同 意 書

医療機関名　　病院長 殿

研究名：「JCOG0911: 初発膠芽腫に対するインターフェロン-β ＋テモゾロミド併用化学放射線療法のランダム化第 II 相試験」の附随研究： 化学療法、放射線療法を施行した膠芽腫例における効果予測因子および予後因子に関する研究

説明内容：

- この研究が JCOG の臨床試験（JCOG0911）の附随研究であること。
- 遺伝子とは
- 遺伝子と疾病
- この研究へのご協力について
- 研究協力の任意性と撤回の自由
- 研究の意義、目的と方法
- 予測される研究結果および提供者等に対して予測される危険や不利益
- 研究計画書の開示
- 試料の提供は無償であること
- 個人情報の保護について
- 研究の倫理審査
- 試料から得られた解析結果は、匿名化された上研究成果として公表
- 研究から生じる知的財産権の帰属
- この研究終了後の検体の取扱いの方針
- 研究資金について
- 遺伝カウンセリングについて
- 研究責任者の氏名と職名
- どんなことでも質問してください
- 担当医師の連絡先、研究代表者、試料解析研究事務局

上記の研究について、私が説明しました。

説明担当者署名：

説明年月日：　　年　　月　　日

上記の研究について、担当医師から説明を受けよく理解しましたので、研究への参加（腫瘍組織、血液、および JCOG0911 試験の診療情報データの提供）に同意します。

患者本人署名：

同意年月日：　　年　　月　　日

別紙４

厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

悪性神経膠腫に対する Temozolomide の治療効果を増強した標準治療確立に関する研究

研究分担者	隈部俊宏	東北大学脳神経外科
	倉津純一	熊本大学脳神経外科
	佐藤慎哉	山形大学医学部附属病院
	杉山一彦	広島大学脳神経外科
	高橋　潤	北野病院脳神経外科
	田中克之	聖マリアンナ医科大学脳神経外科
	永根基雄	杏林大学脳神経外科
	西川　亮	埼玉医科大学脳神経外科
	別府高明	岩手医科大学脳神経外科
	村垣善浩	東京女子医科大学脳神経外科
	若林俊彦	名古屋大学脳神経外科
	角美奈子	国立がん研究センター放射線治療科
	米盛　勧	国立がん研究センター乳腺科・乳腺内科

研究協力者

嘉山孝正（国立がん研究センター、山形大学脳神経外科）、野村和弘（東京労災病院）、淺井昭雄（関西医科大学脳神経外科）、阿部竜也（大分大学脳神経外科）、有田和徳（鹿児島大学脳神経外科）、有田憲生（兵庫医科大学脳神経外科）、岩立康男（千葉大学脳神経外科）、大熊洋輝（弘前大学脳神経外科）、大西丘倫（愛媛大学脳神経外科）、斎藤延人（東京大学脳神経外科）、佐々木富男（九州大学脳神経外科）、田部井勇助（都立駒込病院脳神経外科）、寺崎瑞彦（久留米大学脳神経外科）、中洲庸子（静岡県立静岡がんセンター脳神経外科）、中村博彦（中村記念病院）、橋本直哉（大阪大学脳神経外科）、藤巻高光（埼玉医科大学脳神経外科）、宝金清博（北海道大学脳神経外科）、松尾孝之（長崎大学脳神経外科）、松村　明（筑波大学脳神経外科）、宮本亨（京都大学脳神経外科）、吉田一成（慶應大学脳神経外科）、渡邊学郎（日本大学医学部付属板橋病院）

研究要旨

希少悪性腫瘍のひとつである悪性神経膠腫の中で、最も予後不良の疾患とされる膠芽腫に対し標準治療となった Temozolomide(TMZ)併用化学放射線療法の治療効果を増強する目的で、Japan Clinical Oncology Group(JCOG)脳腫瘍グループとして、TMZ に Interferon-β（INF-β）を併用する化学放射線療法の有効性を評価するランダム化第 II 相臨床試験を計画し、プロトコールの作成を行い、平成 22 年 4 月より登録を開始し、平成 24 年 1 月に目標症例数 120（実登録数 122）の症例登録が終了した。

A～H.の報告内容は研究代表者と同一であるため省略する。

研究成果の刊行に関する一覧

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sunayama J, Shibui S	FoxO3a functions as a key integrator of cellular signals that control glioblastoma stem-like cell differentiation and tumorigenicity	Stem Cells	29	1327-37	2011
Hashimoto K, Shibui S	Comparison of clinical outcomes of surgery followed by local brain radiotherapy and surgery followed by whole brain radiotherapy in patients with single brain metastasis: single-center retrospective analysis	Int J Radiat Oncol	15	e475-8	2011
Ohno M, Shibui S	Reactivation of hepatitis B virus after glio-blastoma treatment with temozolomide	Neurol Med Chir	51	728-31	2011
Terasaki M, Shibui S	Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen --A24 with recurrent or progressive glioblastoma multiforme	J Clin Oncol	29	337-44	2011

Nakamura H, Kuratsu J	Molecular and clinical analysis of glioblastoma with an oligodendroglial component (GBMO)	Brain Tumor Pathology	28	185-90	2011
Takezaki T, Kuratsu J	Essential Role of the Hedgehog signaling pathwayIn human glioma-initiating cells	Cancer Science	102	1306-12	2011
Makino K, Kuratsu J	Does adding FDG-PET to MRI improve the differentiation between primary cerebral lymphoma and glioblastoma? Observer performance study	Ann Nucl. Med	25	432-8	2011
Kawaguchi T, Kumabe T	Early detection of venous thromboembolism in patients with neuroe-pithelial tumor: efficacy of screèning with serum D:-dimer measurements and Doppler ultrasonography	J Neurooncol	101	495-504	2011
Saito R, Kumabe T	Regression of recurrent glioblastoma infiltrate-ing the brainstem after convection-enhanced delivery of nimustine hydrochloride	J Neurosurg Pediatr	7	522-6	2011

Nakamura T, Kumabe T	Local convection-enhanced delivery of chemotherapeutic agent transiently opens blood-brain barrier and improves efficacy of systemic chemotherapy in intracranial xenograft tumor model	Cancer Lett	310	77-83	2011
Shibahara I, Kumabe T	New insights into glio-maclassification based on isocitrate dehydro-genasel and 2 gene status	Brain Tumor Pathol	28	203-8	2011
櫻田香, 佐藤慎哉	術中MRIを用いた脳腫瘍手術	CI研究	1	25-32	2010
櫻田香, 佐藤慎哉	中心溝近傍悪性神経膠腫の手術	脳腫瘍の外科	1	30-4	2010
Yamasaki F, Sugiyama K	Advantages of high b-value diffusion-weighted imaging to diagnosepseudo-responses in patients with recurrent glioma after bevacizumab treatment	Eur J Radiol		Epub ahead of print	2011
Yamasaki F, Sugiyama K	Magnetic resonance spectroscopic detection of lactate is predictive of a poor prognosis in patients with diffuse intrinsic pontine glioma	Neuro Oncol	13	791-801	2011

Doskaliyev A, Sugiyama K	Lymphomas and glioblas-tomas: differences in the apparent diffusion coefficient evaluated with high b-value diffu-sion-weighted magnetic resonance-imaging at 3T	Eur J Radiol	81	339-44	2012
Nagane M	Multidisciplinary prog-ress in neuro-oncology 2010	Lancet Neurol	10	18-20	2011
永根 基雄	高齢者初発膠芽腫に対する放射線化学療法の治療成績と因子解析	Geriatric Neurosurgery	23	3-11	2011
Nagane M	Predominant antitumor effects by fully human anti-TRAIL-receptor2 (DR5) monoclonal antibodies in human glioma cells *in vitro* and *in vivo*	Neuro Oncol	12	687-700	2010
Liu K-W, Nagane M, Nishikawa R	SHP-2/PTPN11 mediates gliomagenesis driven by PDGFRA and Ink4a/Arf aberrations in mice and humans	J Clin Invest	121	906-17	2011
Feng H, Nagane M, Nishikawa R	Activation of Raqc1 by Srcdependent phosphoryla-tion of Dock180(Y1811) mediates PDGFRastimu-lated glioma tumorigen-esis in mice and humans	J Clin Invest	121	4670-84	2011

Adachi J, Nishikawa R	O6-methylguanine-DNA methyltransferase pro-motor methylation in 45 primary central nervous system lymphomas: quantitative assessment of methylation and res-poinse to temozolomide treatment	J Neuro Oncol	107	147-53	2012
Beppu T	Prediction of malignancy grading using computed tomography perfusion imaging in nonenhancing supratentorial gliomas	J Neuro Oncol	103	619-27	2011
Yoshimitsu K, Muragaki Y	Wireless modification of the intraoperative examination monitor for awake surgery	Neurol Med Chir	51	472-6	2011
Ando T, Muragaki Y	Precise comparison of protoporphyrin IX fluorescence spectra with pathological results for brain tumor tissue identification	Brain Tumor Pathol	28	43-51	2011
Natsume A, Wakabayashi T	Girdin maintains the stemness of glioblastoma stem cells	Oncogene		e-pub	2011

