

ORIGINAL ARTICLE

Targeting SPARC expression decreases glioma cellular survival and invasion associated with reduced activities of FAK and ILK kinases

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Secreted protein acidic and rich in cysteine (SPARC) is an extracellular glycoprotein expressed in several solid cancers, including malignant gliomas, upon adoption of metastatic or invasive behaviors. SPARC expression in glioma cells promotes invasion and survival under stress, the latter process dependent on SPARC activation of AKT. Here we demonstrate that downregulation of SPARC expression with short interfering RNA (siRNA) in glioma cells decreased tumor cell survival and invasion. SPARC siRNA reduced the activating phosphorylation of AKT and two cytoplasmic kinases, focal adhesion kinase (FAK) and integrin-linked kinase (ILK). We determined the contributions of FAK and ILK to SPARC effects using SPARC protein and cell lines engineered to overexpress SPARC. SPARC activated FAK and ILK in glioma cells previously characterized as responsive to SPARC. Downregulation of either FAK or ILK expression inhibited SPARC-mediated AKT phosphorylation, and targeting both FAK and ILK attenuated AKT activation more potently than targeting either FAK or ILK alone. Decreased SPARC-mediated AKT activation correlated with a reduction in SPARC-dependent invasion and survival upon the downregulation of FAK and/or ILK expression. These data further demonstrate the role of SPARC in glioma tumor progression through the activation of intracellular kinases that may provide novel therapeutic targets for advanced cancers.

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Introduction

The treatment of patients with primary brain tumors remains one of the greatest challenges in oncology. The

median survival of patients with the most aggressive primary brain tumors, World Health Organization grade IV gliomas (glioblastoma multiforme) has reached only 14.6 months in a trial of concurrent chemotherapy and radiotherapy (Stupp *et al.*, 2005). This high mortality is partially attributable to local invasion of tumor into normal brain, preventing complete surgical resection (Giese *et al.*, 2003). Although relatively little is known about the molecular events underlying glioma invasion, one implicated protein is secreted protein, acidic and rich in cysteine (SPARC/osteonectin/BM-40). SPARC is an extracellular glycoprotein abnormally expressed in many solid tumors, and SPARC expression can be further elevated upon conversion to invasive and metastatic tumors (Framson and Sage, 2004). SPARC is overexpressed in astrocytomas of all grades, but is preferentially expressed at sites of tumor invasion (Rempel *et al.*, 1998; Lal *et al.*, 1999). As SPARC is expressed at low levels in normal adult brain (Mundlos *et al.*, 1992), but highly expressed in gliomas, SPARC is an attractive target in brain tumor therapy. Increased SPARC expression levels have been linked to poor glioma patient survival (Rich *et al.*, 2005), which suggests that the reduction of SPARC expression may have therapeutic benefit. Indeed, expression of antisense oligonucleotides against SPARC in melanoma cells blocked tumor formation (Ledda *et al.*, 1997).

The precise biological and molecular mechanisms through which a reduction in SPARC expression might contribute to improved tumor therapy remain to be investigated. There is no well-established SPARC signal transduction pathway, and SPARC variably affects multiple cell behaviors, including proliferation, apoptosis, angiogenesis and invasion. However, in human glioma cell lines, SPARC increases invasion *in vitro* and *in vivo* (Schultz *et al.*, 2002; Rich *et al.*, 2003). SPARC also promotes cell survival through the activation of AKT/protein kinase B (Shi *et al.*, 2004), an intracellular serine/threonine kinase known to inhibit apoptosis and known to be activated in cancer (Datta *et al.*, 1999). We recently demonstrated that exogenous SPARC protein induces activating phosphorylation of AKT within minutes in a concentration-dependent manner, and stable overexpression of SPARC also increases AKT phosphorylation (Shi *et al.*, 2004). Therefore, we

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hypothesized that, in gliomas, decreasing SPARC expression could reduce AKT phosphorylation to decrease invasion and increase cell death.

We, now, demonstrate that targeting SPARC expression with short interfering RNA (siRNA) decreases cellular survival and invasion, with a concordant decrease in AKT signaling. Further, we demonstrate that SPARC-mediated AKT phosphorylation involves two cytoplasmic non-receptor tyrosine kinases: focal adhesion kinase (FAK) and integrin-linked kinase (ILK). SPARC activates FAK and ILK, and targeting FAK and/or ILK expression with siRNA reduces AKT phosphorylation and the ability of SPARC to promote invasion and survival. Therefore, our studies establish components of a novel signaling pathway through which SPARC regulates pro-tumorigenic cellular behaviors.

Results

siRNA-mediated downregulation of SPARC expression inhibits glioma cell invasion in vitro

siRNA represents both a tool to specifically delineate the function of numerous genes and also a potential

therapeutic modality. We tested the ability of a customized siRNA pool (Dharmacon, Chicago, IL, USA) to downregulate the expression of SPARC in two well-characterized glioma lines, U373MG and D54MG, which both express SPARC. SPARC siRNA was highly effective in decreasing SPARC expression relative to a non-targeting siRNA control: SPARC expression decreased by 72.5% in D54MG cells and 90.7% in U373MG cells (Figures 1a and 2a, and data not shown).

To determine if SPARC siRNA could reduce pro-tumorigenic cellular behaviors associated with SPARC expression, we first determined the effect of decreased SPARC expression on tumor cell invasion. We measured the capacity of glioma cells to invade through Matrigel, an artificial extracellular matrix, after transfection with a non-targeting control siRNA or SPARC siRNA. Decreased SPARC expression led to the inhibition of invasion by 54% and 49% in D54MG and U373, respectively. In addition, introduction of SPARC siRNA inhibited the invasion of D54MG and U373MG by greater than 50% when compared with two further siRNA controls directed against luciferase and lamin A/C (Supplementary Figure 1). Thus, SPARC siRNA can decrease glioma invasion *in vitro*.

