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3. Asymptomatic Cervical/Intracerebral Vascular Stenosis/Occlusion

3-1. Asymptomatic intracranial cerebral artery stenosis

Recommendations

1. Management of risk factors for atherosclerosis is recommendable in the prevention of ischemic stroke in patients with asymptomatic major cerebral artery stenosis and occlusion (Grade C1).
2. Antiplatelet therapy is recommended for asymptomatic major cerebral artery stenotic and occlusive lesions as necessary after evaluation by a specialist (Grade C1).

Evidence

There is no evidence that EC-IC bypass surgery is more effective than drug therapy alone for recurrent cerebral ischemic symptoms in patients with symptomatic internal carotid and middle cerebral artery occlusion or stenosis[66-68] (Ib). In particular, no analysis limited to asymptomatic patients has been conducted, so no recommendable scientific rationales are available. In addition, asymptomatic middle cerebral artery stenosis is not a likely risk factor for ischemic stroke.[69,70]

Bypass surgery has been known to significantly improve vascular reserve in patients with an increased cerebral oxygen extraction fraction[71] (III), whereas there is no evidence of this in asymptomatic patients. Currently, a joint study to evaluate the efficacy of bypass surgery in patients with a markedly low increase rate of cerebral blood flow following the acetazolamide challenge test is ongoing[72] (Ib).

Treatment should be considered for individual patients when they have to undergo surgery requiring general anesthesia or interruption of blood flow due to other diseases or have bilateral lesions. Because there is no evidence regarding performing an EC-IC bypass before or simultaneously with coronary artery revascularization or revascularization of contralateral lesion in patients with major cerebral artery occlusive lesions, patients should be assessed on an individual basis[73] (III). No report on cerebral endovascular treatment, such as vasodilation only, is available in patients with asymptomatic stenosis.

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3. Asymptomatic Cervical/Intracerebral Vascular Stenosis/Occlusion

3-2. Asymptomatic cervical carotid artery stenosis/occlusion

Recommendations

1. For mild or moderate asymptomatic carotid stenosis, management of risk factors for atherosclerosis and, as necessary, medical treatment including antiplatelet therapy is recommended (Grade C1). There is no adequate scientific evidence supporting revascularization, such as carotid endarterectomy and percutaneous angioplasty/stenting (Grade C1).
2. For severe (≥60%) asymptomatic carotid stenosis, in addition to optimal medical treatment including antiplatelet therapy, carotid endarterectomy (CEA) by surgeons and at facilities well experienced in surgery and perioperative management is recommended (Grade B).
3. For patients with severe (≥80%) asymptomatic carotid stenosis who are good candidates for carotid endarterectomy (CEA), in addition to optimal medical treatment, percutaneous carotid artery angioplasty and stenting (CAS) may be appropriate alternatives (Grade B). Nonetheless, based on reported incidences of perioperative complications, cerebral infarction and the mortality rate, no consensus on the indications for CEA or CAS for this group has been reached.

Note 1: See also the Section "II. CEREBRAL INFARCTION/TRANSIENT ISCHEMIC ATTACK (TIA), 4. Cerebral Infarction in the Chronic stage, 4-7. Carotid endarterectomy (CEA)" related to this section.

Evidence

No evidence has as yet been presented indicating that antiplatelet agents are effective for the primary prevention of ischemic stroke in patients with asymptomatic carotid stenosis.[74] The effect of antithrombotic therapy for the primary prevention of ischemic stroke in patients with asymptomatic carotid occlusion or intracerebral vascular stenosis/occlusion has not been studied. In an observational study in patients with asymptomatic carotid stenosis, a multivariate analysis showed that orally administered antiplatelet therapy was related to decreases in the incidences of ischemic stroke and cardiovascular mortality rate.[75] According to a meta-analysis and report on intima-media thickness (IMT), anti-hypertensive drugs,[76] statins[77] (Ia), and pioglitazone[78] (Ib), an oral antihyperglycemic drug, had the effect of delaying in the progress of subsequent IMT enlargement; thus, they may be effective for the prevention of the progress of cervical/intracerebral vascular lesions and associated ischemic stroke, but evidence to illustrate this is currently unavailable.

For severe asymptomatic carotid stenosis with a stenosis rate of ≥60%, in addition to optimal medical treatment including antiplatelet agents and hyperlipidemia-improving drugs, the incidence of stroke is lower when carotid endarterectomy (CEA) is performed[79-81] (Ib). However, the Asymptomatic Carotid Atherosclerosis Study (ACAS) has reported that a high treatment level with an incidence <3% of perioperative complications is required for surgeons and facilities performing CEA in patients with asymptomatic carotid stenosis[80] (Ib). There is no adequate scientific rationale demonstrating the efficacy of CEA in patients at high CEA-associated risk such as those with concurrent ischemic cardiac disease or who have undergone cervical surgery/radiotherapy[79-81] (Ib).

There is no clear rationale to enable recommending CEA in cases of mild or moderate asymptomatic carotid stenosis[80,81] (Ib). A subanalysis in the ACAS showed no difference in the frequency of perioperative complications or ipsilateral cerebral infarction within 5 years in patients with asymptomatic carotid stenosis and contralateral carotid artery occlusion between CEA and medical treatment[82] (Ib).

The evidence of the effect of percutaneous carotid artery angioplasty and CAS on the prevention of stroke in patients with severe asymptomatic carotid stenosis remains inadequate (IIa). Nonetheless, many subgroup analyses in randomized controlled trials and non–randomized controlled trials, particularly in patients with a high CEA-associated risk, have increasingly reported that CAS is "equivalent" or "noninferior" to CEA[83-90] (IIa).

No report has as yet been published on extracranial-intracranial bypass for asymptomatic carotid artery occlusion including patients with decreased cerebrovascular reserve; the scientific basis for recommending the procedure is thus lacking.