Ohka F, Wakabayashi T	The global DNA methylation surrogate LINE-1 methylation is correlated with MGMT promoter methylation and is a better prognostic factor for glioma	PloS ONE	6	E23332	2011
Motomura K, Wakabayashi T	Benefits of Interferon-β and Temozolomide combination therapy for newly diagnosed primary glioblastoma with the unmethylated MGMT promoter: a multicenter study	Cancer	117	1721-30	2011
Sekine I, Sumi M	Phase I Study of Concurrent High-Dose Three-Dimensional Conformal Radiotherapy with Chemotherapy Using Cisplatin and Vinorelbine for Unresectable Stage III Non-Small-Cell Lung Cancer	Int J Radiat Oncol Biol Phys	82	953-9	2012
Minami-Shimmyo Y, Sumi M	Risk factors for treatment-related death associated with chemotherapy and thoracic radiotherapy for lung cancer	J Thorac Oncol	7	177-82	2012
Yonemori K	The notorious "drug lag" for oncology drugs in Japan	Invest New Drugs	29	706-12	2011

Yonemori K	Compliance with Good Clinical Practice in oncology registration trials in Japan	Ann Oncol	22	1451-6	2011
Yonemori K	Participation of elderly patients in registration trials for oncology drug applications in Japan	Ann Oncol	21	2112-8	2010
Yonemori K, Shibui S	Disruption of the blood brain barrier by brain metastases of triple-negative and basal-type breast cancer but not HER2/neu-positive breast cancer	Cancer	116	302-8	2010

STEM CELLS®
CANCER STEM CELLS

FoxO3a Functions as a Key Integrator of Cellular Signals That Control Glioblastoma Stem-like Cell Differentiation and Tumorigenicity

JUN SUNAYAMA,[a,b,c] ATSUSHI SATO,[a,d] KEN-ICHIRO MATSUDA,[a,d] KEN TACHIBANA,[a] ERIKO WATANABE,[a] SHIZUKA SEINO,[a] KAORI SUZUKI,[a,e] YOSHITAKA NARITA,[e] SOICHIRO SHIBUI,[e] KAORI SAKURADA,[d] TAKAMASA KAYAMA,[d] ARATA TOMIYAMA,[a] CHIFUMI KITANAKA[a,b,c]

[a]Department of Molecular Cancer Science and [d]Department of Neurosurgery, Yamagata University School of Medicine, Yamagata, Japan; [b]Oncology Research Center, Research Institute for Advanced Molecular Epidemiology, Yamagata University, Yamagata, Japan; [c]Global COE program for Medical Sciences, Japan Society for Promotion of Science, Tokyo, Japan; [e]Department of Neurosurgery, National Cancer Center Hospital, Tokyo, Japan

Key Words. FoxO3a • Akt • Extracellular signal-regulated kinase • p70S6K • Glioblastoma stem cells

ABSTRACT

Glioblastoma is one of the most aggressive types of human cancer, with invariable and fatal recurrence even after multimodal intervention, for which cancer stem-like cells (CSLCs) are now being held responsible. Our recent findings indicated that combinational inhibition of phosphoinositide-3-kinase/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways effectively promotes the commitment of glioblastoma CSLCs to differentiation and thereby suppresses their tumorigenicity. However, the mechanism by which these two signaling pathways are coordinated to regulate differentiation and tumorigenicity remains unknown. Here, we identified FoxO3a, a common phosphorylation target for Akt and ERK, as a key transcription factor that integrates the signals from these pathways. Combinational blockade of both the pathways caused nuclear accumulation and activation of FoxO3a more efficiently than blockade of either alone, and promoted differentiation of glioblastoma CSLCs in a FoxO3a expression-dependent manner. Furthermore, the expression of a constitutively active FoxO3a mutant lacking phosphorylation sites for both Akt and ERK was sufficient to induce differentiation and reduce tumorigenicity of glioblastoma CSLCs. These findings suggest that FoxO3a may play a pivotal role in the control of differentiation and tumorigenicity of glioblastoma CSLCs by the PI3K/Akt/mTOR and MEK/ERK signaling pathways, and also imply that developing methods targeting effective FoxO3a activation could be a potential approach to the treatment of glioblastoma. STEM CELLS *2011;29:1327–1337*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Glioblastoma multiforme, the most common primary brain tumor in adults, is highly aggressive with a median survival of less than 2 years despite multimodality treatment consisting of surgical resection followed by radiotherapy and chemotherapy [1]. In recent years, many studies have reported the presence of stem cells in glioblastoma, referred to as cancer stem-like cells (CSLCs) or cancer-initiating cells [2–5]. One important property of glioblastoma CSLCs is their highly oncogenic potential, generating tumors that reproduce the characteristics of the original tumor on implantation into nude mice, whereas other cells isolated from the same tumors are nontumorigenic. Other properties include self-renewal capacity, i.e., ability to undergo divisions that allow the generation of more glioblastoma CSLCs, and differentiation capacity, which contributes to tumor cellular heterogeneity [6]. Of therapeutic significance, glioblastoma CSLCs also represent a population of radioresistance and chemoresistance that can survive treatment and repopulate the tumors. Therefore, it has been thought that glioblastoma

Author contributions: J.S.: concept and design, collection and assembly of data, data analysis and interpretation and manuscript writing; A.S.: concept and design, collection and assembly of data, data analysis and interpretation; K.-i.M., K.T., S. Seino, and A.T.: data analysis and interpretation; E.W.: collection and assembly of data; K. Suzuki: provision of study material or patients; Y.N. and S. Shibui, K. Sakurada, and T.K.: provision of study material or patients, data analysis and interpretation; C.K.: concept and design, data analysis and interpretation, manuscript writing and final approval of manuscript. J.S. and A.S. contributed equally to this article.

Correspondence: Chifumi Kitanaka, M.D., Ph.D., Department of Molecular Cancer Science, Yamagata University School of Medicine, Yamagata 990-9585, Japan. Telephone: 81-23-628-5212; Fax: 81-23-628-5215; e-mail: ckitanak@med.id.yamagata-u.ac.jp Received April 19, 2011; accepted for publication July 4, 2011; first published online in STEM CELLS *EXPRESS* July 26, 2011.
1066-5099/2009/$30.00/0 doi: 10.1002/stem.696

CSLCs play a primary role in tumor maintenance and recurrence [7, 8].

A number of therapeutic strategies directed at CSLCs are emerging and are now undergoing experimental validation, among which is differentiation therapy [9]. The possibility that differentiation of CSLCs within a malignancy may lead to tumor degeneration and increased susceptibility to conventional cytotoxic anticancer therapies has been recognized for some time [9]. In this respect, a number of potential strategies has been reported that can promote differentiation of glioblastoma CSLCs. Bone morphogenetic protein can function as a key inhibitory regulator of glioblastoma CSLCs by regulating their differentiation status [10], and the inhibition of the transforming growth factor-β-Sox4 (sex-determining region of Y chromosome-related high mobility group box 4)-Sox2 pathway blocked the tumorigenicity of glioblastoma CSLCs by promoting their differentiation [11]. Knockdown of transformation/transcription domain-associated protein has increased differentiation of glioblastoma CSLCs and suppressed tumor formation in vivo [12]. It has been reported that pleiomorphic adenoma gene like 2 (PLAGL2) executes its oncogenic activities through regulation of the cellular differentiation status, and reduction of PLAGL2 represses their tumorigenic potency [13]. All these reports demonstrate that the promotion of glioblastoma CSLC differentiation can markedly reduce their tumorigenic potential and hence the glioblastoma CSLC population per se, underscoring the idea that differentiation therapy holds promise as an approach to target glioblastoma CSLCs.

Recently, we have shown that targeted inactivation of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway in glioblastoma CSLCs promotes their differentiation into neuronal and glial lineages and this effect is apparently augmented by concurrent inhibition of the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. Importantly, combinational blockade of both pathways more effectively suppressed their tumorigenicity than blockade of either alone [14]. These findings suggest that the PI3K/Akt/mTOR and MEK/ERK pathways coordinately regulate the differentiation and tumorigenicity of glioblastoma CSLCs. However, to date, the critical molecules mediating such effects of these two signaling pathways remain to be identified.

