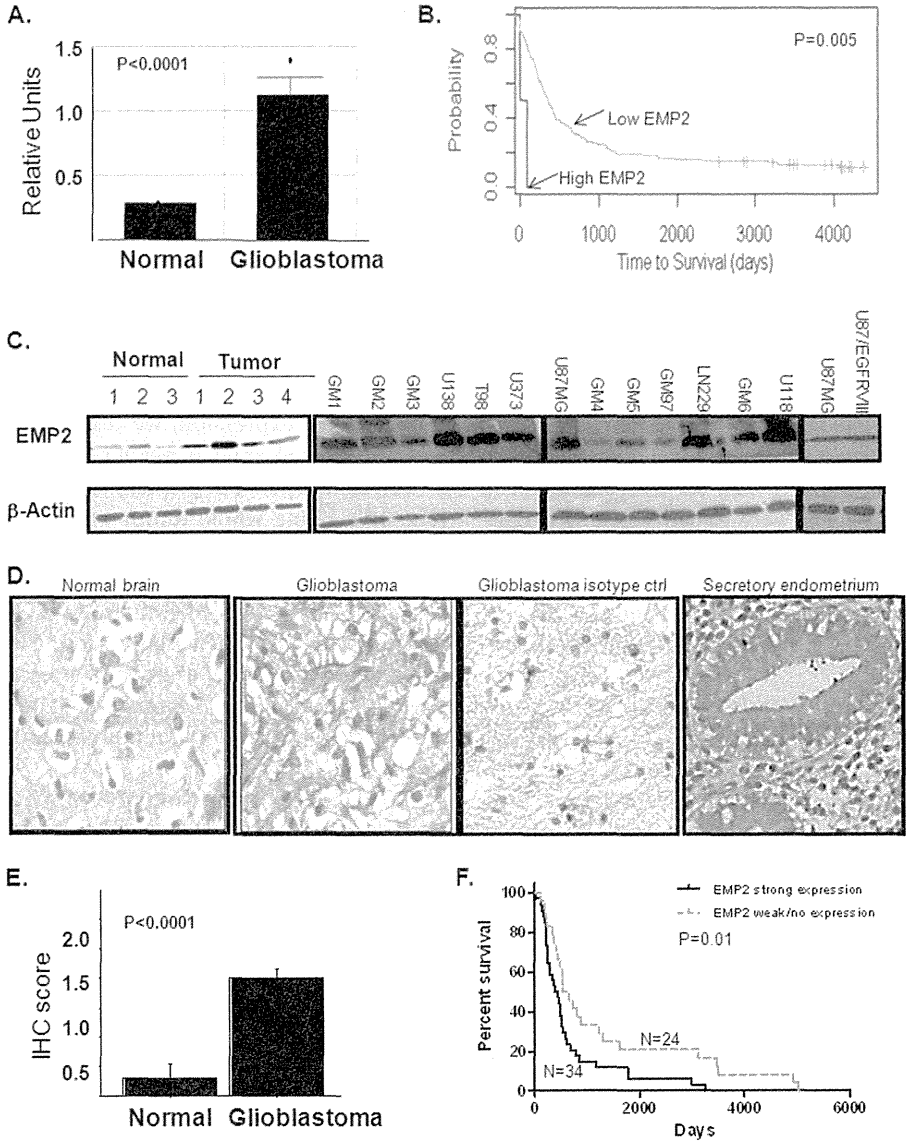


FIGURE 1. **EMP2 expression is increased in GBM.** *A*, EMP2 mRNA expression (Affymetrix microarray) was increased in GBM compared with normal brain. *, $p <$ 0.001. *B*, survival data for high and low EMP2 mRNA expression. *C*, *left*, EMP2 expression was increased in GBM tumors compared with normal regions. *Right*, EMP2 expression was evaluated in a panel of GBM cells lines and in lines derived from patients by Western blotting analysis. β-Actin expression was used as a loading control. *D* and *E*, GBM tissue arrays containing 329 cores from 110 patients were stained for EMP2 expression. *D*, EMP2 protein expression was determined in normal brain, in GBM, and in secretory endometrium (positive control) using an EMP2 polyclonal antibody. To detail nonspecific staining, rabbit preimmune serum was used. Staining was visualized using deNovo Red. Nuclei were counter-stained using hematoxylin. *E*, EMP2 expression was quantitated on a 0–3 histological scale by two independent pathologists, and the average IHC score is shown. *F*, EMP2 expression was dichotomized based on high (histological score, ≥2) or low (histological score, ≥1) expression. High EMP2 expression correlated with a poor survival.

