause of the childhood medulloblastoma (Shh-subtype). Current study focuses on the requirement of Brg1 in mouse model of Shh-type medulloblastoma. In vitro evidences showed that Brg1 is required for mitogenic target gene expression and proliferation in primary SmoM2 CGNP and tumor cultures. In vivo deletion of Brg1 through Math1-Cre dramatically decreased death rate and prolonged the survival resulted from Shh-type medulloblastoma. Induction of Brg1 deletion in subcutaneous transplantation led to tumor aggression significant blocked, the tumor proliferation decreased as well. RT-qPCR and WB confirmed that Shh-dependent mitogenic target genes are decreased by knockout of Brg1. RNA-seq analysis in the primary tumor showed the Brg1 deletion efficiently reversed the SmoM2 oncogenic effects in medulloblastoma development. This study provides evidences that chromatin remodeling complex BAF through Brg1 is a therapeutic target for Shh-type medulloblastoma. Considering H3K27me3 changes by Brg1 deletion, ChIP-seq of Brg1 and together with histone modifications will further uncover the molecular mechanisms underlining Shh-type medulloblastoma.					
15. SUBJECT TERMS Medulloblastoma, Sonic Hedgehog, Chromatin remodeling, BAF complex, Brg1, mouse model of shh-subtype medulloblastoma					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	20	19b. TELEPHONE NUMBER (include area code)

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Introduction

Brain tumors are the leading cause of cancer-related death in children, and medulloblastoma is the most common malignant pediatric brain tumor (1). Although overall survival rates have improved in recent years, the mortality rate remains significant. Hence, new insights into the molecular mechanisms controlling medulloblastoma development are essential for improving clinical trial design, and developing molecularly targeted therapies (2). Shh signaling pathway plays important roles in many development processes and adult homeostasis (3-6). Elevation of Shh target gene expression has been associated with the initiation and /or maintenance of a large spectrum of cancer types, among which medulloblastoma is one of the most well-known Shh-dependent cancer type (3, 7-9). During early postnatal cerebellum development, Shh is required for CGNP proliferation (10-12). However, overactive Shh pathway causes CGNP over-proliferation and medulloblastoma (7, 13, 14). Among all the genetic defects, mutations resulting in an overactive Shh signaling in cerebellum granule neuron precursors (CGNPs) are the leading cause of the childhood medulloblastoma and are responsible for ~25% of occurrences (Shh-subtype) (2). Shh signaling pathway mediated by Patched (Ptch1) and Smoothed (Smo) controls target gene expression by differentially regulating activity of Gli family of transcription factors (3, 4, 12). The regulation of mitogenic target genes by Shh/Gli in cerebellum is critical for CGNP proliferation and medulloblastoma formation.

Mammalian SWI/SNF like BAF (Brg1/Brm associated factors) chromatin remodeling complexes regulate transcription by modulating chromatin structures (15, 16). It has been shown that depending on the tissue contexts, BAF complexes can either promote or suppress tumor development by regulating different sets of target gene transcription in a context-dependent manner (17-20). Recently, we have shown that Brg1, the core subunit of BAF complexes, interacts with Gli transcription factors and is required for activating Shh-induced target gene transcription. *Brg1*-deletion resulted in reduced proliferation of CGNPs in developing cerebellum due to impaired Shh-activated target gene expression, indicating that Brg1 is required for Shh-dependent CGNP proliferation (21). Thus I hypothesize that Brg1 is required for Shh-subtype medulloblastoma growth and progression. In the study I use *SmoM2*-mouse

model and breed with Brg1 conditional knockout allele to test the hypothesis. Those studies for molecular mechanism of medulloblastoma growth at chromatin level will provide insights for drug development and therapy of pediatric brain tumor and other Shh-dependent tumor.

Body

Aim1. Determine function of *Brg1* in *SmoM2*-induced medulloblastoma formation

Two subaims in this part are to determine 1) the function of *Brg1* in *SmoM2*-dependent Shh target gene expression and CGNP proliferation, and 2) *Brg1* function in *SmoM2*-dependent medulloblastoma formation.