There is no scientific basis for recommending simultaneous CEA with heart bypass surgery or CEA as pretreatment in asymptomatic carotid stenosis patients with coronary artery disease.[91,92]

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4. Asymptomatic Cerebral Arteriovenous Malformation (AVM)

Recommendations

The effect of craniotomy or stereotactic radiotherapy to improve the prognosis in patients with asymptomatic cerebral arteriovenous malformation (AVM) remains unclear (Grade C1). Treatment policies for asymptomatic AVMs need to be determined for individual patients while taking into account the risks of the natural course and treatment of AVMs in general.

Evidence

For asymptomatic AVMs, a follow-up examination or treatment, such as craniotomy and stereotactic radiotherapy, should be considered. In terms of the natural history of asymptomatic AVMs, the annual incidence of bleeding has been reported to be 6.44%, equivalent to that in bleeding patients[93] (IIb). For asymptomatic AVMs classified into Spetzler-Martin grade 4 or 5, craniotomy was reportedly not recommended because of its associated high risk[94] (IIa). There is currently no evidence comparing the prognosis after a follow-up examination and that after treatment, nor does evidence exist comparing the effect of surgical treatment and stereotactic radiotherapy. A randomized controlled trial on the effect of treatment in patients with nonhemorrhagic AVMs has been undertaken in the United States[95] (Ib).

In general, the frequency of AVMs is 13.4/million population/year, and it is accompanied by hemorrhage in about 50% of cases.[96] The annual incidence of bleeding in patients with nonhemorrhagic AVMs including asymptomatic cases is approximately 2%-3%.[93] After bleeding, the annual incidence of bleeding elevates to approximately 15%, and then in several years, the annual incidence decreases to approximately 1%-2%.[93] General factors related to hemorrhage in patients with AVMs reportedly include deep-seated nidus,[97,98] deep drainer,[98] age (elderly,[98] young[99]), posterior cranial fossa lesions,[100] nidus size (small,[101] large[102]), and concurrent aneurysms related to the nidus[103] (IIb-III). One report states that the incidence of bleeding is high for AVMs classified into Spetzler-Martin grade 4 or 5[104] (IIb), but another report documents that the annual incidence of bleeding is as low as 1.5%.[94] The mortality rate from the initial bleeding in patients with AVMs is reportedly approximately 10%,[105] and compared with intracerebral hemorrhage (ICH) attributable to other diseases, AVMs have been rarely reported to cause any permanent neurological deficit regardless of initial or recurrent bleeding[106] (IIb). For AVMs classified into Spetzler-Martin grade 1 or 2, good surgical outcomes have been reported overall for general AVM treatment, and particularly better outcomes have been reported in children[99,107] (IIb-III). Stereotactic radiotherapy is more

suitable for patients with deep small lesions who are at a high surgery-associated risk,[108,109] and a better therapeutic effect has been reported in children than in adults[110-112] (IIb-III). Stereotactic radiotherapy is associated with post-treatment bleeding prevention not only during the period after complete occlusion, but also in the latency period until angiographically confirmed complete occlusion has been achieved[113] (IIb). The complete occlusion rate for AVMs achieved by endovascular embolization alone is as low as 22%.[114] It is considered a preoperative adjuvant therapy for surgery patients who are unable for whatever reason to undergo other treatment approaches[115] because it has been reported that the complications associated with preoperative embolization cannot be ignored.[115] There are, for example, reports that embolization before stereotactic radiotherapy decreased the occlusion rate after radiotherapy[94,112] (IIa-IIb).

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5. Unruptured Cerebral Aneurysms

5-1. Diagnosis of and screening for unruptured cerebral aneurysms

Recommendations

In the diagnosis of and screening for unruptured cerebral aneurysms, the use of magnetic resonance angiography (MRA) (≥0.5T) is preferable. For further assessment such as potential surgical indication, a diagnosis based on digital subtraction angiography (by catheterization), 3-dimensional angiography (by catheterization), and 3-dimensional helical computed tomography is more highly recommended (Grade A).

Evidence

The gold standard in the diagnosis of cerebral aneurysms is cerebral angiography by catheterization. However, with the recent advancement of imaging techniques, MRA and 3-dimensional helical computer tomography have enabled adequately accurate diagnoses, with a reported sensitivity and specificity for these 2 approaches of 76%-98% and 85%-100%, respectively[116] (IIa).

The familial frequency of unruptured cerebral aneurysms is several-fold higher than normal for family members of patients who have developed subarachnoid

hemorrhage. There is no clear evidence as to whether or not screening for their presence or absence using MRA or angiography and treatment would be beneficial for predicting the life expectancy and the quality of life of the patients[117] (IIa). Furthermore, the significance of screening for unruptured cerebral aneurysms in adults without any family history of subarachnoid hemorrhage has not been revealed[118] (III).

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5. Unruptured Cerebral Aneurysm

5-2. Early actions in the case of identifying unruptured cerebral aneurysms

Recommendations

1. When a diagnosis of unruptured cerebral aneurysm is made, the patient should be provided with all accurate information such as the natural history (annual incidence of bleeding) of unruptured cerebral aneurysms, and written informed consent should be obtained for subsequent treatment strategies (Grade B).
2. Patients may develop depression or anxiety after a diagnosis of an unruptured cerebral aneurysm. Informed consent fully taking this aspect into account is important, and counseling is advisable when depression symptoms or anxiety are severe (Grade C1).
3. In the case where good communication of the risks between a patient and physician cannot be achieved, obtaining a second opinion from another physician or at another institution is recommended (Grade C1).

Evidence

The natural course of unruptured cerebral aneurysm, candidates for therapy, and selection of the therapeutic strategy remain undetermined in many cases; thus, it is not easy for patients to accurately understand the information their physician has given them. Patients tend to consider both the risk of rupture and risks associated with treatment as being quite high[119,120] (IIb); it has therefore been reported that a diagnosis of an unruptured cerebral aneurysm increases their anxiety levels [121,122] (IIa).