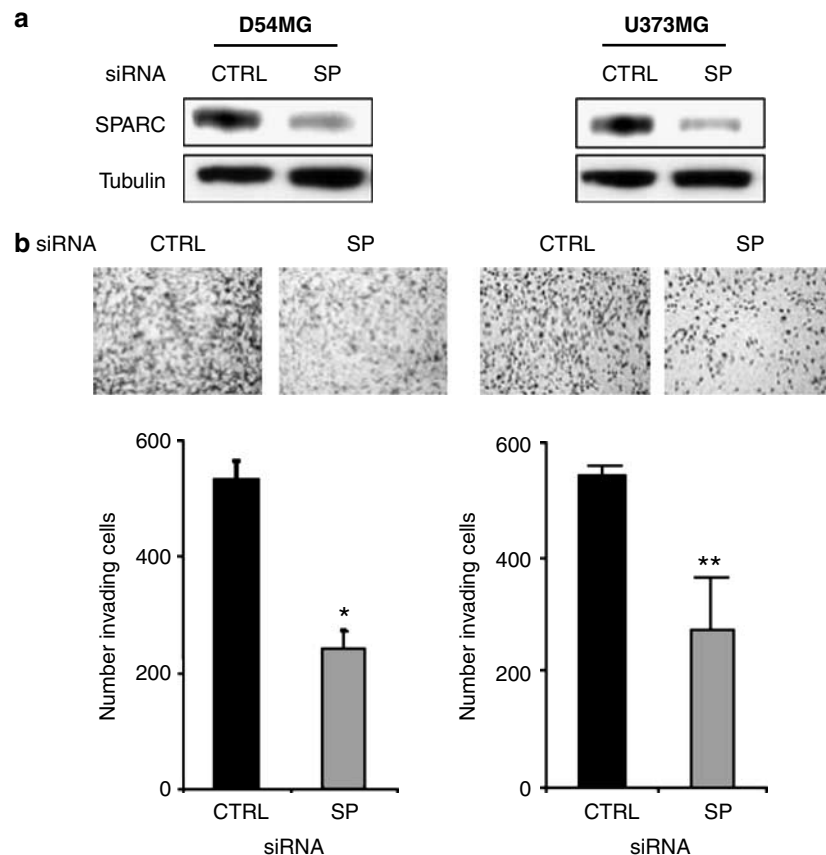


Figure 1 RNA interference of SPARC expression decreases glioma cellular invasion. D54MG and U373MG cells were transfected with equimolar quantities of siRNA of a non-targeting control (CTRL) or SPARC (SP) for 24 h, serum-starved for 24 h and then collected for Matrigel invasion. (a) Analysis by Western confirms successful targeting of SPARC expression. (b) Representative images of invading tumor cells are displayed and numbers of invading tumor cells quantified. * $P=0.0026$, SPARC siRNA compared with control siRNA; ** $P\leq 0.0394$, SPARC siRNA compared with control siRNA.

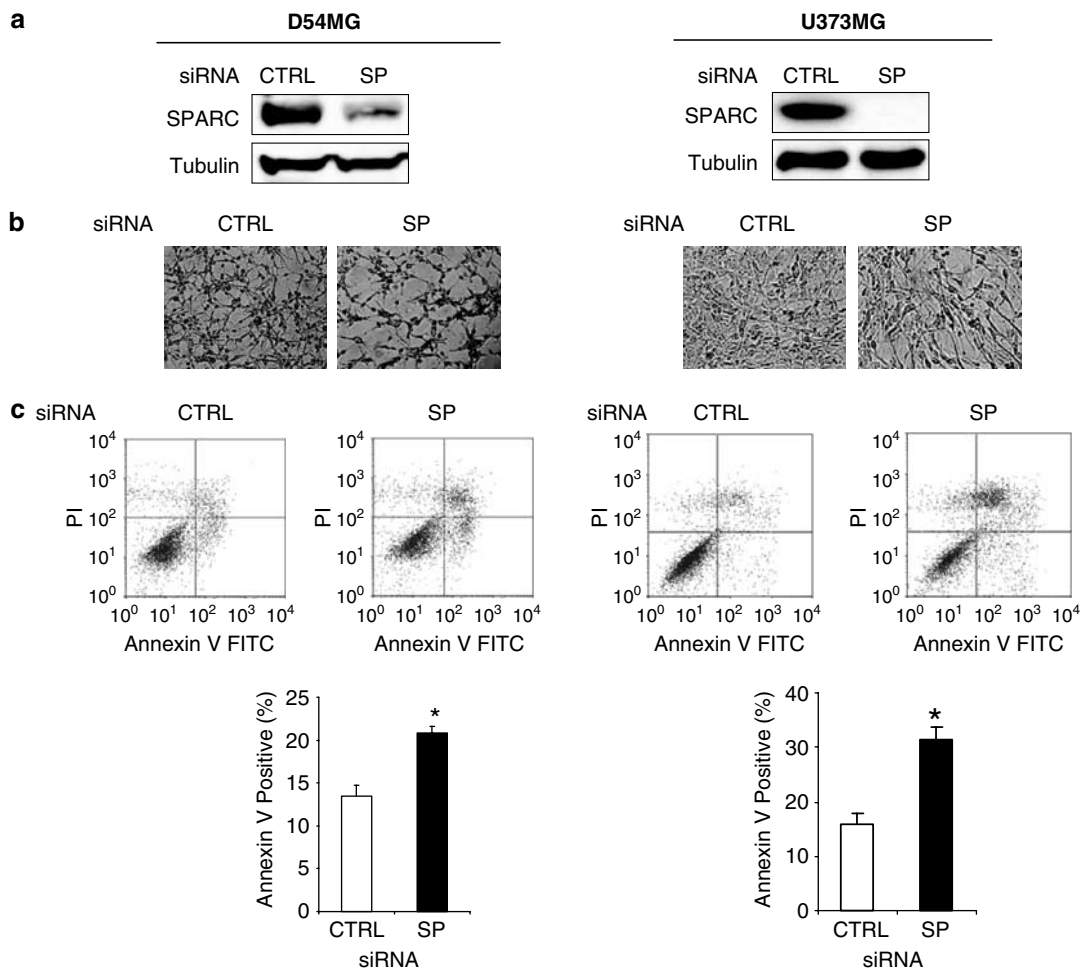


Figure 2 Reduction of SPARC expression by siRNA diminishes glioma cellular survival. D54MG and U373MG cells were transfected with equimolar quantities of siRNA of a non-targeting control (CTRL) or SPARC (SP) for 24 h, serum-starved for 24 h and then collected for Annexin V/propidium iodide FACS analysis or continually serum-starved for 7 days. **(a)** Western analysis confirms successful targeting of SPARC expression. **(b)** Representative images of surviving glioma cells in the survival test are displayed. **(c)** Plots of Annexin V/propidium iodide stain cell population are shown and numbers of apoptotic (Annexin V positive) cells quantified. * $P=0.007$, SPARC siRNA compared with control siRNA.