Using an inducible mouse model of medulloblastoma with a *SmoM2-YFP* mutant gene (a point mutation in *Smoothed*) knocked-in at the *Rosa26* locus downstream of a *LoxP-flanked* stop signal (22) , and an inducible *Actin-CreER* transgene, as well as a conditional *Brg1* null allele, we bred different genotypes: *wt*, *Brg1*^{ikO}, *Smo*, and *Smo Brg1*^{ikO}, to determine *Brg1* function in CGNPs. We have previously shown in the last report that cultured *SmoM2* CGNPs display increased expression of *Gli1* (the most faithful and sensitive Shh target gene) compared to wild-type cultures. Conditional knockout of *Brg1* decreased *Gli1* protein level, and CGNP proliferation indicated by a mitotic marker phosphorylated histone 3 (H3P). *Brg1* deletion reduced the *SmoM2*-dependent mitogenic target gene expression. These experiments suggested that *Brg1* is required for *SmoM2*-induced Shh target gene expression and CGNP proliferation.

To determine the function of *Brg1* in *SmoM2*-dependent medulloblastoma formation, we previously bred above-mentioned mice but using *Nestin-creER*. One injection of tamoxifen is expected to induce the expression of *SmoM2* and deletion of *Brg1*. However, since activity of CreER system depends on the Cre expression level and tamoxifen delivery efficiency, deletion of *Brg1* and expression of *SmoM2* occurred in a

Table 1. Death rate of mice of *Brg1* ^{+/+}, *F/+*, and *F/F* with *SmoM2*, *Math1-cre*

Type w/Math1cre R26-SmoM2	Total number	Death number	Death Percentage
<i>Brg1</i> ^{+/+}	6	4	67%
<i>Brg1</i> <i>F/+</i>	11	3	27%
<i>Brg1</i> <i>F/F</i>	11	1	9%

mosaic pattern. The resulting tumor formation rate and survival curve from *Brg1*^{+/+} and *Brg1*^{F/F} mice has a trend to be different but the difference is not significant. As an alternated plan, we bred *SmoM2 Math1-Cre*, *Brg1*^{F/F}, *F/+* or *+/+* mice to analyze the

function of *Brg1* in *SmoM2*-dependent medulloblastoma initiation and formation. *Math1*-cre is expressed in CGNP cells. It induces *SmoM2* expression and *Brg1* deletion in the same cells. The data showed so far 66% *Brg1* wild type mice died from medulloblastoma, the percentage of dead *Brg1* F/+, F/F mice was down to 27%, 9% respectively (Table 1). The survival curve of *Brg1* F/F mice was significantly different from that of *Brg1* +/+ mice (Figure 1). These data suggested that *Brg1* deletion can efficiently inhibit growth of medulloblastoma *in vivo*.

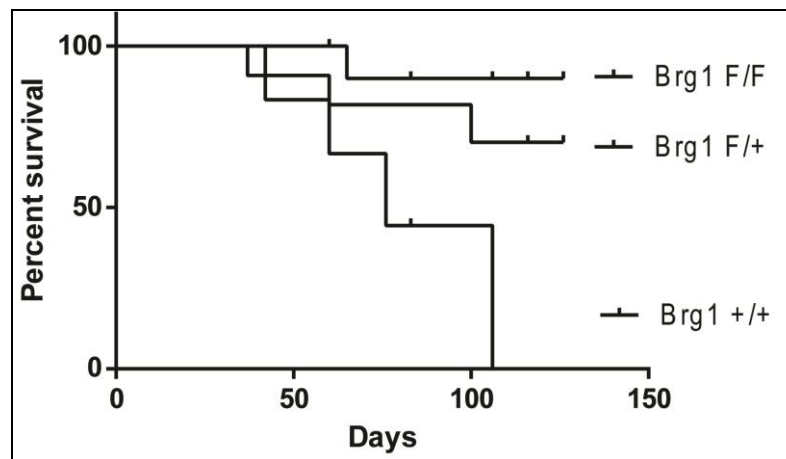


Figure 1 Survival curve of MB mice *SmoM2*, *Math1-Cre*, *Brg1*+/+, F/+ and F/F. n=6 (*Brg1* +/+), 11 (*Brg1* F/+), 11 (*Brg1* F/F). Log-rank test, $P=0.018$; Logrank test for trend $P=0.0084$.