Here in this study, we revealed that FoxO3a receives inputs from the PI3K/Akt/mTOR and MEK/ERK pathways in glioblastoma CSLCs and controls their differentiation and tumorigenicity. Our findings suggest that FoxO3a may function as a key integrator of these cellular signals controlling glioblastoma CSLCs and may as such be a potential therapeutic target in glioblastoma treatment.

Materials and Methods

Cell Culture

Patient-derived glioblastoma (SJ28P3 and #38) and A172 CSLCs were isolated and cultured as described previously [14, 15]. Briefly, primary human glioblastoma cells were derived from surgical specimens obtained after informed consent from glioblastoma patients in accordance with a protocol approved by the Institutional Review Boards of the National Cancer Center and Yamagata University School of Medicine. A172 glioblastoma cells were obtained from the RIKEN Bioresource Center. Cells were cultured in the stem cell culture medium (Supporting Information) in the presence of 20 ng/ml epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Under this culture condition, cell aggregates known as spheres are formed within a few days. Spheres were cultured in the sphere culture condition with EGF and bFGF for a period of time. Subsequently, cells were plated on collagen-coated dishes (IWAKI, Tokyo, Japan, http://atg.ushop.jp) for the monolayer culture of stem-like cells. Monolayer-cultured CSLCs were dissociated by Accutase (Sigma, St. Loius, MO, http://www.sigmaaldrich.com) and reseeded once every 6–7 days. Characterization of the monolayer-cultured CSLCs has been described [14, 15].

Lentiviral Vectors

The cDNA encoding human wild-type FoxO3a was generated by polymerase chain reaction (PCR) using primers 5′-CCC TCG AGT CAG CCT GGC ACC CAG CTC TGA GAT-3′ and 5′-CCC TCG AGT CAG CCT GGC ACC CAG CTC TGA GAT-3′ from a human placenta cDNA library and subcloned into the BamHI-XhoI of pENTR (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Site-directed mutagenesis was performed with the QuikChange kit (Stratagene, Santa Clara, CA, http://www.chem.agilent.com) to introduce Thr/Ser to Ala changes at T32, S253, S294, S315, S344, and S425 of FoxO3a [16, 17]. A dominant-negative FoxO3a, which contains the DNA binding domain but lacks the transactivation domain [18], was amplified by PCR using pENTR-FoxO3a as a template. Each FoxO3a mutant was inserted into lentiviral expression vector pLenti (Invitrogen). Lentiviruses were produced in Lenti-X 293T cells with packing mix (Lenti-X HT Packaging System, Clontech, Mountain View, CA, http://www.clontech.com) according to the manufacturer's instructions.

Knockdown by RNA Interference

After monolayer-cultured patient-derived and A172 CSLCs were seeded at a density of 2×10^5 cells per milliliter in the stem cell culture medium on collagen-coated dishes, they were transfected with siRNAs using Lipofeetamine 2000 Reagent (Invitrogen). The siRNAs used in this study are described in Supporting Information.

Generation of Stable Cell Lines

Patient-derived glioblastoma CSLCs (SJ28P3) stably expressing FoxO3a short hairpin RNA (shRNA) were obtained using the BLOCK-iT RNAi expression vector kits (Invitrogen) according to the manufacturer's instructions. The shRNA of FoxO3a (Hmi405996) and the nontargeting control sequence were purchased from Invitrogen. Cells were transfected with control and FoxO3a shRNA vectors. The transfectants were selected in the stem cell culture medium containing blasticidin (Invitrogen).

Subcellular Fractionation

Cells were homogenized in hypotonic buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 1 mM EDTA) containing 0.1% Triton X-100. The lysates were centrifuged at 3,000 rpm for 10 minutes at 4°C and separated into pellet and supernatant fractions. The pellet was resuspended in hypotonic buffer containing 0.1% Triton X-100, recentrifuged, and used as the nuclear fraction. The supernatant fraction was recentrifuged at 15,000 rpm for 20 minutes at 4°C and was used as the cytoplasmic fraction.

Animal Experiments

Intracranial xenografts: monolayer-cultured SJ28P3 or #38 CSLCs (1×10^4) in 10 μl Dulbecco's modified Eagle's medium/F12 medium were injected stereotactically into the right cerebral hemisphere of a 5-week-old male BALB/cAJcl- nu/nu mice (CLEA Japan, Inc.) at a depth of 3 mm. All animal experiments were performed under a protocol approved by the Animal Research Committee of Yamagata University.

Statistical Analysis

Results are expressed as the means ± SDs and were analyzed using the unpaired Student's t test, while mouse survival was

evaluated by the Kaplan-Meier method and analyzed using the log-rank test.

Sphere Formation Assay, Immunoblot Analysis and Immunoprecipitation, and Immunofluorescence Analysis

These methods are described in Supporting Information.

RESULTS

Tight Association Between Upregulation of FoxO3a and Differentiation Induced by the Inhibition of the PI3K/Akt/mTOR and MEK/ERK Signaling Pathways in Glioblastoma CSLCs

As candidate molecules that have been implicated in cellular differentiation and could also be under the control of both the PI3K/Akt/mTOR and MEK/ERK pathways [16, 17], we investigated the possible involvement of Forkhead Box O (FoxO) transcription factors in the regulation of glioblastoma CSLC differentiation. As we reported previously [14], inhibition of either the PI3K/Akt/mTOR (with a dual PI3K/mTOR inhibitor NVP-BEZ235) or the MEK/ERK (with a MEK inhibitor SL327 or U0126) pathway caused modest, and inhibition of both caused marked, induction of glioblastoma CSLC differentiation as indicated by the increased expression of differentiation markers, βIII-tubulin, and glial fibrillary acidic protein (GFAP) (Fig. 1A, 1F). Under these conditions, we found that the expression level of FoxO3a, but not those of FoxO1 and FoxO4, is increased in close association with the expression of the differentiation markers in both glioblastoma patient-derived CSLCs (SJ28P3 CSLCs) as well as in those derived from an established glioblastoma cell line, A172 (A172 CSLCs) (Fig. 1A, 1F; Supporting Information Fig. 1). A subcellular fractionation study further indicated that nuclear FoxO3a expression is closely associated with the differentiation status of glioblastoma CSLCs. FoxO3a, which was localized predominantly in the cytoplasm in the control (vehicle-treated) condition, accumulated in the nucleus as the PI3K/Akt/mTOR and/or MEK/ERK pathways were inhibited (Fig. 1B, 1G). The results of the fractionation study were also confirmed by immunocytochemistry: vehicle-treated cells showed a perinuclear pattern of FoxO3a expression, whereas cells treated concomitantly with the dual PI3K/mTOR inhibitor and the MEK inhibitor showed nuclear accumulation of FoxO3a and became positive for βIII-tubulin expression (Fig. 1C, 1H). Consistent with the shift of FoxO3a expression and localization, the expression of p27, a major transcriptional target of FoxO3a, was induced by inhibition of the PI3K/Akt/mTOR and/or MEK/ERK pathways in a FoxO3a expression-dependent manner (Fig. 1D, 1E, 1I, 1J).

FoxO3a is Required for Glioblastoma CSLC Differentiation Induced by Inhibition of the PI3K/Akt/mTOR and MEK/ERK Signaling Pathways

Given the close association between FoxO3a function and glioblastoma CSLC differentiation induced by PI3K/Akt/mTOR and/or MEK/ERK pathway inhibition, we next examined whether FoxO3a is required for glioblastoma CSLC differentiation induced by inhibition of the signaling pathways. In patient glioblastoma CSLCs (SJ28P3) in which FoxO3a expression is knocked down, the induction of βIII-tubulin and GFAP expression by combinational treatment with NVP-BEZ235 and SL327 was substantially impaired when compared with the control cells (Fig. 2A). Immunocytochemical analysis also revealed that βIII-tubulin- and GFAP-positive cells increased markedly after the combinational inhibitor treatment in control cells but only marginally in FoxO3a knockdown cells (Fig. 2B). Essentially identical results were obtained from A172 CSLCs (Fig. 2D), except that A172 CSLCs, like the original A172 cells from which they were derived, did not express GFAP under any culture condition [15]. In addition, we noted that FoxO3a knockdown also suppressed the increase of βIII-tubulin and GFAP expression induced by individual inhibition of either the PI3K/Akt/mTOR or MEK/ERK pathway (Supporting Information Fig. 2). Together, these data indicate that the promotion of glioblastoma differentiation by inhibition of the PI3K/Akt/mTOR and/or MEK/ERK pathways requires FoxO3a expression. However, in contrast to the differentiation markers, FoxO3a knockdown had no appreciable effect on the expression change of the stem/progenitor markers (Nestin, Musashi, Bmi1, and Sox2) induced by the combinational inhibitor treatment (Fig. 2A, 2D). In line with these results, sphere formation by FoxO3a knockdown cells was inhibited as efficiently as that by control cells in the presence of the inhibitors (Fig. 2C, 2E), suggesting that, in contrast to its essential role in differentiation, FoxO3a may not necessarily be required for "initial" inhibition of self-renewal by concurrent inhibition of the PI3K/Akt/mTOR and MEK/ERK pathways (see Discussion section).