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5. Unruptured Cerebral Aneurysm

5-3. Treatment of unruptured cerebral aneurysms

Recommendations

1. When a patient is diagnosed with an unruptured cerebral aneurysm, his or her suitability as a treatment candidate must first be carefully considered, taking into account their relevant background characteristics such as age and health condition; characteristics of the lesion such as size, site and form; the natural history of unruptured cerebral aneurysms; and treatment outcomes at the facility and by the surgeon. It is advisable to obtain adequate informed consent from the patient before determining whether or not therapy is applicable and the treatment strategies (Grade B).
2. Based on the natural history (ie, risk of rupture) of unruptured cerebral aneurysms, when the patient's life expectancy is 10-15 years or more, it is recommended in principle to consider treatment for the following lesions (Grade C1):
   (1) Unruptured cerebral aneurysm s of 5-7 mm or larger
   (2) Even if the size is <5 mm, treatment can be considered if the lesion is
      A) a symptomatic cerebral aneurysm
      B) a cerebral aneurysm located at a site such as the posterior circulation, anterior communicating artery, and internal carotid-posterior communicating artery
      C) a cerebral aneurysm with morphological characteristics such as a high dome/neck aspect ratio, irregular shape and blebbing.

   However, as mentioned in the previous section (5-2. Early actions in the case of identifying unruptured cerebral aneurysms), a diagnosis of unruptured cerebral aneurysm is reported to cause depressive symptoms and/or anxiety. It is not appropriate to judge the appropriateness of treatment only based on a benefit-risk analysis on a rupture rate and risk of complications or a cost-effectiveness analysis. Therefore,

Recommendations, *continued*

treatment should be considered for individual patients if they do not meet the foregoing criteria, and adequate informed consent should be obtained.

3. When following up lesions without surgical treatment such as craniotomy and endovascular treatment, the patient should be advised to avoid smoking and heavy alcohol consumption, and hypertension should be treated (Grade A). During such surgery-free follow-up, imaging studies should be performed regularly, approximately every 6 months to 1 year (Grade C1).
4. In the case of endovascular treatment, during the entire follow-up period incomplete occlusion and recurrence after the treatment must be very carefully and regularly assessed (Grade B).
5. After craniotomy with clipping, a very long-term follow-up is highly recommended (Grade C1).

Evidence

1. Risks associated with rupture

The number of high evidence-level reports on the rupture rate of unruptured cerebral aneurysm is rather low. The natural history of unruptured cerebral aneurysms varies according to the size and site. Large aneurysms and symptomatic unruptured cerebral aneurysms have been reported to rupture at a higher rate. In addition to these, age, female, multiplicity, history of subarachnoid hemorrhage, smoking, irregular aneurysm shape, presence of blebbing, and high dome neck aspect ratio have been reported to promote rupture[123-132] (IIa-III).

The International Study of Unruptured Intracranial Aneurysms (ISUIA) study conducted at 53 Western sites published its interim report in 1998[125] (IIb), and a report on prospective data was added in 2003[126] (IIa). In a prospective follow-up study (1692 patients, 2686 aneurysms; mean age, 4.1 years; 6544 patients/year) reported in 2003, rupture rates for unruptured cerebral aneurysms measuring ≤7 mm were as follows. In a group without previous subarachnoid hemorrhage (Group 1), 0% during 5 years in Group A (internal carotid, anterior communicating artery and middle cerebral aneurysm) and 2.5% (annual, 0.5%) in the Group P (vertebrobasilar aneurysm and internal carotid-posterior communicating aneurysm); and in a group with concurrent ruptured cerebral aneurysms (Group 2), 1.5% (annual, 0.3%) in Group A and 3.4% (annual, 0.7%) in Group P. For larger cerebral aneurysms, no clear difference was seen according to the presence or absence of previous subarachnoid hemorrhage (SAH). The rupture rates were as follows: for a size of 7-12 mm, 2.6% (annual, 0.5%) in Group A and 14.5% (annual, 2.9%) in Group P; for a size of 13-24 mm, 14.5% (annual, 2.9%) in Group A and 18.4% (annual, 3.7%) in Group P; for a size of ≥25 mm, 40% (annual, 8%) in the Group A and 50% (annual, 10%) in the Group P. The 5-year mortality rate was 12.7%, and 33 (65%) of 51 patients died in whom rupture was noted.

No racial difference in the frequency of unruptured cerebral aneurysms has as yet been revealed. Nonetheless, because the incidence of SAHs is higher in Finland and Japan than other regions, the rupture rate of unruptured cerebral aneurysms may vary among races. In a meta-analysis (4795 patients, 26,122 patients/year from 19 articles) by Wermer et al, the annual rupture rates of unruptured cerebral aneurysms were 1.2% during a follow-up period of ≤5 years, 0.6% for 5-10 years, and 1.3% for ≥10 years. The rupture rates slightly differed according to the duration of follow-up and also varied according to size: 0.5% for ≤5 mm, 1.2% for 5-10 mm, and 1.5% for ≥10 mm. Factors with a significant difference were a size of ≥5 mm, posterior circulation, symptomatic, and Japanese and Finnish studies.[130,133]

In Japan, the following data have been reported on unruptured cerebral aneurysms: The annual rupture rate is 1.9-2.7%. A large size, posterior circulation, symptomatic, multiplicity, and multilocular form are reportedly factors for a high risk of rupture. However, it has been also reported that there is no difference in the rupture rate according to associated medical complications or the site of the aneurysm [127-129] (III).

The UCAS Japan is still in the intermediary stage so that no accurate rupture rate has been announced. However, the following information has been reported: The overall annual rupture rate is approximately 0.9%; the size and site of the cerebral aneurysm are important factors associated with rupture; and although no significant differences have been identified at this point, female, multiplicity, smoking and elderly are risk factors[134] (IIa).

Yonekura et al[135] conducted the SUAVe Study to prospectively follow up all patients with small unruptured cerebral aneurysms measuring <5 mm (329 patients, 380 lesions), and reported that rupture occurred in 3 patients (0.8%/year, 95% confidence interval, 0.2-3%), and ≥2 mm enlargement was noted for 18 lesions (4.7%) during the follow-up in 375 patients/year. They have reported that factors associated with enlargement and rupture include multiplicity, female sex, age ≥70 years, and anterior communicating and basilar aneurysms[135] (IIa).