SPARC siRNA inhibits the survival of glioma cells

As our laboratory previously demonstrated that overexpression of SPARC in glioma cell lines promotes survival under serum starvation (Shi *et al.*, 2004), we investigated whether SPARC siRNA could decrease the survival of glioma cells under this stress. D54MG and U373MG glioma cells transfected with SPARC siRNA survived at decreased rates relative to matched cells transfected with a non-targeting control siRNA (Figure 2). As expected, the decreased survival of the cells transfected with SPARC siRNA was associated with increased rates of apoptosis as measured by the Annexin V assay. Decreasing SPARC expression increased apoptosis by 54% in D54MG and 99% in U373MG (Figure 2c). Similarly, siRNA directed against SPARC increased the rate of apoptosis in D54MG and U373MG cells when compared with two additional siRNA controls directed against luciferase and lamin A/C (Supplementary Figure 2). Thus, SPARC siRNA negatively impacts tumor cell behavior through two related biologic processes—invasion and survival.

SPARC siRNA decreases activation of AKT, FAK and ILK intracellular kinases

We next sought to define the molecular mechanisms through which SPARC siRNA could alter tumor cell invasion and survival. Our laboratory previously demonstrated that SPARC activates the phosphoinositide-3-kinase-AKT pathway, a key mediator of cellular survival (Shi *et al.*, 2004). Therefore, we first determined whether SPARC siRNA could alter AKT phosphorylation at key residues reflecting kinase activation. An siRNA-mediated decrease in SPARC expression reduced the activating phosphorylation of AKT at Serine 473 without affecting total AKT levels (Figure 3a, Supplementary Figure 3).

To further elucidate the molecular mechanisms through which SPARC siRNA could reduce AKT phosphorylation, we screened molecular targets linked to glioma invasion and survival that are known to activate AKT. We determined that reducing SPARC expression could influence the mRNA expression of multiple genes involved in invasion and survival

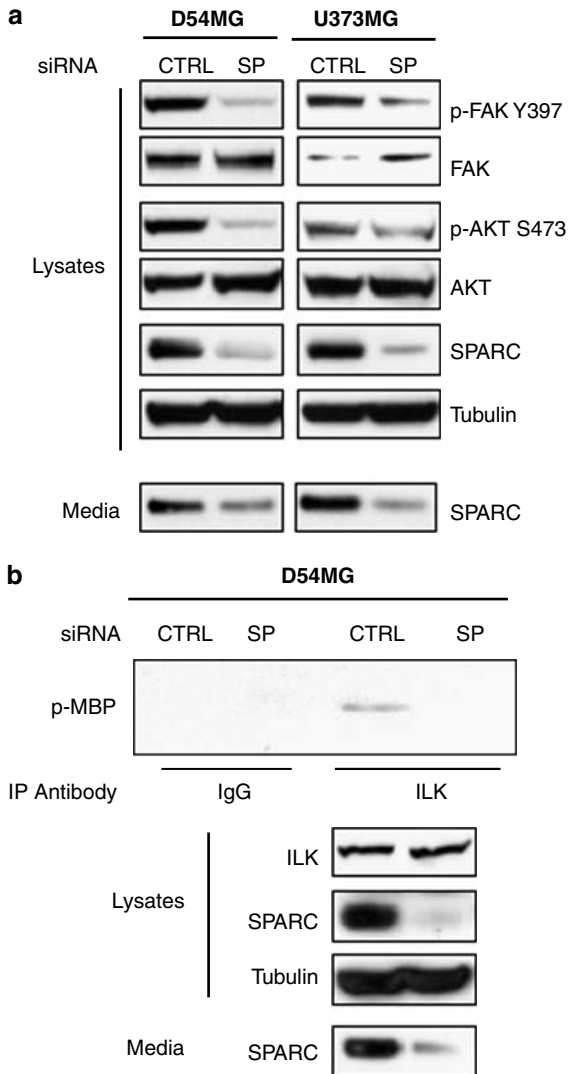


Figure 3 Downregulated SPARC expression is associated with decreased phosphorylation of cellular signaling effectors. D54MG or U373MG cells were transfected with equimolar quantities of siRNA of a non-targeting control (CTRL) or SPARC (SP) for 24 h, serum starved for 24 h and then collected for Western analysis and non-radioactive ILK activity assay. **(a)** Phosphorylated and total levels of FAK and ILK, as well as levels of intracellular and secreted SPARC were assessed by immunoblotting. **(b)** ILK activity was assayed by *in vitro* kinase assay with Western analysis of phospho-MBP levels. Equal ILK and tubulin expression in lysates, as well as downregulation of intracellular and secreted SPARC expression with siRNA, was confirmed by Western blotting.

(Table 1), but we detected no changes in the phosphorylation states of several growth factor receptors after transfection with SPARC siRNA (data not shown). These data correlated with our prior studies, which demonstrated that exogenous SPARC protein treatment or overexpression of SPARC in glioma cell lines did not alter the activation of several growth factor pathways. We rather found that SPARC expression changes correlated with decreased activation of FAK (Figure 3 and Supplementary Figure 3) and ILK (Figure 3), two non-receptor tyrosine kinases whose activities have been

linked to neoplastic cell invasion. In cells transfected with SPARC siRNA, the activating phosphorylation of FAK on Tyrosine 397 (Y397) decreased (Figure 3a), as did the kinase activity of ILK (Figure 3b). As phosphorylation-specific antibodies of ILK residues that reflect protein activation are currently unavailable, we measured the impact of SPARC on ILK activity through an ILK kinase assay. ILK protein was immunoprecipitated and assessed for kinase activity on myelin basic protein (MBP) as a substrate. Whereas an IgG control antibody did not select for any kinase activity, immunoprecipitation, using an anti-ILK antibody, identified kinase activity in glioma cells transfected with a non-targeting control siRNA that was absent in matched cells transfected with siRNA targeting SPARC (Figure 3b). Thus, downregulation of SPARC expression decreases the activity of key intracellular kinases linked to cancer invasion and survival.