FoxO3a is Controlled by Akt- and ERK-Mediated Phosphorylation and is Under the Influence of mTOR/p70S6K-Mediated Negative Feedback in Glioblastoma CSLCs

Although the data presented thus far clearly indicate that FoxO3a, essential for glioblastoma CSLC differentiation, is under the control of the PI3K/Akt/mTOR and MEK/ERK pathways, it remains to be shown whether these signaling pathways control FoxO3a indirectly or directly through Akt and/or ERK-mediated phosphorylation of FoxO3a in glioblastoma CSLCs. To address this issue, we first examined the phosphorylation status of FoxO3a at sites presumed to be phosphorylated by Akt and ERK. In support of the idea that FoxO3a is under the control of the PI3K/Akt/mTOR and MEK/ERK pathways through direct phosphorylation by Akt and ERK, the dual PI3K/mTOR inhibitor NVP-BEZ235 inhibited FoxO3a phosphorylation at Ser253, known to be phosphorylated by Akt [16], and the MEK inhibitors SL327 and U0126 inhibited its phosphorylation at consensus sequences for ERK phosphorylation [17] (Fig. 3A, left; Supporting Information Fig. 3A, left). We next examined whether Akt- and ERK-mediated phosphorylation has a functional role in the control of FoxO3a. To this end, we exogenously expressed in glioblastoma CSLCs three types of FoxO3a mutants, 3A (Akt), 3A (ERK), and 6A, in which the Akt phosphorylation sites (T32/S253/S315), ERK phosphorylation sites (S294/S344/S425), and both (T32/S253/S315, S294/S344/S425) are substituted for alanine residues, respectively [16, 17] (Supporting Information Fig. 4). Of note, FoxO3a phosphorylation at ERK consensus sequences was abolished in the 3A (ERK) mutant, indicating that ERK does phosphorylate FoxO3a at these serine residues mutated in the 3A (ERK) mutant (Fig. 3B). Subcellular fractionation and immunocytochemical studies clearly demonstrated that the mutations at the Akt and ERK phosphorylation sites had effects on FoxO3a localization equivalent to the inhibition of PI3K/mTOR and MEK, respectively: the 6A mutant was localized predominantly in the

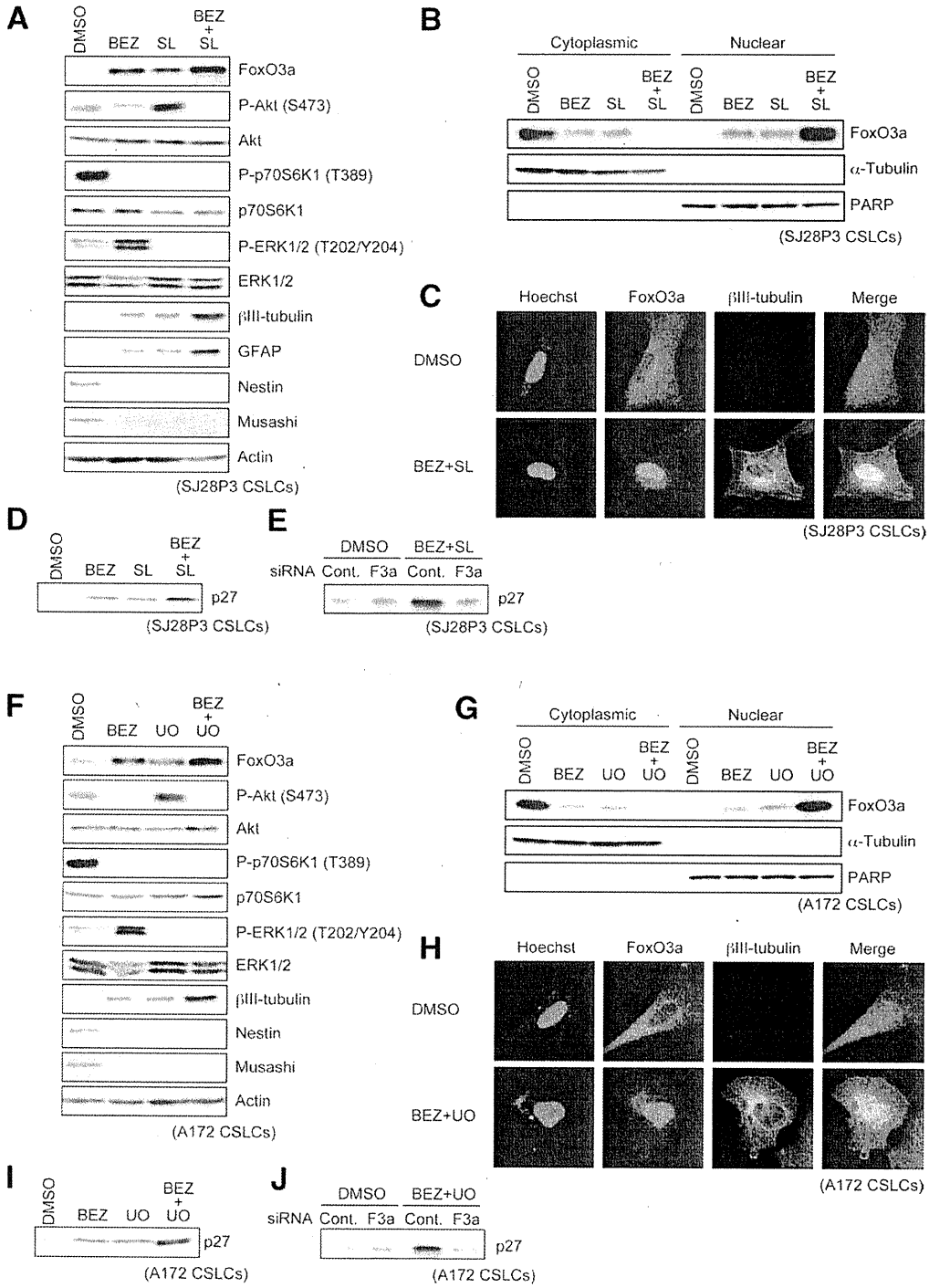

Figure 1. The expression and activity of FoxO3a are closely correlated with glioblastoma cancer stem-like cell (CSLC) differentiation induced by concurrent inhibition of the PI3K/Akt/mammalian target of rapamycin and MEK/extracellular signal-regulated kinase pathways. SJ28P3 (**A–E**) and A172 (**F–J**) CSLCs were cultured in the absence or presence of NVP-BEZ235 (BEZ, 1 μM) and/or SL327 (SL, 10 μM)/U0126 (UO, 10 μM) for 3 days. (**A, D, F, I**): Cell lysates were subjected to immunoblot analysis with the indicated antibodies. Alternatively, the cells were subjected to subcellular fractionation, with the amount of FoxO3a in the cytoplasmic and nuclear fractions being assessed by immunoblot analysis (**B, G**) or immunocytochemistry (**C, H**) with the indicated antibodies. (**E, J**): CSLCs transfected with the control or FoxO3a (F3a) siRNAs were treated, 10 hours after transfection, with BEZ (1 μM) and SL (10 μM)/UO (10 μM) for 3 days, and then the cell lysates were subjected to immunoblot analysis with the indicated antibodies. Abbreviations: CSLC, cancer stem-like cell; DMSO, dimethyl sulfoxide; PARP, poly(ADP-ribose) polymerase; siRNA, short-interfering RNA.