Aim2. Determine function of *Brg1* in *SmoM2*-dependent tumor progression and maintenance.

In this part we determined the roles of *Brg1* in primary cultured medulloblastoma and in tumor progression by allograft transplantation.

Development of Shh-dependent medulloblastoma requires an active Shh pathway for maintenance and progression. It has been reported that 40% of *SmoM2*, *Actin-CreER* mice develop medulloblastoma due to leakage of the CreER activity (22). Indeed we have observed the occurrence of similar tumors in the *Brg1*^{ikO} *SmoM2* *Actin-CreER* mice without tamoxifen induction. However, the weak Cre activity without tamoxifen is not sufficient for *Brg1* deletion. Hence, the weak Cre activity gives us the chance to further delete *Brg1* after medulloblastoma is formed through tamoxifen treatment.

To determine the role of *Brg1* in medulloblastoma growth, we previously cultured primary cells of medulloblastoma formed due to Cre leakage in *Brg1*^{ikO} *SmoM2* *Actin-CreER* mice at P60. 4-hydroxy tamoxifen (4OHT) was added to the culture to induce *Brg1*

deletion. After 3 days in culture, *Brg1* was effectively deleted in 4OHT-treated cultures. Deletion of *Brg1* led to significant reduction of *Gli1*, *Ptch1* expression, and the mitogenic target gene *CcnD1* in medulloblastoma cultures. Deletion of *Brg1* also inhibited medulloblastoma growth as shown by an ATP viability assay in the cultured cells.

To determine the requirement of *Brg1* for *SmoM2* medulloblastoma allograft tumor formation ability, freshly prepared small tumor pieces of *Brg1^{F/+} SmoM2 Actin-CreER* was injected/transplanted subcutaneously into the flank regions of immunodeficient SCID-NOD mice. After tumor is visible, the recipient SCID-NOD mice were injected with tamoxifen every other day for 10 times to induce *Brg1* deletion in allograft tumors. The *Brg1^{F/F}* tumor size after injection of tamoxifen was statistically significant smaller than the