SPARC activates the non-receptor tyrosine kinases, FAK and ILK

To validate the link between SPARC and the activation of FAK and ILK, we performed the reverse experiments to those targeting SPARC expression: we activated the SPARC pathway either through the addition of exogenous SPARC protein or through genetic modification to increase SPARC expression. Treatment of a glioma cell line responsive to SPARC and D54MG with exogenous SPARC protein rapidly induced the phosphorylation of a key regulatory FAK residue, Y397, within minutes, in time and concentration-dependent manners (Figure 4a and b). The activation of FAK by exogenous SPARC protein is self-limited, with a return to baseline phosphorylation levels by 60 min of incubation. These data demonstrate, for the first time, that FAK is a downstream target of SPARC and that the biological responses mediated by SPARC could be due, at least in part, to the phosphorylation and activation of FAK.

Cancer cells and tumor-associated stromal elements produce SPARC to provide cancer cells long-term exposure to SPARC. To mimic these effects, we previously created D54MG glioma cell lines transduced to overexpress SPARC (Rich *et al.*, 2003). In SPARC-overexpressing D54MG cells, FAK phosphorylation was increased relative to vector control cells (Figure 4c). Exogenous SPARC protein treatment specifically induced ILK kinase activity in a rapid manner relative to the bovine serum albumin control (Figure 5a). In concordance with these results and those with FAK detailed above, D54MG cells, engineered to overexpress SPARC, also display a constitutive increase in ILK kinase activity (Figure 5b). Thus, FAK and ILK are potential components of the SPARC intracellular signaling pathway upstream of AKT.

SPARC-mediated AKT phosphorylation is mediated through FAK and ILK

To define the roles of FAK and ILK in SPARC-mediated activation of AKT, we targeted FAK and/or

Table 1 Genes differentially regulated in glioma cells expressing SPARC siRNA compared to non-targeting siRNA

Name of gene (mRNA)	Gene abbreviation	GeneBank ID	Fold change
<i>Decreased with SPARC siRNA</i>			
Potential mediators of invasion			
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	NM_003118	13.3689
Integrin, beta 2	ITGB2	NM_000211	21.598
Semaphorin 3B	SEMA3B	NM_004636	2.90697
Disintegrin-like and metalloprotease with thrombospondin motif, type 4 variant	ADAMTS13	NM_139028	2.325581
SPARC-like 1 (mast9, hevin)	SPARCL1	NM_004684	2.1934
Potential mediators of survival			
Extracellular signal-regulated kinase 8	ERK8	NM_139021	95.2381
TP53TG3 protein	TP53TG3	NM_016212	10.72961
V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	NM_000222	6.1349
Aryl hydrocarbon receptor	AHR	NM_001621	4.310345
Mitogen-activated protein kinase kinase kinase 8	MAP3K8	NM_005204	3.115265
Hypoxia-inducible protein 2	HIG2	BC008573	2.564103
RAB40B, member RAS oncogene family	RAB40B	NM_006822	2.277904
Protein phosphatase 5, catalytic subunit	PPP5C	NM_006247	2.04918
BCL2 binding component 3	BBC3	NM_014417	2.028398
<i>Increased with SPARC siRNA</i>			
Potential mediators of invasion			
Transforming growth factor, beta receptor II	TGFBR2	NM_003242	6.848
Semaphorin 4F	SEMA4F	NM_004263	6.351
Semaphorin 6A	SEMA6A	AK027654	4.668
Membrane metallo-endopeptidase	MME	NM_000902	3.482
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	NM_002421	2.871
A disintegrin-like and metalloprotease with thrombospondin motif, type 2 variant	ADAMTS13	NM_139027	2.515
Semaphorin 6D	SEMA6D	NM_024966	2.201
Semaphorin 3C	SEMA3C	NM_006379	2.013
Potential mediators of survival			
Proapoptotic caspase adaptor protein	PACAP	NM_016459	6.472
Transformed 3T3 cell double minute 2, p53 binding protein	MDM2	NM_006879	6.355
V-abl Abelson murine leukemia viral oncogene homolog 2	ABL2	NM_005158	3.436
Rho GTPase-activating protein 23	ARHGAP23	AB040934	3.059
RAB27B, member RAS oncogene family	RAB27B	NM_004163	3.03
Ankyrin repeat domain 11	ANKRD11	AK093762	2.933
DNA-damage-inducible transcript 3	DDIT3	NM_004083	2.93
Rho guanine nucleotide exchange factor (GEF) 5	ARHGEF5	NM_005435	2.737
Growth arrest and DNA-damage-inducible, beta	GADD45B	NM_015675	2.331
Programmed cell death 6	PDCD6	NM_020731	2.245
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	PPP1R3C	NM_005398	2.225
Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	PIK3R1	M61906	2.118
Protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	NM_014330	2.1
Protein kinase C, beta 1	PRKCB1	NM_002738	2.036
Mitochondrial ribosome recycling factor	MRRF	BC002814	2.033
RAB2B, member RAS oncogene family	RAB2B	NM_032846	2.004

Abbreviation: SPARC, secreted protein acidic and rich in cysteine. Total RNA was extracted from 10⁷ U373MG or D54MG cells transfected with siRNA directed against SPARC or non-targeting siRNA. Genes similarly regulated by loss of SPARC expression between the two glioma lines are displayed. Spotted array studies were completed at the Duke University Microarray Facility.

ILK expression through transient transfection of D54MG cells with specific siRNAs targeting either FAK or ILK expression. Decreased FAK and ILK protein levels after siRNA transfection, relative to a non-targeting control siRNA, were confirmed by Western analysis (Figure 6a). The ILK siRNA transfection was modestly more potent in decreasing ILK expression than the FAK siRNA was in decreasing FAK expression (Figure 6a). Under serum-starved conditions, the addition of SPARC to D54MG cells, transfected with control siRNA, increased AKT phosphorylation, as previously described by us (Shi *et al.*, 2004) (Figure 6a). Transfection with ILK siRNA decreased SPARC induction of AKT phosphorylation by 67%, whereas transfection with FAK

siRNA reduced SPARC-induced AKT phosphorylation by 24% (Figure 6a and data not shown). These results suggest that both FAK and, perhaps more significantly, ILK are downstream of SPARC but upstream of AKT in a signal transduction pathway. The greater effects of targeting ILK expression on SPARC activation of AKT may be due to the greater knockdown efficiency of ILK expression and/or a more direct regulation of AKT activity by ILK. FAK and ILK activation of AKT appears to be additive, as the combined targeting of both FAK and ILK signaling dramatically blocks SPARC activation of AKT phosphorylation by 93% (Figure 6b and data not shown). These results confirm the roles of FAK and ILK in mediating SPARC activation of AKT phosphorylation.