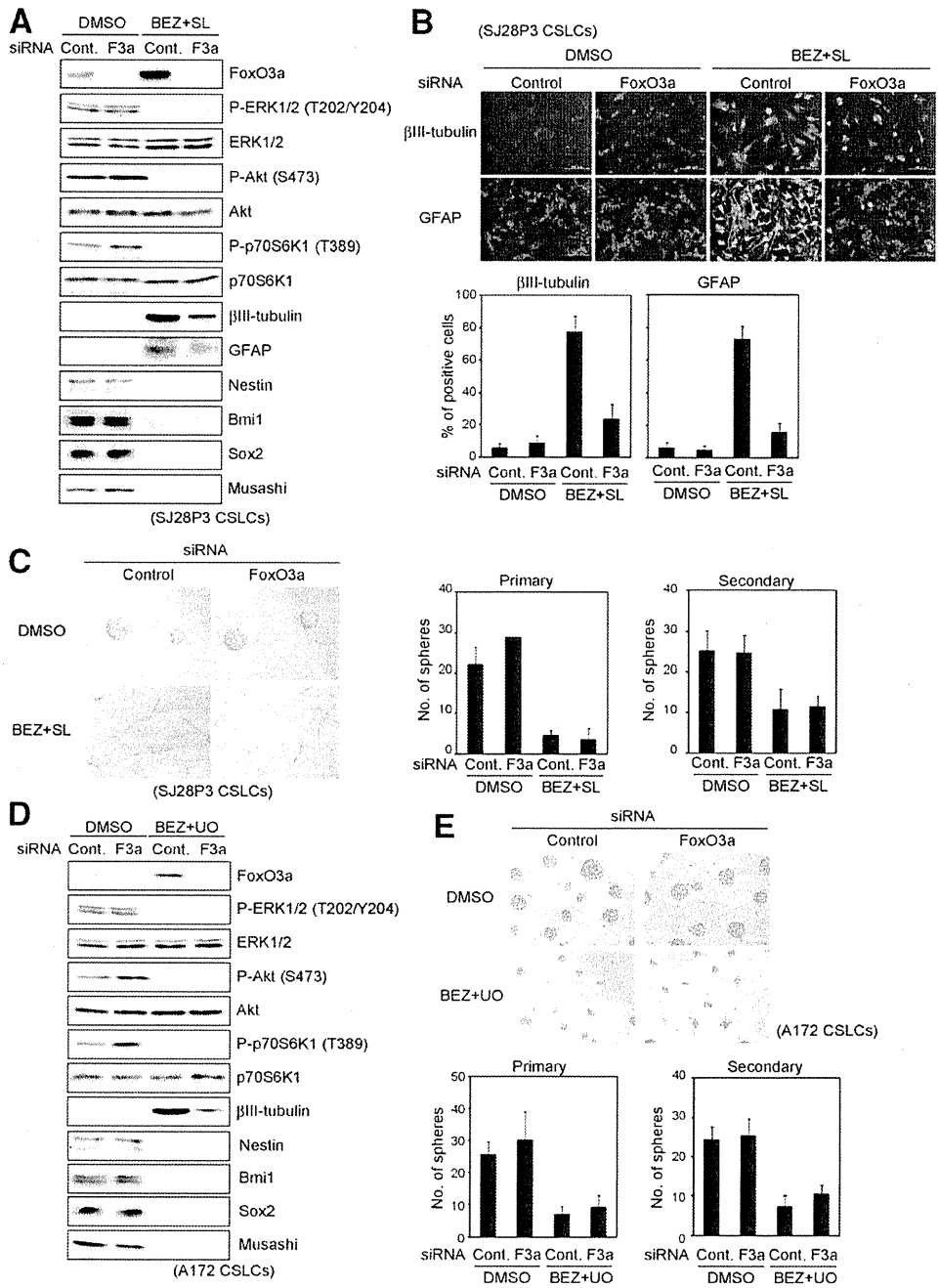

Figure 2. FoxO3a activation is required for differentiation of glioblastoma cancer stem-like cell (CSLC) induced by concurrent inhibition of the PI3K/Akt/mammalian target of rapamycin and MEK/extracellular signal-regulated kinase pathways. SJ28P3 **(A–C)** and A172 **(D, E)** CSLCs transfected with the control or FoxO3a (F3a) siRNAs were treated, 10 hours after transfection, with NVP-BEZ235 (BEZ, 1 μM) and SL327 (SL, 10 μM)/U0126 (UO, 10 μM) for 3 days. The cells were then subjected to immunoblot analysis with the indicated antibodies **(A, D)** or to immunocytochemistry with the indicated antibodies **(B)**. Alternatively, the cells were subjected to the primary and secondary sphere formation assays **(C, E)**. Note that the primary and secondary sphere formation assays were performed in the presence and absence (i.e., after washout) of the indicated inhibitors, respectively. Abbreviations: Cont., control; CSLC, cancer stem-like cell; DMSO, dimethyl sulfoxide; GFAP, glial fibrillary acidic protein; siRNA, short-interfering RNA.

nucleus, whereas the two 3A mutants were present both in the cytoplasm and the nucleus. Importantly, the 3A (Akt) mutant accumulated in the nucleus in the presence of the MEK inhibitor, whereas the 3A (ERK) mutant did so in the presence of the PI3K/mTOR inhibitor (Fig. 3C–3E), indicating that the lack of Akt and ERK phosphorylation sites, respectively, mimics the effect of PI3K/Akt/mTOR and MEK/ERK pathway inhibition. Collectively, these results suggest that direct phosphorylation of FoxO3a by Akt and ERK controls its subcellular localization and that FoxO3a efficiently