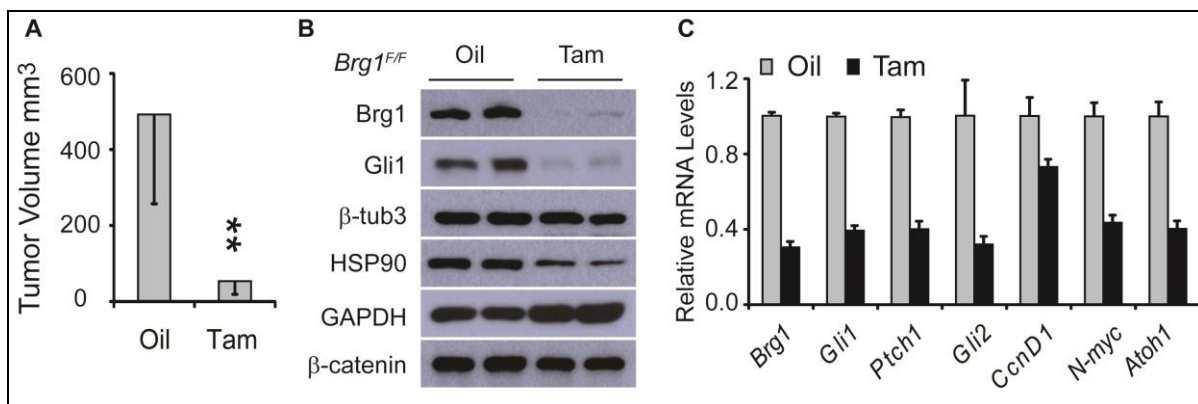


Figure 2 Brg1-deletion in SCID-NOD mice by subcutaneously transplantation decreased the tumor volume, mitogenic target gene expression at protein and mRNA level. A) Tumor volume significantly decreased by knockout of Brg1 in transplantation. B) Protein level in transplanted tissue with injection of tamoxifen showed Brg1 was efficiently deleted by tamoxifen treatment, and the Shh target gene Gli1 decreased dramatically at protein level. C) RT-qPCR analysis showed the decrease of mitogenic target genes at the mRNA level in transplanted tumor tissue. Student's t-test: **, $P < 0.01$.

control in which oil was injected (Figure 2A). In contrast, no such changes were found in *Brg1^{+/+}* tumor transplantation. Biochemistry analyses showed Brg1 was deleted by tamoxifen treatment, and Shh target gene Gli1 was dramatically decreased by deletion of Brg1 (Figure 2B). RT-qPCR confirmed the mRNA change of *Brg1* and *Gli1*. At the same time we found the other Shh target genes *Ptch1*, *CcnD1* and *N-myc* decreased as well. Interestingly, we found the important transcription factor *Gli2* and *Atoh1* decreased by Brg1 deletion (Figure 2C), suggesting transcription circuits was greatly changed after Brg1 deletion.

These data together indicated *Brg1* reduction inhibits medulloblastoma progression

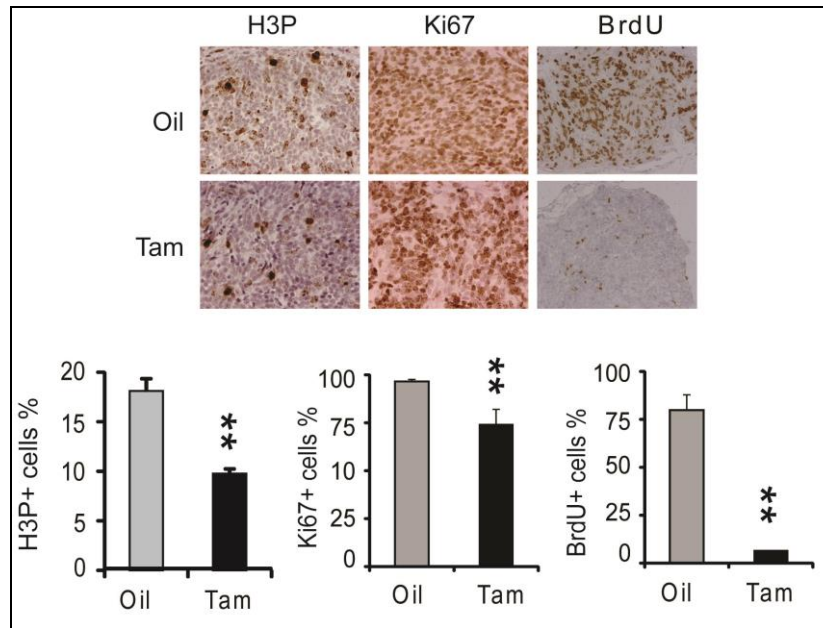


Figure 3 Brg1 is required for tumor cell proliferation in subcutaneous transplants in SCID-NOD mice. Significance was determined by Student's t-test. **: $p < 0.01$.

and maintenance. We then investigated whether the proliferation was decreased by *Brg1* deletion. The transplanted tumor tissue was stained with proliferation marker H3P, Ki67 and BrdU. All these markers were significantly decreased in *Brg1* deleted tumors induced by tamoxifen treatment (Figure 3).

Aim3. Identify molecular mechanisms of Brg1/BAF coactivation in medulloblastoma through Shh signaling.