The amount of data on enlargement rates of aneurysm is even smaller than that on rupture rates. Observational studies using magnetic resonance angiography and 3-dimensional helical computed tomography reported that approximately 7% of masses enlarged, and showed that time course changes in the enlargement rate based on the Kaplan-Meier method were 2.5% in the first year, 8% in the second year, and 17.6% in the third year; the risk for enlargement increased as time passed[136,137] (III). Factors associated with enlargement included size, multiform lesion, and basilar aneurysm and anterior communicating aneurysm[135-141] (IIa).

2. Complications associated with treatment

The incidence of complications associated with treatment is also a significant factor for determining treatment candidates for unruptured cerebral aneurysm. The incidence of complications associated with treatment is reportedly 1.9-12%[126,134,142,143] (IIa-III).

In the prospective ISUIA Study reported in 2003, the incidence of serious complications (modified Rankin scale ≥3; MMSE <24) and the mortality rate at 1 month after craniotomy were 12% and 1.5%, respectively, and those after endovascular treatment were 7.3% and 1.8%, respectively[126] (IIa). The following factors were reportedly associated with the aggravation of treatment outcomes: aneurysm size (≥12 mm) and site (posterior circulation) and history of symptomatic cerebral ischemia and symptomatic aneurysm in patients who underwent craniotomy, and aneurysm size (≥12 mm) and site (posterior circulation) in patients who received endovascular treatment.

Another study identified anterior communicating aneurysm and aneurysms at the bifurcation of the internal carotid artery as risk factors associated with craniotomy with clipping[142] (III). In another report, the mortality rate in patients with nongiant anterior circulation cerebral aneurysms was 0.8%, and the incidence of complications was 1.9%, so that the risk associated with treatment was low[143] (III). No significant difference was detected based on sample size, reported year of survey, age, sex, or aneurysm size or site[144] (III). In the interim report from UCAS Japan, a decrease of 2 or more points on the modified Rankin scale was noted in ≤5% of treatment outcomes in more than 2600 patients[134] (IIb).

With regard to the effect of craniotomy on higher brain functions in patients with unruptured cerebral aneurysms, the ISUIA Study has reported that decreased higher brain functions are particularly frequent in elderly patients[126] (IIa). However, there is a study showing that IQ actually improved, and that there was no difference in changes in other numerical values after craniotomy in patients with unruptured cerebral aneurysms based on detailed examinations including intellectual and memory tests and blood flow before and after the surgery[145] (IIb). It was further demonstrated that careful surgical techniques could minimize the development of complications related to higher brain functions.

The impact of the identification of unruptured cerebral aneurysm and treatment on the quality of life (QOL) have been also reported,[146-148] but there has been no large-scale study or clear evidence. An analysis using the Short Form-36 (SF-36) and depression scale reported the SF-36 score in preoperative patients with unruptured cerebral aneurysm was lower than for the general population, and the score might temporarily decrease immediately after treatment, but the QOL in all the patients returned to the level equivalent to that of the general population after 3 years. This report demonstrated that the detection of unruptured cerebral aneurysm decreased patient QOL, and treatment improved it.[148] It was also reported in another study that the patient QOL decreased for a short period after the diagnosis of an unruptured cerebral aneurysm, but recovered considerably within 1 year after surgery; however, there was also a complaint of a decreased QOL[146] (III).

3. Effectiveness analysis of treatment

Multiple cost-effectiveness analyses of treatment have been made by inputting conditions such as the annual rupture rate of unruptured cerebral aneurysm, the incidence of complications associated with treatment and patients' age. The analysis results vary according to input conditions. Consequently, these cost-effectiveness analyses or risk-benefit analyses cannot be rationales for providing concrete recommendations[149-155] (III).

For example, if the annual rupture rate is 1%, and the incidence of treatment-related complications is 5% or less, treatment is effective in patients aged ≤70 years. If the rupture rate is 2%, treatment is effective in patients aged ≤65 years even if the incidence of treatment-related complications is 5%-10%. It has been also reported that treatment is not beneficial if the incidence of complication is >10%.[149] There is also an analysis using input conditions based on prospective data from the ISUIA study that treatment of anterior circulation aneurysms measuring <7 mm is not useful in terms of the risk-benefit relationship[152] (III). For other aneurysms, the efficacy changes according to the patient age[153] (III). In a cost-effectiveness analysis on screening of unruptured cerebral aneurysm, the results have shown that when assuming the incidence of treatment-related complication is 10%, the mortality rate of SAH is 50%, and the prevalence of unruptured cerebral aneurysm is 3%, screening is valuable if the annual rupture rate of unruptured cerebral aneurysm is 2%, and the screening has no effect if the rate is 0.5%[154] (III).

4. Selection of therapy and long-term results

Studies comparing endovascular treatment and craniotomy with respect to the selection of therapy, treatment outcomes, duration of hospitalization and costs have been reported[156] (IIb),[157] (III). Both of these studies represent data from overseas countries having different healthcare systems and they were moreover non-randomized clinical studies; thus, they cannot be used to provide clear evidence.

Reports on the long-term results after treatment of unruptured cerebral aneurysm are scarce. With regard to a radical cure after endovascular treatment of unruptured cerebral aneurysm, one report stated that the use of Guglielmi detachable coils achieved complete or quasi-complete embolization for 91% of lesions, whereas the complete embolization rate was 54% based on an evaluation including 46 reports[158,159] (III); thus, a radical cure remains unidentified. Consequently, a thorough and regular postoperative follow-up regimen should be established to check for incomplete embolization and recurrence.

During long-term follow-up in patients with unruptured cerebral aneurysm treated with craniotomy, the incidence of subarachnoid hemorrhage due to recurrence of treated cerebral aneurysm and rupture of new aneurysms was reportedly 1.4% for 10 years and 11.4% for 20 years[160] (IIb); therefore, even if clipping is perfect, long-term follow-up is still necessary.