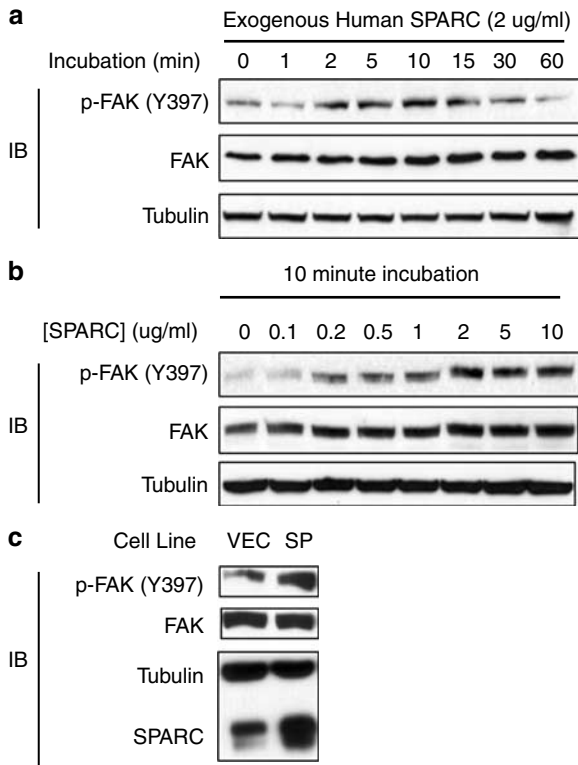


Figure 4 SPARC induces activating FAK phosphorylation. D54MG cells were cultured in 10% serum until 60–75% confluent and then serum-starved overnight. FAK phosphorylation was measured over a time course after incubation with a final concentration of 2 µg/ml (46 nM) human platelet SPARC (a) or over a concentration range with SPARC for 10 min (b). (c) Serum-starved D54MG glioma cells expressing SPARC display increased FAK activation. Glioma cell lines transduced with a control retroviral vector (VEC) or a retrovirus encoding SPARC (SP) were plated in 10% serum overnight, serum-starved overnight and lysed before Western blotting.

SPARC-mediated invasion and cellular survival are dependent on FAK and ILK

Similar to the effects of SPARC, the effects of FAK and ILK have been linked to augmented survival and invasion (Troussard *et al.*, 2000; Hecker *et al.*, 2002; Obara *et al.*, 2004; Xie *et al.*, 2004). Therefore, we investigated whether the activation of FAK and ILK by SPARC regulates tumor cell survival and invasion by modulating FAK and/or ILK expression through transfection with specific siRNAs utilized in our prior experiments. Transfection of vector control D54MG cells with either FAK or ILK siRNA did not impact tumor cell invasion through an artificial extracellular matrix (Figure 7). In contrast, siRNA targeting the expression of FAK partially inhibited the pro-invasive effects of SPARC. There was a 30% decrease in the ability of SPARC-expressing D54MG cells to invade through the Matrigel with the transfection of FAK siRNA (Figure 7). The disruption of ILK expression by siRNA transfection inhibited the effects of SPARC on invasion by 63% (Figure 7). Combined siRNA targeting of FAK and ILK expression reduced the invasive potential of SPARC-expressing cells by 73% to levels

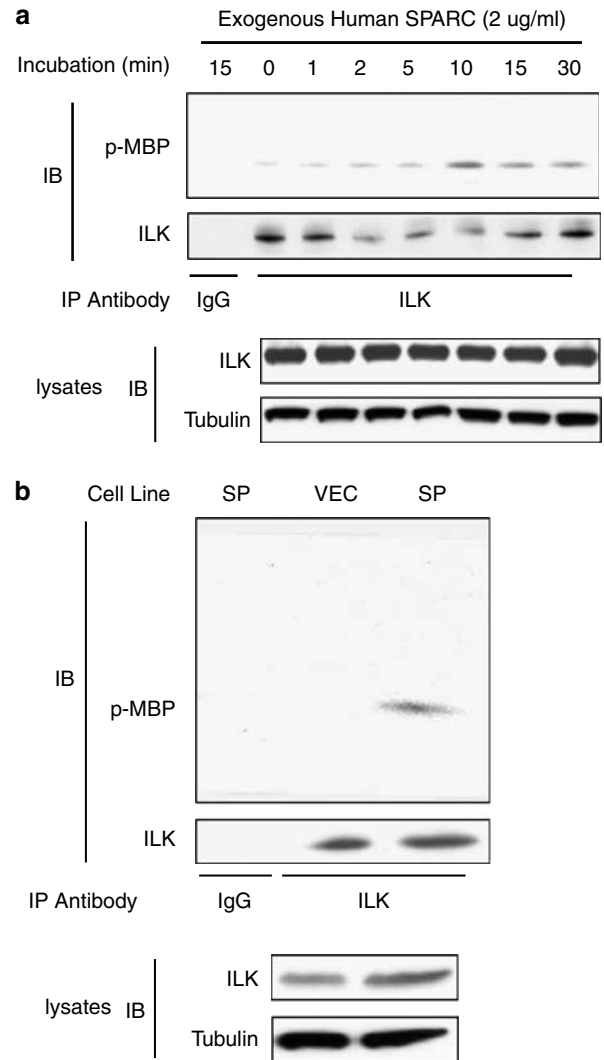


Figure 5 SPARC increases ILK kinase activity. (a) Exogenous SPARC treatment induces ILK kinase activity. D54MG cells were cultured in 10% serum until 60–75% confluent and then serum-starved overnight. ILK activity was measured over a time course after incubation with a final concentration of 2 µg/ml (46 nM) of human platelet SPARC. ILK kinase assays were performed using a rabbit immunoaffinity-purified ILK antibody and MBP as a substrate. (b) Serum-starved D54MG glioma cell lines expressing SPARC display increased ILK kinase activity. Glioma cell lines, transduced with a control retroviral vector (VEC) or a retrovirus encoding SPARC (SP), were plated in 10% serum overnight, serum-starved overnight and lysed. ILK kinase activity assays were performed as in (a).