Our previous studies have suggested that BAF complex activates Shh-induced transcription by recruiting other unidentified co-activators to Shh target genes, and our previous aim was to investigate the Brg1/BAF complex interacting protein in medulloblastoma development. However, based on the current data, *Brg1* deletion already made systematic changes to limit medulloblastoma growth. To understand the mechanisms underlying Brg1 function in Shh target gene activation and Shh-dependent medulloblastoma, we have modified subaims to use RNA-seq to determine know the global changes of all genes and to use a proteomic approach (23) to identify BAF-interacting proteins in Shh-activated medulloblastoma. Regarding proteomic analysis, the BAF-interacting co-activators will be good candidates for mediating Shh-induced gene

activation and for Shh-dependent tumor formation. We confirmed interaction between Brg1 and Gli1 in the medulloblastoma tissue, indicating the tissue is a feasible resource to probe the interaction proteins important in medulloblastoma development.

As to RNA-seq, we injected the mice *Brg1*^{+/+} and *Brg1* *F/F* suffering medulloblastoma with tamoxifen every another days for 20 days. The primary tumor tissue was confirmed with the Brg1 deletion and the decrease of mitogenic genes. Then, total RNA was extracted from the tissue and ran for RNA-seq. Results showed that 1517 genes were changed by deleting Brg1 (Figure 4A). Almost half of the genes overlapped with SmoM2-regulated genes in medulloblastoma formation (22). Among these 1517 changed genes, the downregulated genes by Brg1 deletion matched well with the upregulated genes by SmoM2 transgene, and *vice versa* (Figure 4B). GO analysis clearly showed those downregulated genes by Brg1 deletion consisted of proliferation genes, such as cell cycle, DNA binding, hedgehog, Wnt and Notch signaling. The upregulated genes are most neuronal associated genes (Figure 4C). Hence, Brg1 deletion efficiently inhibits the SmoM2 effects in medulloblastoma formation.

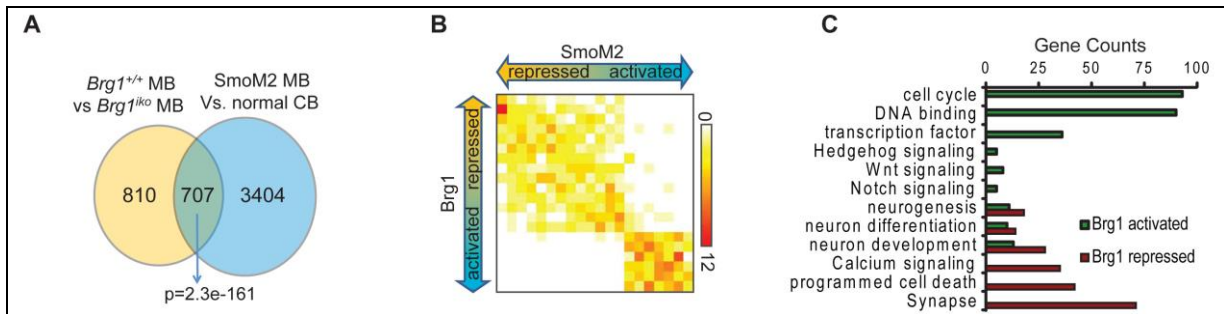


Figure 4 RNA-seq showed Brg1 deletion efficiently reverses SmoM2 effects in medulloblastoma formation. A) Significant overlap between Brg1 regulated genes and genes differentially expressed in SmoM2 tumors and normal cerebellum tissues (ref Mao 2006). Brg1 regulated genes are determined by comparing gene profiles between SmoM2 MB with or without Brg1 deletion. B) Brg1 activates and represses gene sets specifically activated or repressed in SmoM2 tumors. Overlapping genes between Brg1 and SmoM2 regulated genes are placed in a 20x20 matrix with ranked fold changes on both axes. The color key indicates the number of genes falling into each unit. C) Go analysis of the Brg1 regulated gene sets indicates the main categories of genes activated or repressed by Brg1 in SmoM2 MB.