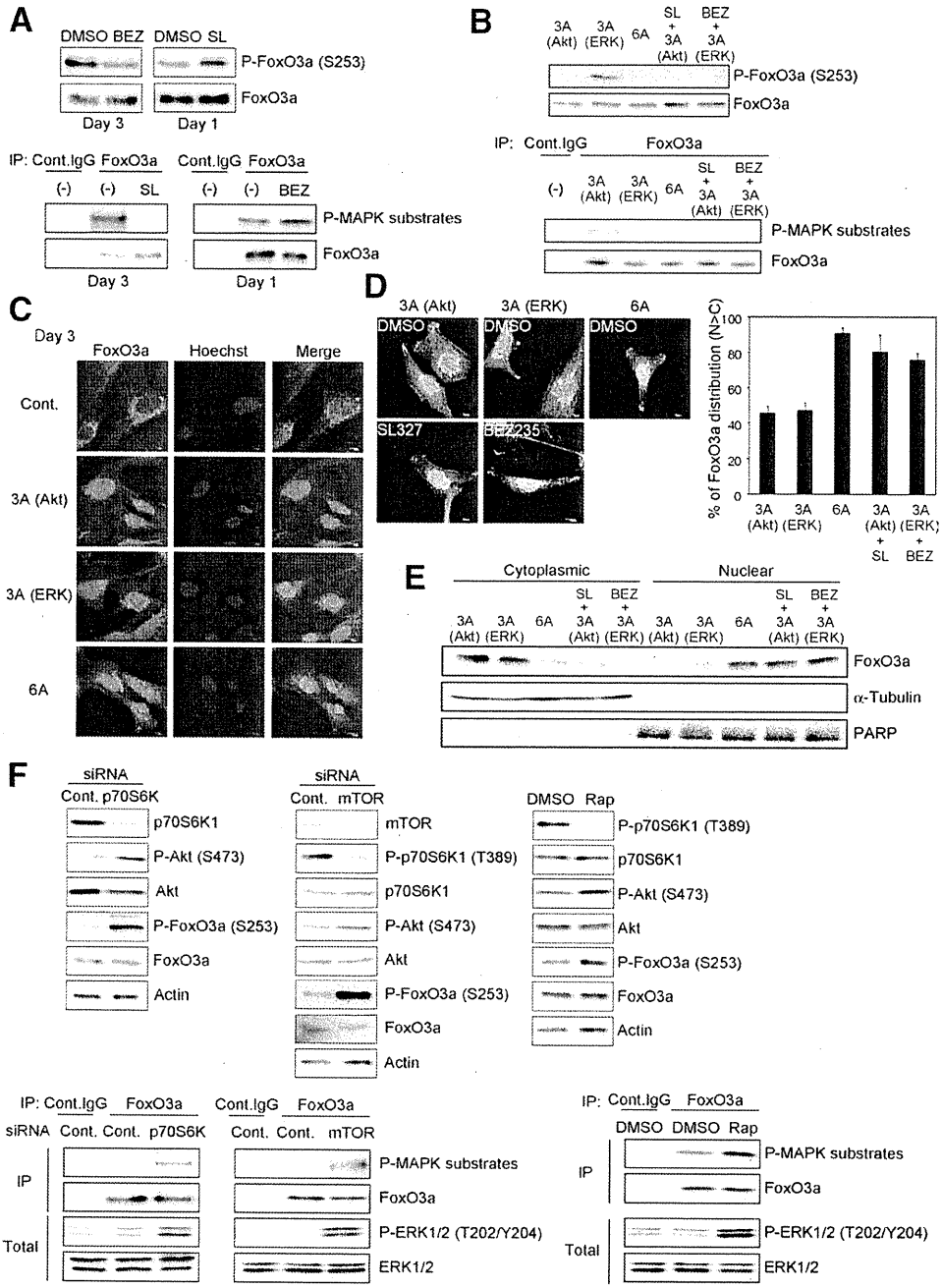

Figure 3. The PI3K/Akt/mammalian target of rapamycin (mTOR) and MEK/extracellular signal-regulated kinase (ERK) pathways, which are under cross-inhibitory regulation via mTOR-p70S6K, directly control FoxO3a phosphorylation at the Akt and ERK phosphorylation sites as well as its subcellular localization. SJ28P3 cancer stem-like cells (CSLCs) were cultured in the absence or presence of NVP-BEZ235 (BEZ, 1 μM) or SL327 (SL, 10 μM) for 3 days (**A**, left) or 1 day (**A**, right). The cells were then subjected to immunoblot analysis with the indicated antibodies (**A**, upper) or to immunoprecipitation (IP) with control IgG (Cont. IgG) or an anti-FoxO3a antibody, followed by immunoblot analysis with the indicated antibodies (**A**, lower). **(B–E):** SJ28P3 CSLCs were infected with an empty control vector or with lentiviral vectors expressing the 3A (Akt) (T32A/S253A/S315A), 3A (ERK) (S294A/S344A/S425A), or 6A (T32A/S253A/S315A, S294A/S344A/S425A) FoxO3a mutant. After 1-day, cells were treated with BEZ (1 μM) or SL (10 μM) for 3 days and subjected to immunoblot analysis with the indicated antibodies (**B**, upper), to IP with control IgG or an anti-FoxO3a antibody followed by immunoblot analysis with the indicated antibodies (**B**, lower) or to subcellular fractionation, with the amount of FoxO3a in the cytoplasmic and nuclear fractions being assessed by immunoblot analysis (**E**). Alternatively, cells were immunolabeled for FoxO3a (**C, D**, left). **(F):** SJ28P3 CSLCs were transfected with the indicated siRNAs or treated with rapamycin (50 nM). After 2 days, the cells were subjected to immunoblot analysis with the indicated antibodies (upper) or to IP with control IgG or an anti-FoxO3a antibody, followed by immunoblot analysis with the indicated antibodies (lower). Abbreviations: Cont., control; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; siRNA, short-interfering RNA; IP, immunoprecipitation; PARP, poly(ADP-ribose) polymerase; Rap, rapamycin.

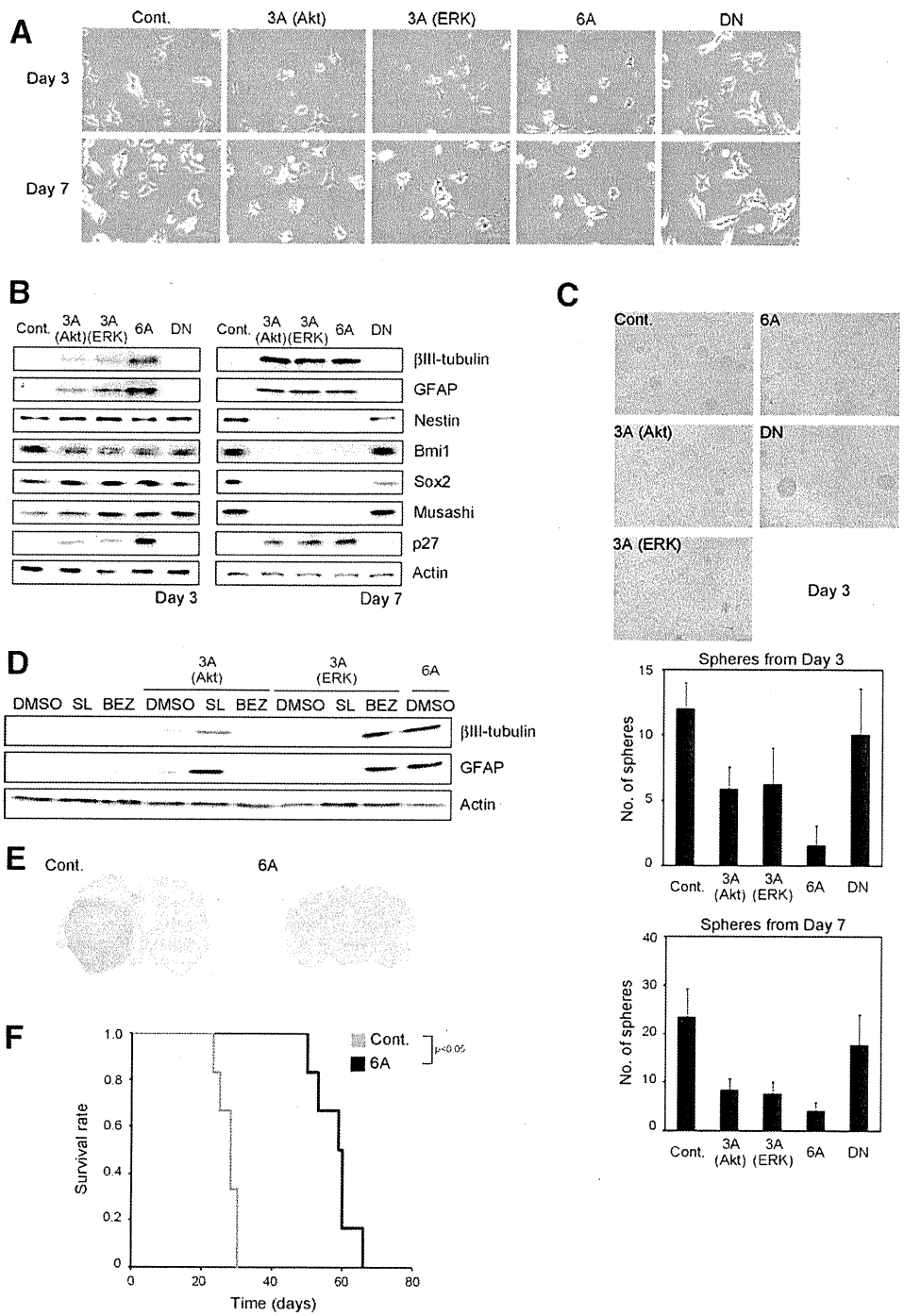

Figure 4. Expression of the Akt and extracellular signal-regulated kinase (ERK) phosphorylation site-defective FoxO3a mutant is sufficient to induce differentiation and inhibit tumorigenicity of glioblastoma cancer stem-like cells (CSLCs). SJ28P3 CSLCs were infected with an empty, control vector or with lentiviral vectors expressing the 3A (Akt) (T32A/S253A/S315A), 3A (ERK) (S294A/S344A/S425A), 6A (T32A/S253A/S315A, S294A/S344A/S425A), or a dominant-negative FoxO3a mutant. Approximately 3 or 7 days after infection, cells were observed under a phase-contrast microscope (**A**), subjected to immunoblot analysis with the indicated antibodies (**B**) or to sphere formation assays (**C**). Alternatively, cells were treated, 1-day after infection, with NVP-BEZ235 (BEZ, 1 μM) or SL327 (SL, 10 μM) for 3 days, and cell lysates were subjected to immunoblot analysis with the indicated antibodies (**D**). (**E, F**): SJ28P3 CSLCs were infected with a control lentiviral vector or with a lentiviral vector expressing the 6A FoxO3a mutant. Approximately 7 days after infection, the cells (1×10^4) were injected intracranially into BALB/c-nu/nu mice. The mice were sacrificed 30 days after intracranial injection, and brain tissue sections were stained with H&E (**E**). Survival of mice (six mice per group) was evaluated by Kaplan-Meier analysis (**F**). Abbreviations: Cont., control vector; DMSO, dimethyl sulfoxide; DN, dominant-negative; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein.