EMP2 Increases αvβ3 Integrin Surface Expression—Previously, we have shown that EMP2 up-regulates αvβ3 integrin surface expression in endometrial cancer cells (19). To determine whether EMP2 could alter integrin expression in GBM, cells were created with modified EMP2 levels. In U373, GM97, and U87MG cells, forced overexpression of EMP2 significantly increased αvβ3 integrin surface expression, whereas a reduction in EMP2 decreased it in both U373 and GM97 (Fig. 2*C*). The reduction in αvβ3 levels was also observed in U87MG cells stably transfected with a ribozyme, although this reduction was not significant. The effects of EMP2 on αvβ3 integrin expression appear to be specific as no significant changes in αvβ5 integrin expression were observed in any of the three cell lines (data not shown).

Integrins are known to transmit signals enhancing cancer cell proliferation and invasion (31); hence, we first examined whether EMP2 levels altered GBM proliferation. BrdU incorporation assays over 24 h revealed that cell proliferation was unaffected by overexpression or reduction in EMP2 in both U373 and T98 cells (data not shown). We next focused on tumor cell migration and invasion. Using U373 cells, ectopic overexpression of EMP2 increased wound healing (Fig. 3*A*) and cell migration (Fig. 3*B*) compared with vector control-expressing cells. Concordantly, a reduction of EMP2 expression using ribozymes decreased wound healing and cell migration, suggesting a role of EMP2 in the regulation of GBM cellular motility. Integrins typically show specificity for select extracellular matrices. It is known that αvβ3 integrin adheres to vitronectin