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

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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

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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 Lipofectamine 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

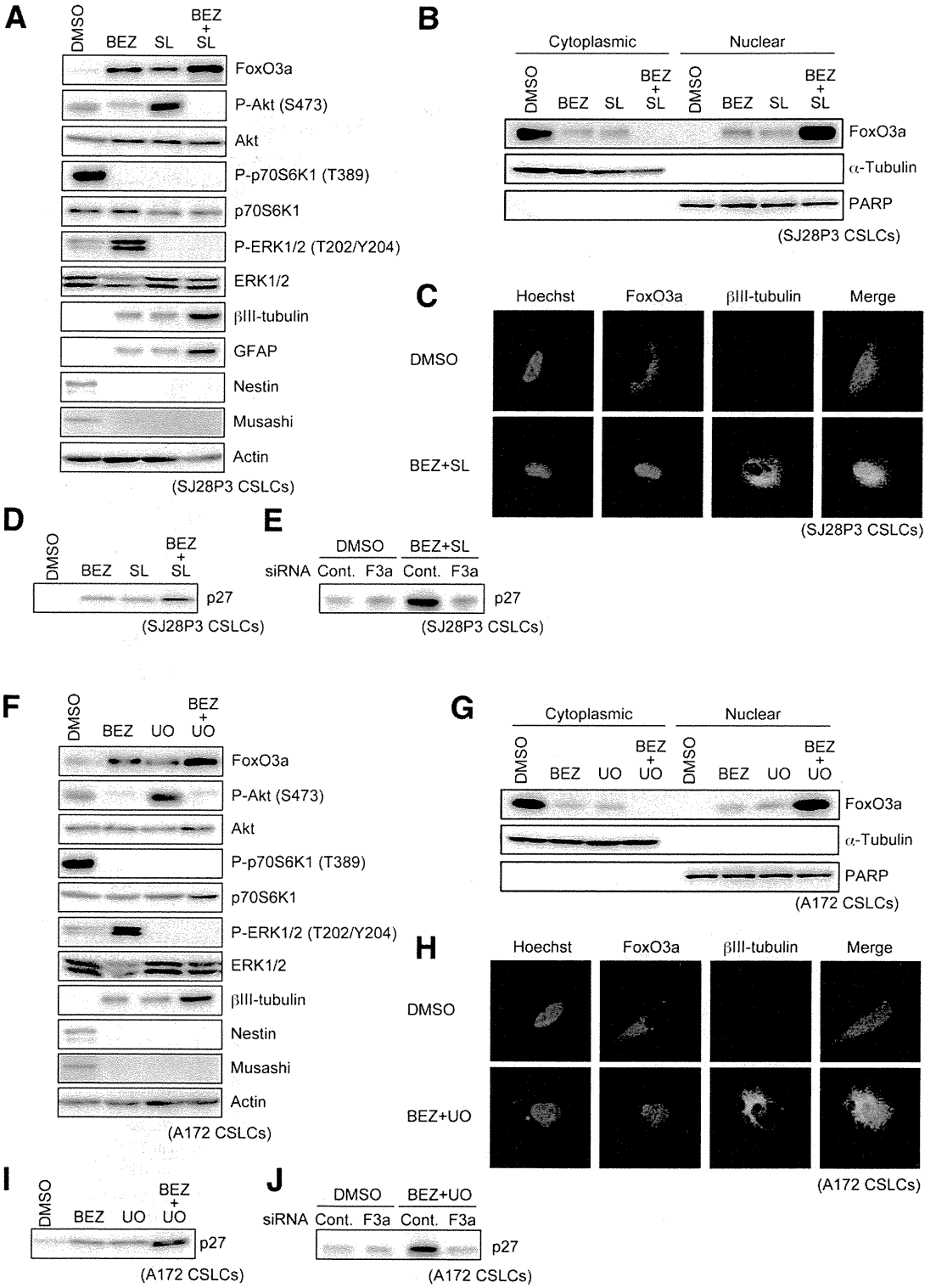

Figure 1. The expression and activity of FoxO3a are closely correlated with glioblastoma cancer stem-like cell (CSLC) differentiation induced by concurrent inhibition of the PI3K/Akt/mammalian target of rapamycin and MEK/extracellular signal-regulated kinase pathways. SJ28P3 **(A–E)** and A172 **(F–J)** 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.