In addition, recently we found that an H3K27me3 demethylase Jmjd3 is required for Shh signaling pathway in development and medulloblastoma growth through modulating histone modification (Shi et al., *Nature communications*, accepted). We hypothesized that

Brg1 may coordinate with Jmjd3 to maintain the H3K27me3 around Shh target gene regulatory regions. Indeed, we found that the H3K27me3 levels at global and the *Gli1* promoter regions were significantly upregulated (Figure 5A, B). It was reported that Brg1 interacts with Jmjd3 to regulate target gene expression (24). To determine whether Jmjd3 recruitment was affected by BAF complex through *Brg1*, we carried out ChIP-Jmjd3 experiment in primary CGNP cultures treated with 4OHT to delete *Brg1*. Results indicated that Jmjd3 binding at *Gli1* regulatory region was significantly decreased when *Brg1* was deleted (Figure 5C). These data taken together showed Brg1 cooperates with histone modifiers, such as Jmjd3, to regulate Shh target genes in medulloblastoma development.

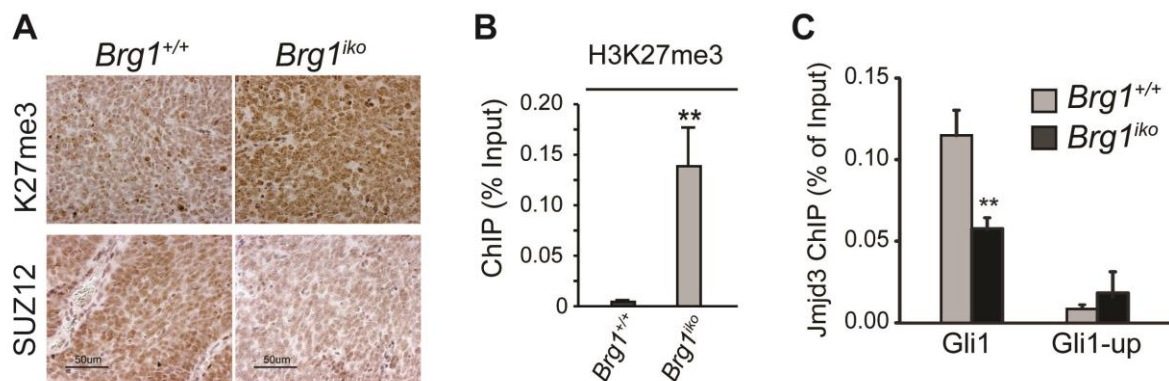


Figure 4 Brg1 coordinates Jmjd3 to maintain the low H3K27me3 level around Shh target gene in medulloblastoma. A) Brg1 deletion in primary SmoM2 MB led to increased global H3K27me3 and decreased PRC2 subunits as shown by immunostaining. B) ChIP-qPCR indicates increased H3K27me3 levels at the *Gli1* regulatory region in SmoM2 MB upon Brg1 deletion. C) Decreased Jmjd3 binding to the *Gli1* gene regulatory region in Shh-treated Brg1 deleted CGNPs as shown by ChIP-qPCR. A region upstream of *Gli1* gene was used as a negative control for Brg1 binding. **: $P < 0.01$.

In summary, Brg1 is required for *SmoM2*-dependent CGNP mitogenic target gene expression and proliferation in both cultures and in tumors. Through conditional knockout Brg1 in primary cultured medulloblastoma cells, tumor growth was inhibited. Induction of Brg1 deletion in subcutaneous transplantation led to significantly blocked tumor aggression. RT-qPCR and Western Blot showed that Shh-dependent mitogenic target genes are decreased by knockout of Brg1. Immunostaining showed the tumor cell proliferation was significantly decreased by deletion of *Brg1*. RNA-seq data suggested that Brg1 deletion efficiently antagonized the *SmoM2* oncogenic effects in medulloblastoma development. Hence, Brg1 coordinates histone modifiers and other factors in regulating

series transcription circuit to form medulloblastoma. This study together showed a viable therapeutic target for medulloblastoma development.