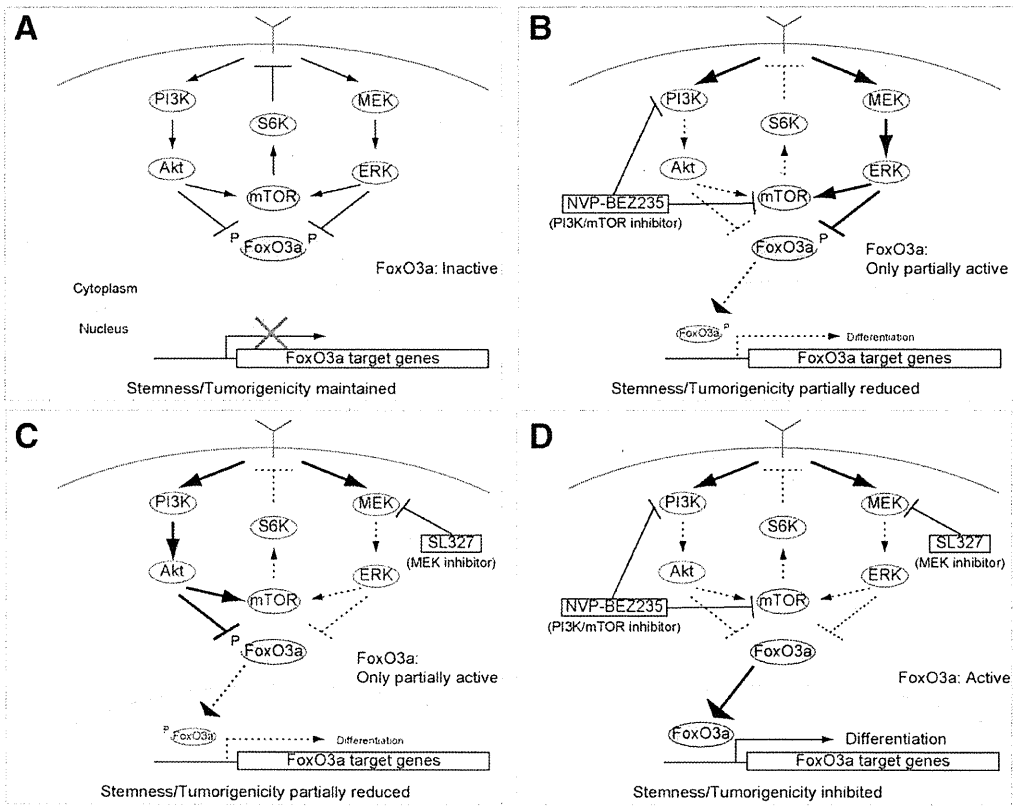

Figure 5. Schematic summary for FoxO3a-mediated control of glioblastoma cancer stem-like cell (CSLC) differentiation and tumorigenicity by the PI3K/Akt/mammalian target of rapamycin (mTOR) and MEK/extracellular signal-regulated kinase (ERK) pathways. **(A):** The PI3K/Akt/mTOR and MEK/ERK pathways, which negatively regulate themselves and each other via p70S6K (S6K), are active in glioblastoma CSLCs. FoxO3a phosphorylated by both Akt and ERK is efficiently retained in the cytoplasm and remains inactive. **(B):** When the PI3K/Akt/mTOR pathway is selectively inhibited, FoxO3a phosphorylated by ERK can still be retained in the cytoplasm. Loss of PI3K/Akt/mTOR pathway-mediated inhibition further activates the MEK/ERK pathway, which contributes to increased phosphorylation of FoxO3a at the ERK sites and consequently, to the maintenance of the stem cell state. **(C):** The same is true when the MEK/ERK pathway is selectively inhibited. **(D):** Upon concurrent inhibition of the PI3K/Akt/mTOR and MEK/ERK pathways, FoxO3a is no longer phosphorylated either by Akt or ERK. Nonphosphorylated FoxO3a efficiently translocates to the nucleus, where it activates its target genes associated with differentiation of glioblastoma CSLCs and suppresses their tumorigenicity. Abbreviations: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; mTOR, mammalian target of rapamycin.

accumulates in the nucleus when it is phosphorylated neither by Akt nor ERK.

We have previously demonstrated that crosstalk between the PI3K/Akt and MEK/ERK pathways through an mTOR-p70S6K axis-dependent feedback loop is involved in the maintenance of self-renewal and tumorigenicity of glioblastoma CSLCs [14]. Therefore, we investigated the possibility that Akt and ERK phosphorylation of FoxO3a is under the control of this crosstalk in glioblastoma CSLCs. In support of this possibility, the MEK inhibitors SL327 and U0126 increased FoxO3a phosphorylation at Ser253, and the PI3K/mTOR inhibitor NVP-BEZ235 did so at ERK consensus sequences (Fig. 3A, right; Supporting Information Fig. 3A, right). Essentially identical results were obtained when MEK1/2 or PI3K isoforms and mTOR were knocked down (Supporting Information Fig. 5). As reported earlier [14], inactivation of the mTOR-p70S6K axis, by means of siRNA-mediated knockdown or a pharmacological inhibitor, induced the phosphorylation of upstream Akt and ERK. Under these conditions, FoxO3a phosphorylation at the Akt and ERK phosphorylation sites was apparently increased (Fig. 3F; Supporting Information Fig. 3B). Thus, the data suggest that, in glioblastoma CSLCs, FoxO3a is regulated through phosphorylation by Akt and ERK, both of which are under the control of the negative feedback loop involving the downstream mTOR-p70S6K axis.

Expression of FoxO3a Lacking Akt- and ERK-Mediated Phosphorylation is Sufficient to Induce Differentiation and to Inhibit Self-Renewal and Tumorigenicity of Glioblastoma CSLCs

We then questioned the impact of Akt- and/or ERK-mediated phosphorylation of FoxO3a on the maintenance of stem cell-like properties of glioblastoma CSLCs. To this end, we again took advantage of the FoxO3a mutants lacking Akt and/or ERK phosphorylation sites. On day 3, glioblastoma CSLCs transduced with the expression vector encoding the 6A mutant showed prominent morphological changes characterized by extension of cellular processes (Fig. 4A), suggesting that the cells may be undergoing differentiation. Cells expressing the 3A mutants at comparable levels to the 6A mutant (Supporting Information Fig. 4) showed essentially similar morphological changes but apparently in a much more modest manner (Fig. 4A). Immunoblot analysis revealed the parallel increase of differentiation markers, GFAP and βIII-tubulin, together

with p27 in cells expressing the 3A and 6A mutants, quite consistent with the morphological changes (Fig. 4B). In addition, the 3A (Akt) and 3A (ERK) mutants, which were by themselves less efficient inducers of differentiation than the 6A mutant, efficiently induced the expression of βIII-tubulin and GFAP in the presence of the MEK and PI3K/mTOR inhibitors, respectively (Fig. 4D). In contrast, increased expression of wild-type FoxO3a, which was not overexpressed as efficiently as the mutants probably due to increased protein degradation when compared with the nonphosphorylatable FoxO3a mutants [19], did not induce differentiation under the experimental condition (Supplementary Information Fig. 6). Thus, the results together suggest that the absence of Akt- and ERK-mediated phosphorylation cooperatively activates FoxO3a to induce differentiation of glioblastoma CSLCs. Although the differences between the 3A and 6A mutants became less prominent on day 7, this could be explained by the fact that these mutants are overexpressed and by saturation of the differentiation-inducing effect (Fig. 4A, 4B). Significantly, we found that the expression of neural stem/progenitor cell markers such as Nestin, Bmi1, Sox2, and Musashi remains unchanged on day 3 but is inhibited on day 7, which may imply that FoxO3a activation is sufficient to inhibit stem/progenitor cell marker expression but induces differentiation independently of their inhibition. We also examined the effect of FoxO3a mutant expression on the self-renewal capacity of glioblastoma CSLCs. In close correlation to their ability to inhibit the expression of stem/progenitor cell markers, the three FoxO3a mutants inhibited sphere formation by glioblastoma CSLCs (Fig. 4C). Similar results were obtained when glioblastoma CSLCs (#38) derived from another patient were used (Supporting Information Fig. 7).

Since the 6A FoxO3a mutant was so efficient at inducing differentiation as well as in depleting the pool of self-renewing glioblastoma CSLCs, we next asked whether the 6A mutant expression could also inhibit their tumorigenic potential. To test this idea, glioblastoma CSLCs transduced with the expression vector for the 6A mutant were injected intracranially into immunodeficient mice, and the animals were monitored for brain tumor formation and survival (Fig. 4E, 4F). Although all animals injected with cells transduced with the control vector died within one month after injection (median survival is 28 days), animals injected with cells transduced with the 6A mutant expression vector survived significantly longer (median survival is 59.5 days). In a parallel experiment, mice were sacrificed 30 days after injection, and the brains were examined for the presence of tumors. While massive tumor growth was confirmed in control animals, no visible tumor growth was detected in animals receiving glioblastoma CSLCs transduced with the 6A mutant expression vector. Collectively, the results suggest that forced activation of the FoxO3a pathway via 6A mutant expression by itself is sufficient to induce differentiation and reduce the tumorigenic potential of glioblastoma CSLCs without requiring inhibition of the PI3K/Akt/mTOR and MEK/ERK pathways.

Discussion

We have recently shown that the PI3K/Akt/mTOR and MEK/ERK pathways, which are aberrantly activated in glioblastomas, crosstalk via a p70S6K-mediated negative feedback mechanism and coordinately regulate differentiation, self-renewal, and tumorigenicity of glioblastoma CSLCs [14]. Here in this study, we have provided evidence that FoxO3a is at least one of the missing links connecting the two pathways. We have shown that concurrent inhibition of the PI3K/Akt/mTOR and MEK/ERK pathways, which negatively regulate each other in glioblastoma CSLCs, causes efficient loss of phosphorylation at Akt and ERK phosphorylation sites, nuclear accumulation, and increased transcriptional activity of FoxO3a, that FoxO3a is required for the differentiation of glioblastoma CSLCs induced by the inhibition of these signaling pathways, and that forced activation of the FoxO3a pathway, conversely, is sufficient to induce differentiation and inhibit self-renewal and tumorigenicity of glioblastoma CSLCs. These findings demonstrate that FoxO3a functions at the convergence of the PI3K/Akt/mTOR and MEK/ERK pathways controlling glioblastoma CSLCs (schematically summarized in Fig. 5). Intriguingly, we have also discovered in the course of this study that oxidative stress activates FoxO3a, induces differentiation, and inhibits self-renewal as well as the tumorigenicity of glioblastoma CSLCs at least in part in a FoxO3a-dependent manner yet without affecting the PI3K/mTOR and MEK/ERK pathways (Supporting Information Fig. 8). This additional observation further suggests the possibility that FoxO3a may function not only as a signal integrator specific to the PI3K/Akt/mTOR and MEK/ERK pathways but may also have a more general and pivotal role in the control of glioblastoma CSLCs.