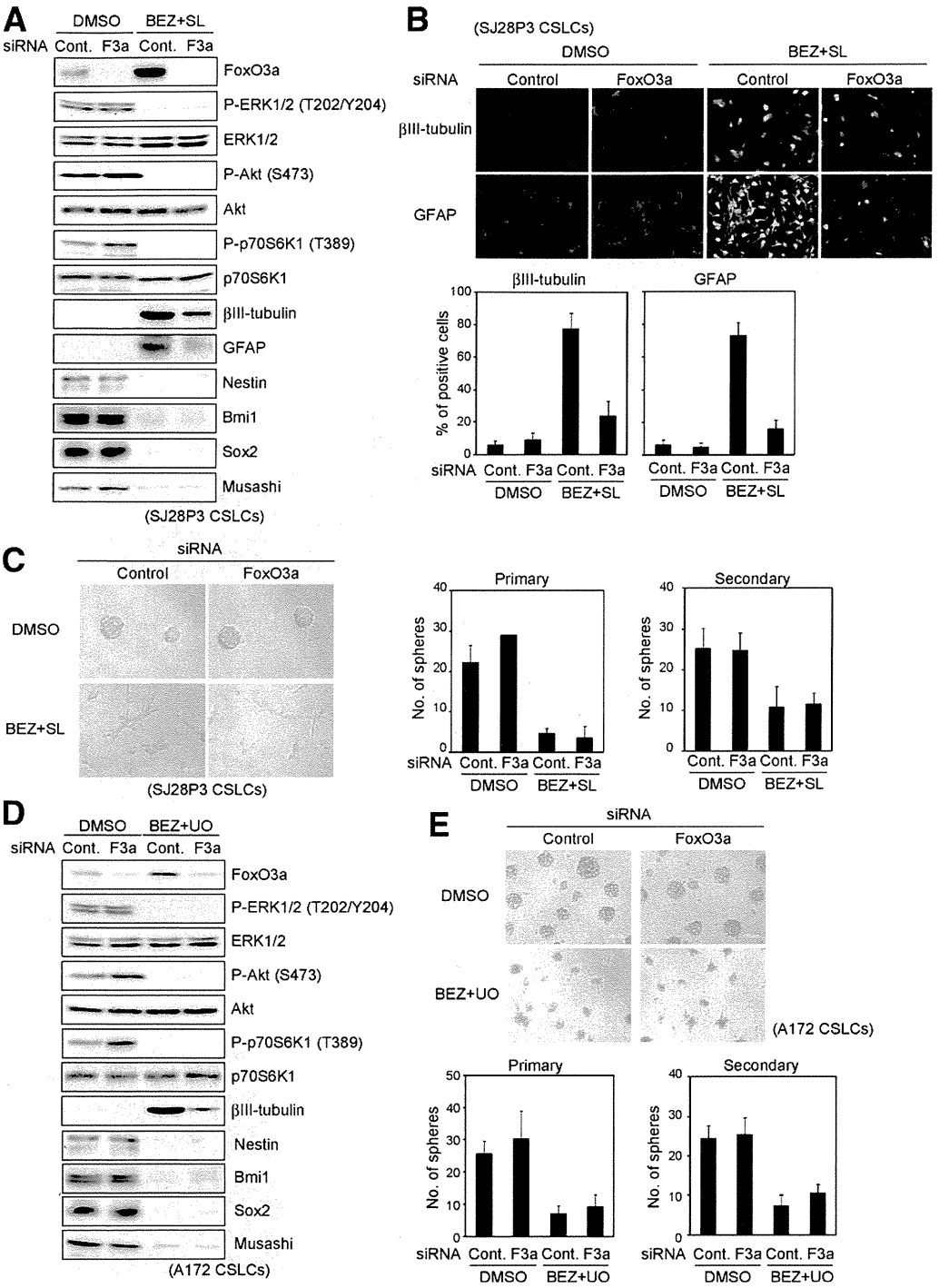

Figure 2. FoxO3a activation is required for differentiation of glioblastoma cancer stem-like cell (CSLC) induced by concurrent inhibition of the PI3K/Akt/mammalian target of rapamycin and MEK/extracellular signal-regulated kinase pathways. SJ28P3 **(A–C)** and A172 **(D, E)** CSLCs transfected with the 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

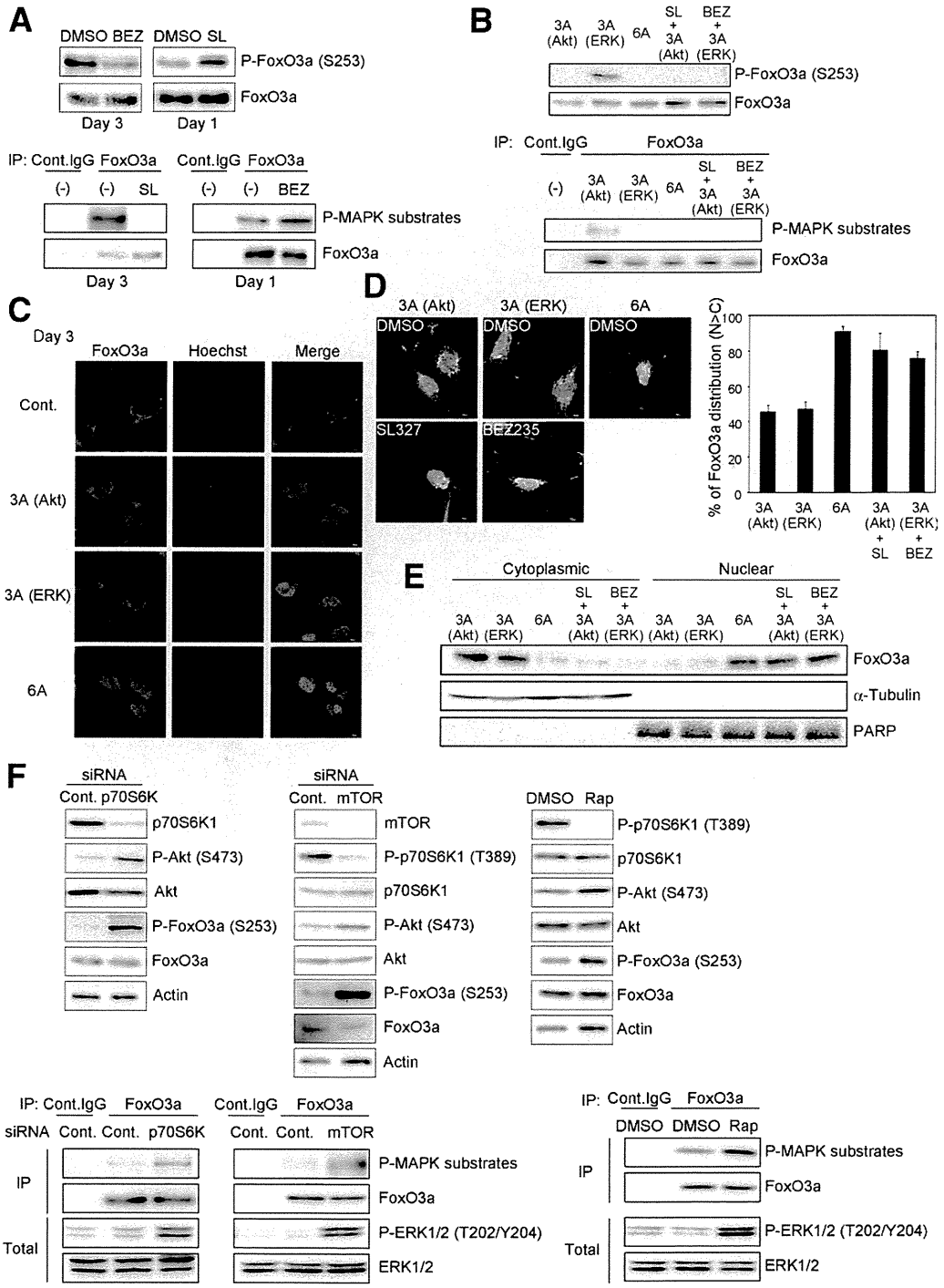

Figure 3. The PI3K/Akt/mammalian target of rapamycin (mTOR) and MEK/extracellular signal-regulated kinase (ERK) pathways, which are under cross-inhibitory regulation via mTOR-p70S6K, directly control FoxO3a phosphorylation at the Akt and ERK phosphorylation sites as well as its subcellular localization. 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.