Key Research Accomplishments

- Brg1 deletion can efficiently inhibit growth of medulloblastoma in vivo.
- Deletion of Brg1 in subcutaneous transplantation decreases tumor growth, mitogenic target gene expression, and tumor cell proliferation.
- Brg1 deletion efficiently reverses the SmoM2 ongenic effects at transcriptional level in medulloblastoma development.
- Brg1 coordinates Jmjd3 to regulate H3K27me3 level in medulloblastoma growth.

Reportable Outcomes

1. **Abstract:** Shi X, Zhang Z, Wang Q, Wu J. Function of Brg1 chromatin remodeling factor in sonic hedgehog-dependent medulloblastoma development [abstract]. Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 Apr 5-9; San Diego, CA. Philadelphia (PA): AACR; 2014. Abstract nr 404.
2. Shi, X., Zhang, Z., Zhan, X., Cao, M., Satoh, T., Akira, S., Shpargel, K., Magnuson, T., Wang, R., Wang, C., Ge, K., Wu, J. (2014). An Epigenetic Switch Induced by Sonic Hedgehog Signaling Regulates Gene Activation during Neural Development and Medulloblastoma Growth. *Nature communications* (Accepted).
3. Shi, X., Wang, Q., Xuan, Z., Wu, J., (2014) Brg1 chromatin remodeling factor is essential for the transcriptional circuits controlling Shh-type medulloblastoma growth. (Submitted).

Conclusions

We have confirmed hypotheses that the chromatin remodeler Brg1 is required for *SmoM2*-dependent mitogenic target gene expression, cerebellum granular neural precursor proliferation, and tumor cell proliferation. We showed the evidence that Brg1 is required for medulloblastoma growth in primary cultures, primary tumor and transplanted tumor. Importantly, deletion of *Brg1* in subcutaneous transplantation inhibits medulloblastoma progression, through decreasing the mitogenic target genes and tumor cell proliferation. Brg1 deletion efficiently reversed *SmoM2* oncogenic effects at transcriptional level in medulloblastoma development. To further understand molecular mechanisms underlining medulloblastoma development, ChIP-seq for Brg1 and histone modifications may systematically give the information how Brg1 together with other cofactors such as histone modifiers regulates tumor growth.

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Appendices

1. Curriculum Vitae.
2. Meeting abstract of Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 Apr 5-9; San Diego.

Biographical Sketch

Provide the following information for each individual included in the Research & Related Senior/Key Person Profile (Expanded) Form.

NAME XUANMING SHI		POSITION TITLE POSTDOCTORAL RESEARCHER II	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Anhui University, Hefei, China	B.S.	1999	Biochemistry
Sichuan Agricultural University & Institute of Microbiology, Chinese Academy of Science, China	M.S.	2005	Biochemistry & Molecular Biology
University of Rostock & Research Institute for the Biology of Farm Animals, Germany	Ph.D.	2009	Molecular Biology
University of Texas Southwestern Medical Center at Dallas	Postdoctoral	2010	Developmental Biology
RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 4 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.			
<u>Positions and Employment</u>			
1999-2001	Assistant Engineer, BBKA Biochemistry Group Company, Bengbu, China.		
2001-2002	Technician, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.		
2002-2005	Graduate student, Sichuan Agricultural University and Institute of Microbiology, Chinese Academy of Sciences. Mentor: Prof. Dr. Yubi Huang & Prof. Dr. Keqian Yang		
2005-2009	PhD student, University of Rostock & Research Institute for the Biology of Farm Animals, Germany. Mentor: Prof. Dr. Hans-Martin Seyfert		
2010-	Postdoctoral researcher, UT Southwestern Medical Center at Dallas, Texas. Mentor: Dr. Jiang Wu		

HONORS AND AWARDS

Visionary Postdoctoral Fellowship Award (2011): Department of Defense, U.S. Army Medical Research and Materiel Command, Congressionally Directed Medical Research Programs, 2011 Peer Reviewed Cancer Research Program.