essentially equivalent to the baseline of vector control cells (Figure 7). Additionally, targeting either FAK or ILK through the transfection of specific siRNAs reversed the survival advantage of SPARC overexpression under serum-starved conditions (Figure 8). Simultaneous transfection with both FAK and ILK siRNA offered no advantage in reversing SPARC-mediated prosurvival effects over either single siRNA (data not shown). These data suggest that the sensitivity of the survival assay differs from that of the Matrigel invasion assay in response to ILK and FAK targeting, or that

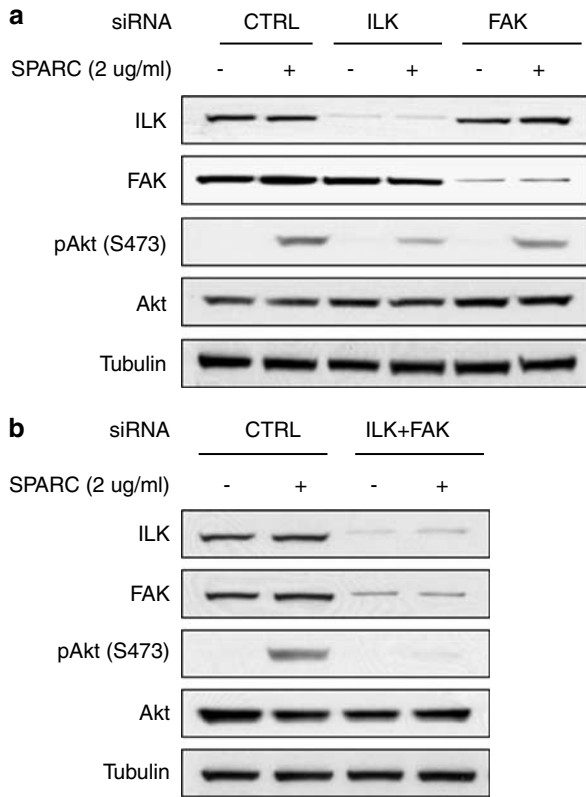


Figure 6 SPARC induction of AKT phosphorylation is dependent on ILK and FAK. siRNA-transfected cells were serum-starved for 24 h and then treated, when indicated, with SPARC protein (2 µg/ml) for 15 min. Cells were harvested for Western to examine the effect of ILK or FAK knockdown on SPARC-mediated AKT phosphorylation. (a) Single treatment with FAK or ILK siRNA partially inhibited SPARC-induced AKT phosphorylation. (b) The combined targeting of FAK and ILK more profoundly inhibited SPARC-induced AKT phosphorylation.

there are differential functions of ILK and FAK in mediating survival and invasion. Thus, SPARC requires ILK and FAK expression to contribute to the tumor biology associated with SPARC expression.

Discussion

Infiltrative glioma cells are a particular therapeutic challenge owing to their diffuse localization, distance from the initial site of resection, protection by an intact blood–brain barrier (Kenney *et al.*, 1992) and low frequency of mitosis (Chicoine and Silbergeld, 1995). The targeting of these invasive glioma cells remains elusive because of a deficiency in our understanding of the molecular events underlying tumor invasion. We now demonstrate the involvement of a novel signal transduction pathway involving SPARC, FAK, ILK and AKT in the control of glioma cell invasion and survival. As elevated SPARC expression is associated with decreased glioma patient survival (Rich *et al.*, 2005), we believe that our results, demonstrating decreased invasion and increased cell death with siRNA

directed against SPARC, suggest that decreasing SPARC expression may have therapeutic benefit for glioma patients. However, as SPARC has the potential to function both as a tumor promoter and tumor suppressor, decreasing SPARC expression may not be beneficial for all tumor types or stages. For example, SPARC induces apoptosis in ovarian cancer cells (Yiu *et al.*, 2001) and inhibits proliferation and metastasis of breast cancer cells (Koblinski *et al.*, 2005), but breast tumor expression of SPARC is linked to increased patient metastases and poor patient survival (Jones *et al.*, 2004; Watkins *et al.*, 2005). When we over-expressed SPARC in HeLa cells or a genetically engineered human breast cancer model (HMEC), SPARC-expressing cells suffered a dramatic inhibition of tumor formation when implanted into immunocompromised rodents (data not shown). Of note, treatment with exogenous SPARC protein or overexpression failed to activate AKT in HeLa or HMEC cells (data not shown). These cell-specific results further suggest that SPARC-induced phosphorylation and activation of AKT contribute to tumor promotion.

We have now determined that the phosphorylation of AKT by SPARC involves the activation of two intracellular tyrosine kinases, FAK and ILK, which have been previously implicated in the control of glioma invasion (Troussard *et al.*, 2000; Hecker *et al.*, 2002; Obara *et al.*, 2004; Xie *et al.*, 2004). Although the precise mechanism through which SPARC activates FAK and ILK remains to be determined, and will be investigated further, we have detected a novel activation of FAK by both exogenous SPARC treatment and stable SPARC expression. In addition, exogenous SPARC and SPARC overexpression increased the kinase activity associated with ILK. SPARC-mediated activation of FAK and ILK is important to SPARC signaling, as siRNA targeting FAK and ILK inhibited the activation of AKT. Furthermore, FAK and ILK activation contribute to SPARC phenotypic effects, as SPARC-mediated glioma cell invasion and survival were blocked through inhibition of FAK and/or ILK expression. Although a recent study showed that SPARC regulates ILK-related activities in fibroblasts (Barker *et al.*, 2005), our results are the first to demonstrate that ILK activity mediates oncogenic effects of SPARC. The requirement of ILK and FAK activation by SPARC for SPARC-mediated changes in cellular behavior suggests that the expression of some invasive and/or survival genes, disregulated with decreased SPARC expression, may depend upon ILK and/or FAK for transcriptional regulation, and we continue to explore this possibility in studies beyond the scope of this investigation.