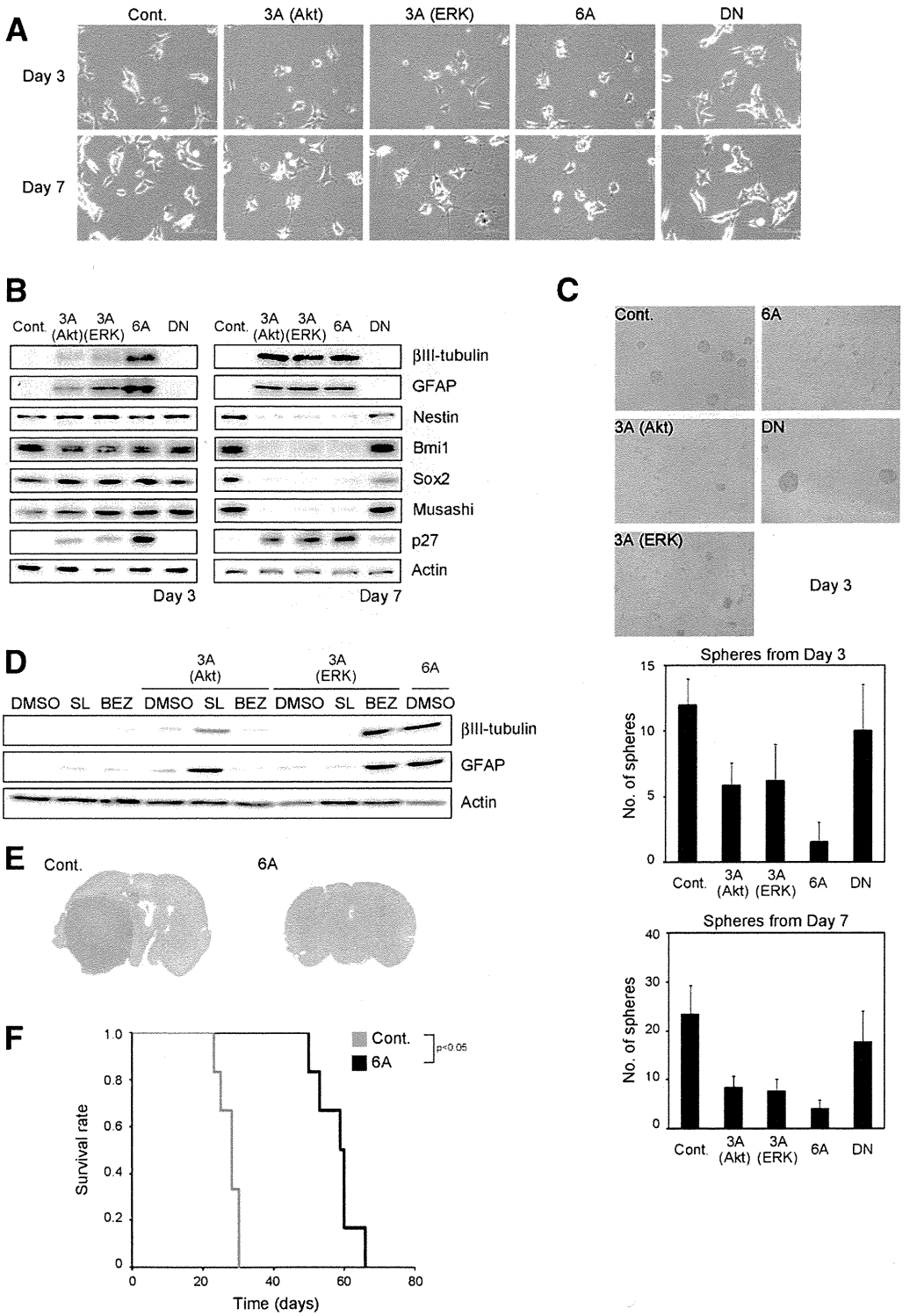

Figure 4. Expression of the Akt and extracellular signal-regulated kinase (ERK) phosphorylation site-defective FoxO3a mutant is sufficient to induce differentiation and inhibit tumorigenicity of glioblastoma cancer stem-like cells (CSLCs). SJ28P3 CSLCs were infected with an empty, control vector or with 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.