Professional Associations/Affiliations

American Association of Cancer Research 2013-2014

Peer-reviewed Publications (in chronological order)

1. **Shi, X.**, Wang, Q., Gu, J., Xuan, Z., Wu, J. (2014). Brg1 chromatin remodeling factor is essential for the transcriptional circuits controlling Shh-type medulloblastoma growth. (Submitted).
2. **Shi, X.**, Zhang, Z., Zhan, X., Cao, M., Satoh, T., Akira, S., Shpargel, K., Magnuson, T., Wang, C., Ge, K., Wu, J. (2014). An Epigenetic Switch Induced by Sonic Hedgehog Signaling Regulates Gene Activation during Neural Development and Medulloblastoma Growth. Nature Communications (Accepted).
3. **Shi, X.**, Metges, C.C., and Seyfert, H.-M. (2013) Characterization of a far upstream located promoter expressing the acetyl-CoA carboxylase-alpha in the brain of cattle. Gene. 2013 Feb 25;515(2):266-71.
4. **Shi, X.**, Metges, C.C., and Seyfert, H.-M. (2012). Interaction of C/EBP and NF-Y factors constrains the promoter IA activity of the bovine acetyl-CoA carboxylase-alpha gene. BMC Molecular Biology 2012 Jun 27;13(1):21 doi:10.1186.
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7. **Shi, X.**, Liu, S., Metges, C.C., and Seyfert, H.-M. C/EBP-beta drives expression of the nutritionally regulated promoter IA of the acetyl-CoA carboxylase-alpha gene in cattle. Biochim. Biophys. Acta-gene regulatory mechanism 1799 (2010) 561-567.
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Print


**ANNUAL
MEETING 2014**
**HARNESSING
BREAKTHROUGHS
TARGETING
CURES**

**April 5-9, 2014
San Diego, CA**
Category: Molecular and Cellular Biology
Session Title: Epigenetics 2

#404 Function of Brg1 chromatin remodeling factor in sonic hedgehog-dependent medulloblastoma development. Xuanming Shi, Zilai Zhang, Qiu Wang, Jiang Wu. UT Southwestern Medical Ctr., Dallas, TX.

Medulloblastoma is the most common malignant pediatric brain tumor. Overactive Shh signaling in cerebellum granule neuron precursors (CGNPs) is the leading cause of the childhood medulloblastoma (Shh-subtype). Previously we have shown that chromatin remodeler Brg1 is required for Shh target gene expression, and Brg1 deletion reduced CGNP proliferation in developing cerebellum. Current study focuses on the function of Brg1 in mouse model of Shh-subtype medulloblastoma. In CGNP cultured from SmoM2 transgenic mice where Shh pathway is constitutively active, we found Brg1 is required for CGNP mitogenic target gene expression and proliferation. In SmoM2 medulloblastoma cultures, we observed that tumor cell growth was inhibited by conditional knockout of Brg1. In subcutaneous transplantation, we found that tumors were significantly shrunk upon induction of Brg1 deletion. Detailed analysis indicated that Shh-dependent mitogenic target genes decreased by loss of Brg1. Further evidences showed that medulloblastoma cell proliferation was significant inhibited by conditional knockout of Brg1. Effect of Brg1 on the chromatin environment of target genes during medulloblastoma development will be discussed. Our study will provide insights to the epigenetic mechanism of Shh-dependent tumor development and new therapeutic targets.

Citation Format

Shi X, Zhang Z, Wang Q, Wu J. Function of Brg1 chromatin remodeling factor in sonic hedgehog-dependent medulloblastoma development [abstract]. Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 Apr 5-9; San Diego, CA. Philadelphia (PA): AACR; 2014. Abstract nr 404.

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