Our demonstration of a novel signal transduction pathway involving SPARC, FAK, ILK and AKT, in the control of glioma cell invasion and survival, has additional therapeutic importance, as low-molecular-weight inhibitors of the kinase activities of FAK, ILK and AKT are each in preclinical or early clinical development. Our results suggest that tumors expressing SPARC may display particular sensitivity to the antitumor effects of these inhibitors. Thus, SPARC

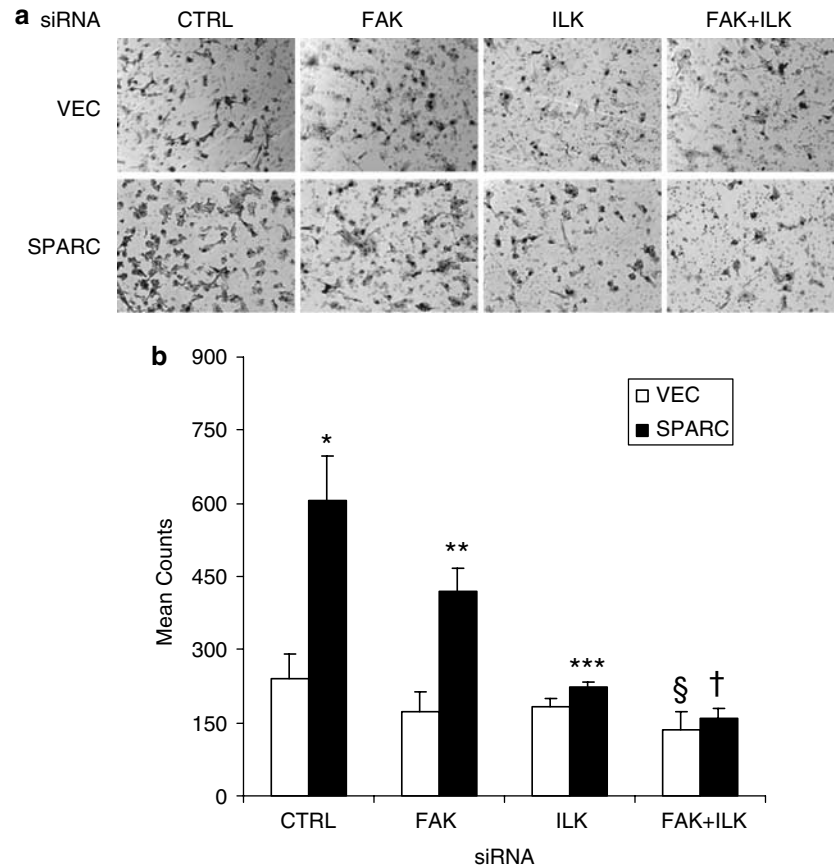


Figure 7 SPARC-induced invasion is ILK and FAK dependent. D54MG glioma cells, transduced with viral vector backbone (VEC) or human SPARC (SPARC), were transfected with equimolar amounts of siRNA of a non-targeting control, FAK, ILK or a combination of both FAK and ILK in media with serum for 24 h. After an additional 24-h incubation without serum, 50 000 cells were added in triplicate to 24-well Transwell inserts coated with the Matrigel and allowed to invade overnight. Parallel siRNA transfections were performed and analysed by Western analysis to confirm successful targeting of ILK and/or FAK expression, as in Figure 4 (data not shown). (a) Representative images of invading tumor cells are displayed. (b) Numbers of invading tumor cells are quantified. * $P=0.0038$ relative to vector cells with non-targeting siRNA control; ** $P=0.0362$, *** $P=0.002$ relative to SPARC expressing cells with non-targeting siRNA control; [§] $P=0.0495$ relative to vector cells with non-targeting siRNA control; [†] $P<0.006$ relative to SPARC expressing cells with non-targeting siRNA control, FAK siRNA or ILK siRNA.

expression levels may permit pre-selection or stratification of patients in clinical trials of these agents. For example, tumor SPARC expression levels in breast cancers have provided markers of sensitivity to an albumin-bound paclitaxel (Gradishar, 2006), potentially because of SPARC binding of albumin, or another molecular mechanism. In addition, FAK, ILK and AKT inhibitors may display combinatorial efficacy owing to the joint activities of these kinases in tumor cells expressing SPARC. Finally, targeting SPARC expression has already been demonstrated to be useful in the control of melanomas (Ledda *et al.*, 1997; Alvarez *et al.*, 2005), which further suggests that decreasing SPARC expression in some other cancer types, such as gliomas, may be beneficial for patient therapy.

Materials and methods

Cell lines and culture

D54MG is the Duke University subline of A-172. U373MG was purchased from the American Type Culture Collection

(Manassas, VA, USA). Vector control or SPARC-overexpressing D54MG cells were created as previously described (Rich *et al.*, 2003). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) media with 10% fetal bovine serum (FBS, Invitrogen) and L-glutamine.

Reagents

Human platelet osteonectin (SPARC) and bovine osteonectin (SPARC) were purchased from Haematologic Technologies (Essex Junction, VT, USA). siRNAs (Dharmacon, Chicago, IL, USA) were used as per the manufacturer's instructions with control non-targeting siRNA (CTRL). Unless otherwise noted, all chemicals were purchased from Sigma (St Louis, MO, USA).

Transfection of siRNA

150 000 cells were plated per six-well in DMEM with 10% FBS and were allowed to attach overnight. Equimolar amounts of siRNAs were incubated with TRANSIT-TKO Transfection Reagent from Mirus (Madison, WI, USA) as per the manufacturer's instructions. Cells were maintained for 48 h before experiments, unless otherwise described.