To our knowledge, there are only a limited number of, and seemingly conflicting, reports on the role of FoxO3a in the control of stem cell-like properties of CSLCs. In prostate cancer, the FoxO3a pathway was more activated in the non-CSLC population than in the CSLC population, and FoxO3a knockdown led to expansion of the CSLC pool as well as to increased self-renewal and tumorigenic capacity [20]. In contrast, in chronic myeloid leukemia, leukemia-initiating cells (LICs) were enriched in cells exhibiting nuclear localization of FoxO3a, and FoxO3a deficiency impaired the leukemia-initiating potential of LICs [21]. Apparently, the contrasting roles of FoxO3a in the maintenance of CSLC properties documented in these reports suggest that FoxO3a may have different functions in CSLCs of different cancer types. In this regard, we have demonstrated for the first time in this study that, in glioblastoma CSLCs, FoxO3a has a "negative" role in the maintenance of stem cell-like properties. This finding may be in line with the recent observation that FoxO3a expression in human glioma samples is correlated with the malignant grade and that low FoxO3a expression is associated with poor patient outcome [22]. Of note, conversely, high expression of FoxO3a has been reported to be associated with a poor prognosis in acute myeloid leukemia [23], in agreement with its "positive" role in the maintenance of stem cell-like properties in LICs, again underscoring the different roles of FoxO3a in different cancer types. The opposite roles of FoxO3a in these two different cancer types may be reflected by the fact that LICs display a quiescent phenotype whereas glioblastoma CSLCs display a proliferative phenotype [21, 24]. At present, it remains totally unknown what underlies such heterogeneity of CSLCs, but identification of FoxO3a transcriptional targets involved in the control of each cancer type might provide clues to understand the underlying mechanism at the molecular level.

Strikingly, the function of FoxO3a in glioblastoma CSLCs was in sharp contrast to its function reported for neural stem cells [25]. Although FoxO3a function was required for the differentiation of glioblastoma CSLCs in our study, it was essential for the maintenance of neural stem cells in adult mice. Indeed, FoxO3a was active and localized in the nucleus in self-renewing neural stem cells, while it was active and localized in the nucleus in differentiated glioblastoma CSLCs. FoxO3a phosphorylation patterns were also highly contrasting: FoxO3a was phosphorylated by Akt in differentiated neural stem cells, whereas it was phosphorylated by Akt in

self-renewing glioblastoma CSLCs [25] (this study). These findings are rather surprising in that the same molecule functions in an entirely opposite manner in neural stem cells and glioblastoma CSLCs, which are generally presumed to share common mechanisms of regulation [26]. Although it currently remains unknown what causes this contrasting difference between neural stem cells and glioblastoma CSLCs in terms of FoxO3a function, it could be a great advantage when FoxO3a is considered as a therapeutic target (see below).

We found in this study that FoxO3a is required for the differentiation but not for the inhibition of self-renewal, both of which were induced by inhibition of the PI3K/Akt/mTOR and MEK/ERK pathways in glioblastoma CSLCs. This finding indicates that, under the control of the two signaling pathways, distinct molecular mechanisms govern the maintenance/loss of stem cell-like properties and the acquisition of differentiation phenotypes by glioblastoma CSLCs, the former being independent of and the latter being dependent on FoxO3a. Importantly, forced activation of FoxO3a not only induced the expression of differentiation markers but also subsequently inhibited stem/progenitor marker expression, and consequently the self-renewal capacity of glioblastoma CSLCs as indicated by decreased sphere formation. This suggests that FoxO3a may not be required for the initial loss of stem cell-like properties of glioblastoma CSLCs preceding the expression of differentiation markers but may contribute to ensuring and establishing a cellular condition in which cells can never restore the stem cell-like state. Therefore, it seems that the exact role of FoxO3a in glioblastoma CSLCs is to promote their "irreversible commitment" to differentiation. However, it is unlikely that FoxO3a is the sole transcription factor for the differentiation of glioblastoma CSLCs, because FoxO3a expression was not required for the differentiation of glioblastoma CSLC induced by serum. As the members of the FoxO family are known for their overlapping functions [19], it is possible that other FoxO family members, for instance FoxO1, might compensate for the lack of FoxO3a function to promote differentiation of glioblastoma CSLCs.

The results of this study suggest that, although they do not necessarily exclude the involvement of other redundant mechanisms as discussed above, FoxO3a is at least in part responsible for the inhibition of tumorigenic potential of glioblastoma CSLCs by combinational inhibition of the PI3K/Akt/mTOR and MEK/ERK pathways. These results give rise to a novel and important notion from a therapeutic perspective that any measures that activate FoxO3a would be sufficient to promote differentiation of glioblastoma CSLCs and thereby inhibit their self-renewal and tumorigenic potential. Indeed, we found in support of this notion that oxidative stress induced by H_2O_2 treatment effectively deprives glioblastoma CSLCs of their tumorigenic potential independently of the PI3K/Akt/mTOR and MEK/ERK pathways (Supplementary Information Fig. 8). To date, a number of other mechanisms have been reported to regulate FoxO3a. For example, IkappaB kinase (IKKβ) or serum-glucocorticoid-related kinases phosphorylate FoxO3a, which triggers nuclear export and cytoplasmic sequestration, thereby inhibiting access to DNA binding sites [27, 28]. Intriguingly, in the case of acute myeloid leukemia, IKKβ overcomes PI3K/Akt and ERK/MAPK to control FoxO3a activity, and blockade of the IKK/nuclear factor kappa B (NFκB) signaling pathway has already been proposed as a possible therapeutic strategy [29–31]. It has also been reported that metformin, which activates AMP-activated kinase (AMPK) by increasing the cellular AMP/ATP ratio, inhibits cancer cell growth and regulates FoxO3a through AMPK [32, 33]. Therefore, targeting these molecules involved in the regulation of FoxO3a function could be a potential and attractive way to control glioblastoma CSLCs. Significantly, the role of FoxO3a in glioblastoma CSLCs and adult neural stem cells appears to be entirely different as described above. Therefore, it might be possible to selectively inhibit the tumorigenicity of glioblastoma CSLCs while sparing the function of neural stem cells, making FoxO3a an ideal candidate of molecular targeting therapy.

Summary

In summary, we have disclosed in this study that FoxO3a is kept in check under the control of the PI3K/Akt/mTOR and MEK/ERK pathways to maintain the stem cell-like state of glioblastoma CSLCs and that unleashing FoxO3a from this restraint is sufficient to commit them to differentiation and suppress their tumorigenicity. These findings will contribute to the development of novel treatment strategies for glioblastoma.

Acknowledgments

We thank Dr. Kimishige Ishizaka for invaluable comments on the manuscript. This work was supported by Grants-in-Aid for Scientific Research, Challenging Exploratory Research, Young Scientists and for Scientific Research on Priority areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; by a Grant-in-Aid from the Global COE program of the Japan Society for the Promotion of Science; by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan; and by a grant from the Japan Brain Foundation.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to declare.

References

1 Stupp R, Mason WP, van den Bent MJ et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–996.
2 Ignatova TN, Kukekov VG, Laywell ED et al. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia 2002;39:193–206.
3 Hemmati HD, Nakano I, Lazareff JA et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA 2003;100:15178–15183.
4 Singh SK, Hawkins C, Clarke ID et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401.
5 Galli R, Binda E, Orfanelli U et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004;64:7011–7021.
6 Park DM, Rich JN. Biology of glioma cancer stem cells. Mol Cells 2009;28:7–12.
7 Liu G, Yuan X, Zeng Z et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer 2006;5:67.
8 Blazek ER, Foutch JL, Maki G. Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia. Int J Radiat Oncol Biol Phys 2007;67:1–5.
9 Frank NY, Schatton T, Frank MH. The therapeutic promise of the cancer stem cell concept. J Clin Invest 2010;120:41–50.
10 Piccirillo SG, Reynolds BA, Zanetti N et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 2006;444:761–765.