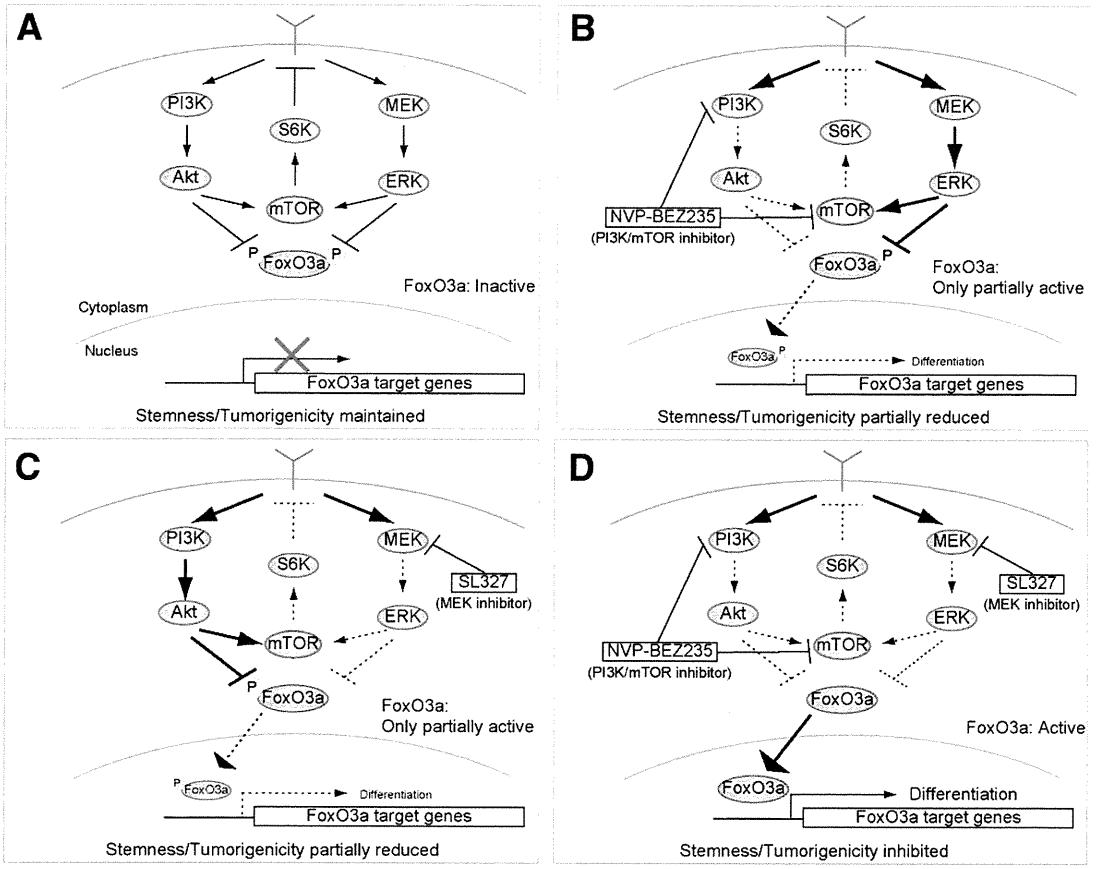

Figure 5. Schematic summary for FoxO3a-mediated control of glioblastoma cancer stem-like cell (CSLC) differentiation and tumorigenicity by the PI3K/Akt/mammalian target of rapamycin (mTOR) and MEK/extracellular signal-regulated kinase (ERK) pathways. **(A):** The PI3K/Akt/mTOR and MEK/ERK pathways, which 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.

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Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to declare.

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Intraoperative Magnetic Resonance Imaging in the Successful Surgical Treatment of an Arteriovenous Malformation

—Case Report—

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Abstract

A 44-year-old female presented with left occipital arteriovenous malformation (AVM) manifesting as sudden onset of severe headache. Magnetic resonance (MR) imaging and conventional angiography showed the left occipital AVM with hemorrhage. Intraoperative MR imaging (iMR imaging) and intraoperative time-resolved imaging of contrast-kinetics (iTRICKS) at 1.5 T revealed complete removal of the nidus of the AVM without conventional catheter angiography. Conventional catheter angiography is commonly used in preoperative and intraoperative examination of AVMs, and for documentation of the surgical outcome, but less-invasive techniques are desirable for both preoperative screening and intraoperative examination. iMR imaging with iTRICKS is less invasive and safer than conventional angiography for both brain tumor surgery and AVM surgery.

Key words: arteriovenous malformation, conventional angiography, intraoperative magnetic resonance imaging, time-resolved imaging of contrast-kinetics, surgery

Introduction

The complication rate of conventional angiography is not very high, approximately 1–2%, but long-term cannulation can cause embolisms.[18] Therefore, less-invasive tools are preferable for intraoperative examination, especially for intracranial arteriovenous malformations (AVMs). The successful use of different intraoperative visualization techniques for AVM surgery has been widely reported: catheter angiography, ultrasonography, indocyanine green (ICG) videoangiography, and time-of-flight (TOF) magnetic resonance (MR) angiography. Ultrasonography is easy to use, but the resolution is not satisfactory.[2,17] ICG videoangiography is not satisfactory if the AVM vessels are deep seated or not on the surface.[7,16] TOF MR angiography is also not adequate for AVM surgery due to poor temporal and spatial resolution.[15] Intraoperative MR imaging (iMR imaging) and neuronavigation have substantially changed the principles of neurosurgery.[1,3-6] iMR imaging can provide updated information on anatomy and on unanticipated brain events, so allowing safer and more accurate surgery.[10-12] Interoperative time-resolved imaging of contrast-kinetics (iTRICKS) can provide high spatial and temporal resolution during iMR imaging. We describe the efficacy of iMR imaging and iTRICKS during AVM surgery with a fully integrated 1.5 T neurosurgical system with neuronavigation and intraoperative imager.

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Case Report

A 44-year-old female presented with sudden onset of severe headache. MR imaging showed left occipital hemorrhage (Fig. 1A, B). Cerebral angiography disclosed a Spetzler-Martin grade 2 AVM in the left occipital lobe. The diameter of the nidus was approximately 3 cm. The AVM was fed by the posterior cerebral artery (Fig. 1D). Preoperative TRICKS provided excellent visualization of the AVM (Fig. 1C).

Left occipital craniotomy was performed and the nidus was removed. After completion, iMR imaging was performed using an integrated 1.5 T neurosurgical system with neuronavigation and intraoperative imager using flexible heart coils (Surgical Suite®; BrainLAB AG, Munich, Germany and GE Healthcare, Chalfont St. Giles, United Kingdom). The imaging parameters for TRICKS were as follows: repetition time 3.5 msec, echo time 1.4 msec, flip angle 15°, field of view 240 × 240 mm, acquisition matrix 256 × 160, section thickness 3.0 mm (resolution doubled using zero fill interpolation processing) to obtain 6.0-cm volume coverage, bandwidth 62.5 kHz, and excitations per scan 0.5. TRICKS reconstruction generated