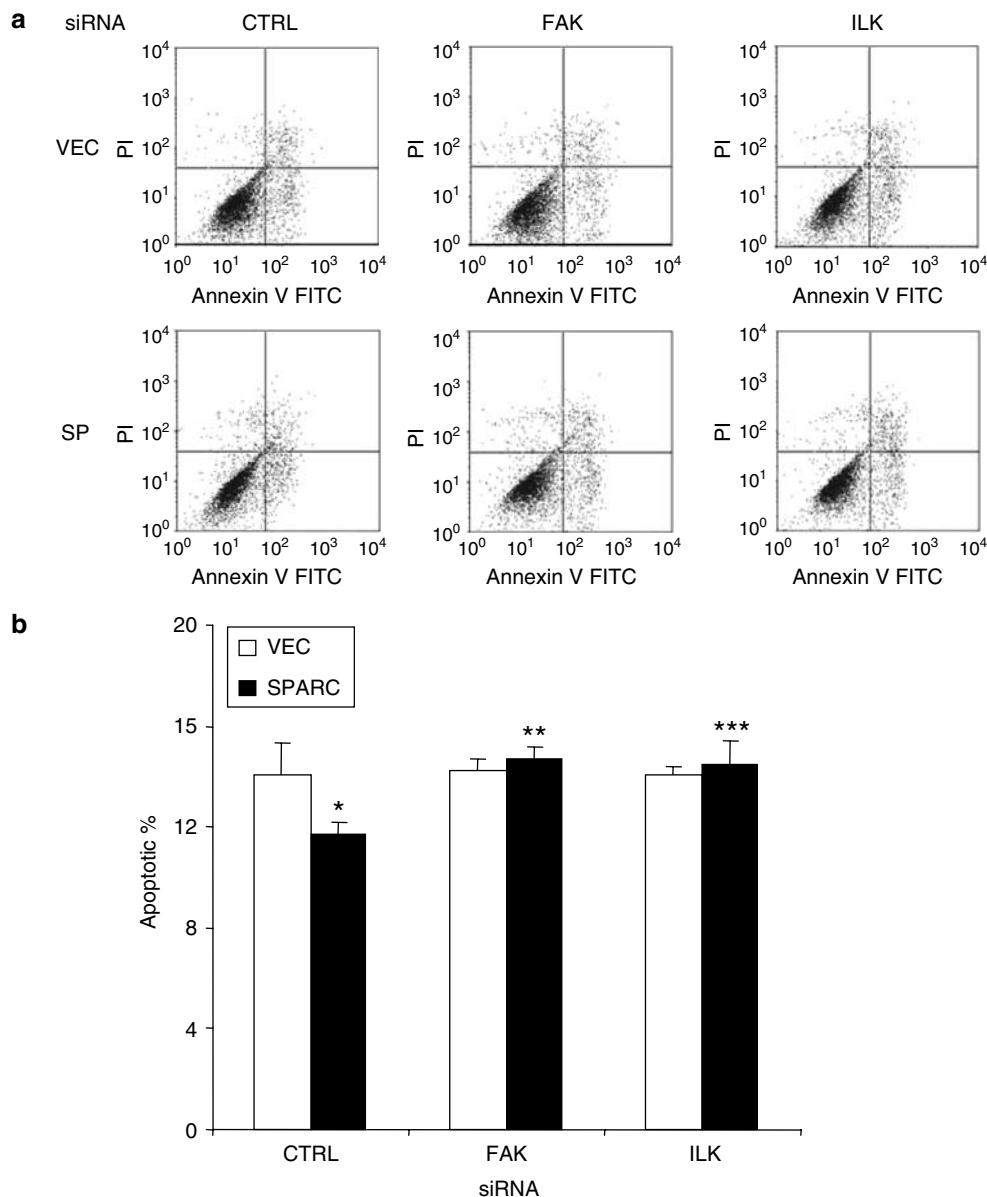


Figure 8 SPARC-induced survival upon serum withdrawal is FAK and ILK dependent. D54MG cells ($n = 150\,000$) transduced with viral vector backbone (VEC) or human SPARC (SPARC) were plated in 6-well tissue culture plates containing 10% serum and allowed to attach for 24 h. Cells were then transfected with equal amounts of non-targeting control, FAK or ILK siRNA. Cells were incubated for an additional 48 h in the absence of serum and the apoptotic index was assessed with Annexin V staining. (a) Representative plots of D54MG cultures stained with Annexin V and propidium iodide are displayed. (b) Quantification of the apoptotic fractions (upper and lower right quadrants) of cell populations is displayed. * $P = 0.036$ relative to vector cells with non-targeting siRNA control; ** $P = 0.0015$, *** $P = 0.0105$ relative to SPARC cells with non-targeting siRNA control.

Immunoprecipitation

Two hundred micro grams of whole-cell lysates was incubated with $2\ \mu\text{g}$ of ILK antibody (Upstate, Charlottesville, VA, USA) or isotype control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 1 h. Complexes were purified on Protein A/G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA) and analysed by Western blotting.

Western analysis

Westerns were completed as previously described (Shi *et al.*, 2004). Membranes were probed with antibodies against phospho-specific AKT (Ser 473), total AKT (Cell Signaling Technology, Beverly, MA, USA), phospho-specific FAK (Tyr

397) (BioSource, Carlsbad, CA, USA), MBP (Thr 98), total FAK, ILK (Upstate), osteonectin/SPARC (Haematologic Technologies, Essex Junction, VT, USA) and α -tubulin (Sigma). Expression levels were measured using the gel analyser option on *ImageJ* software (<http://rsb.info.nih.gov/ij>).

Annexin V assays

Annexin V and propidium iodide staining was completed as previously described (Shi *et al.*, 2004).

Non-radioactive ILK assay

For the ILK assay, $200\ \mu\text{g}$ of cell lysate from serum-starved cells, treated as indicated, was added to $5\ \mu\text{g}$ of rabbit

polyclonal ILK antibody (Upstate) or IgG isotype control (Santa Cruz) and Protein A/G-Sepharose (Amersham Biosciences). Complexes were washed and kinase assays performed by using 0.1 μ g of MBP as a substrate according to manufacturer's instructions (Nonradioactive IP-Kinase assay kit, Cell Signaling Technology). Complexes were analysed by Western for phosphorylated MBP protein.

In vitro matrigel invasion assay

Boyden chamber assays were completed as previously described (Rich *et al.*, 2003).

Spotted array experiments

Total RNA was extracted from 10⁷ D54MG and U373MG cells transfected with siRNA directed against SPARC or a non-targeting control siRNA using a QIAGEN RNeasy Kit (Qiagen Inc., Valencia, CA, USA). MicroArray experiments were performed at the Duke University Microarray Facility using the human Operon V3.0 array (Operon, Biotechnologies Inc., Huntsville, AL, USA) on a GeneMachine OmniGrid Microarray (GeneMachines, San Carlos, CA, USA). Data

were analysed using GeneSpringGX software (Agilent Technologies, Palo Alto, CA, USA).

Statistical analysis

Quantitative data are presented as mean \pm s.d. Significance was assessed by two-tailed Student's *t*-test for two-way analyses. Analysis of variance was used for multiple comparisons.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).