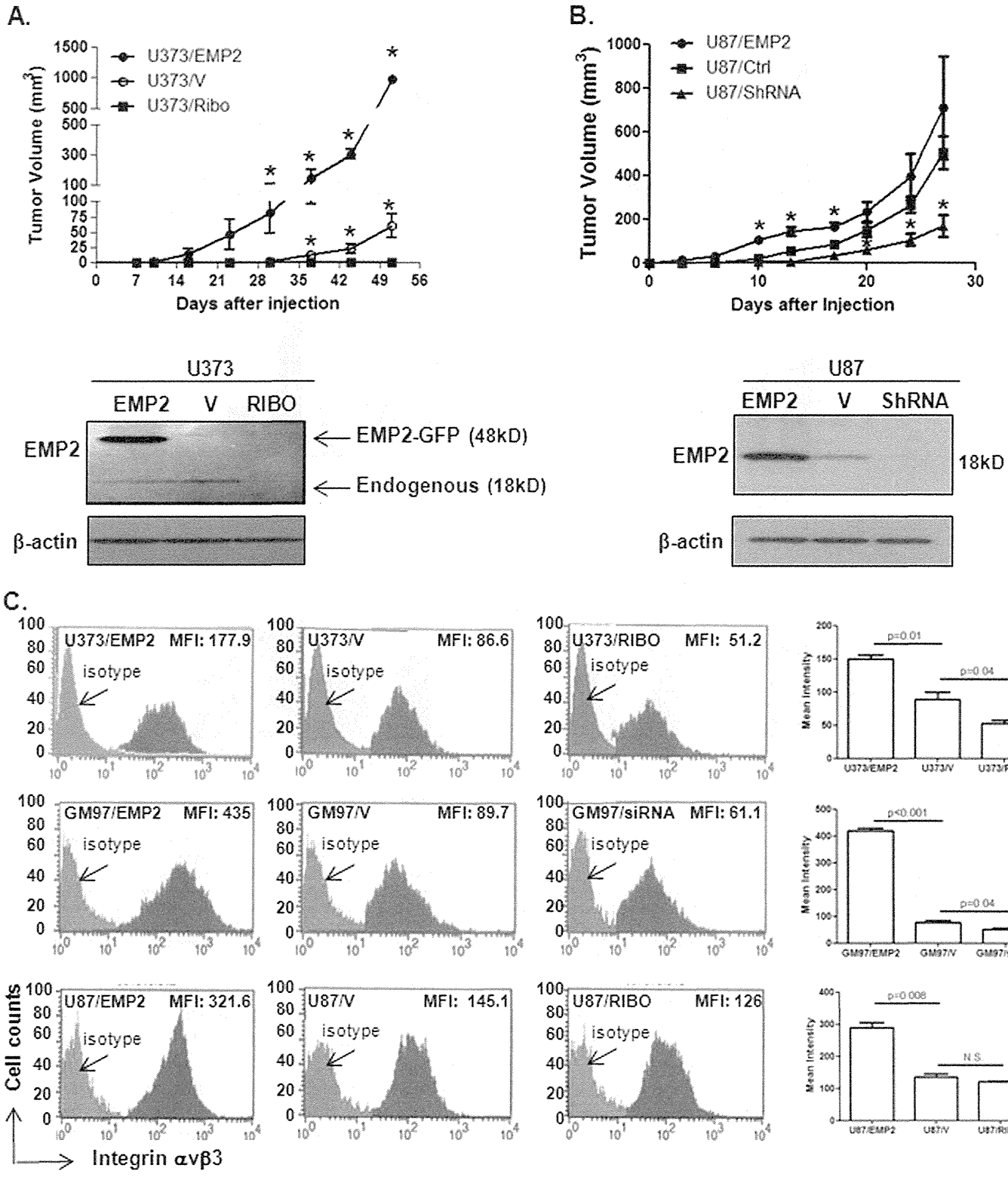


FIGURE 2. **EMP2 expression promotes GBM tumorigenicity.** *A,* U373 modified cell lines were created that overexpress EMP2, express a vector control, or express reduced levels of the protein. U373/EMP2, U373/V, and U373/RIBO cells were then inoculated subcutaneously into athymic nude mice. Tumor volume was calculated twice a week for 50 days. $n = 4$ per group. *, comparison of U373/EMP2 with U373/V or U373/V with U373/RIBO by Student's *t* test, $p < 0.05$. *Bottom,* EMP2 expression was determined using Western blot analysis in U373/EMP2, U373/V, and U373/RIBO cells. Overexpression of EMP2 in these cells is via a GFP-EMP2 fusion protein (48 kDa). *B,* U87/EMP2, U87/V, and U87/shRNA cells were created and injected as above. Tumor volume was monitored for 27 days. $n = 4$ per group. *, $p < 0.05$ by Student's *t* test. *Bottom,* expression of EMP2 in the U87/EMP2, U87/V, and U87/shRNA cells was determined by Western blot analysis. In U87MG cells, EMP2 was overexpressed using a bicistronic vector (18 kDa). *C,* EMP2 expression was modified in U373, U87MG, and GM97 cells. To reduce EMP2 levels, GM97 were transiently transfected with a control or EMP2-specific siRNAs. In U373 and U87MG cells, EMP2 expression was stably reduced using a ribozyme (*RIBO*). αvβ3 integrin surface expression was measured using flow cytometry, and a representative histogram is shown. *Right,* values represent the average mean fluorescent intensity (*MFI*) ± S.E. from three experiments. *N.S.,* not significant.

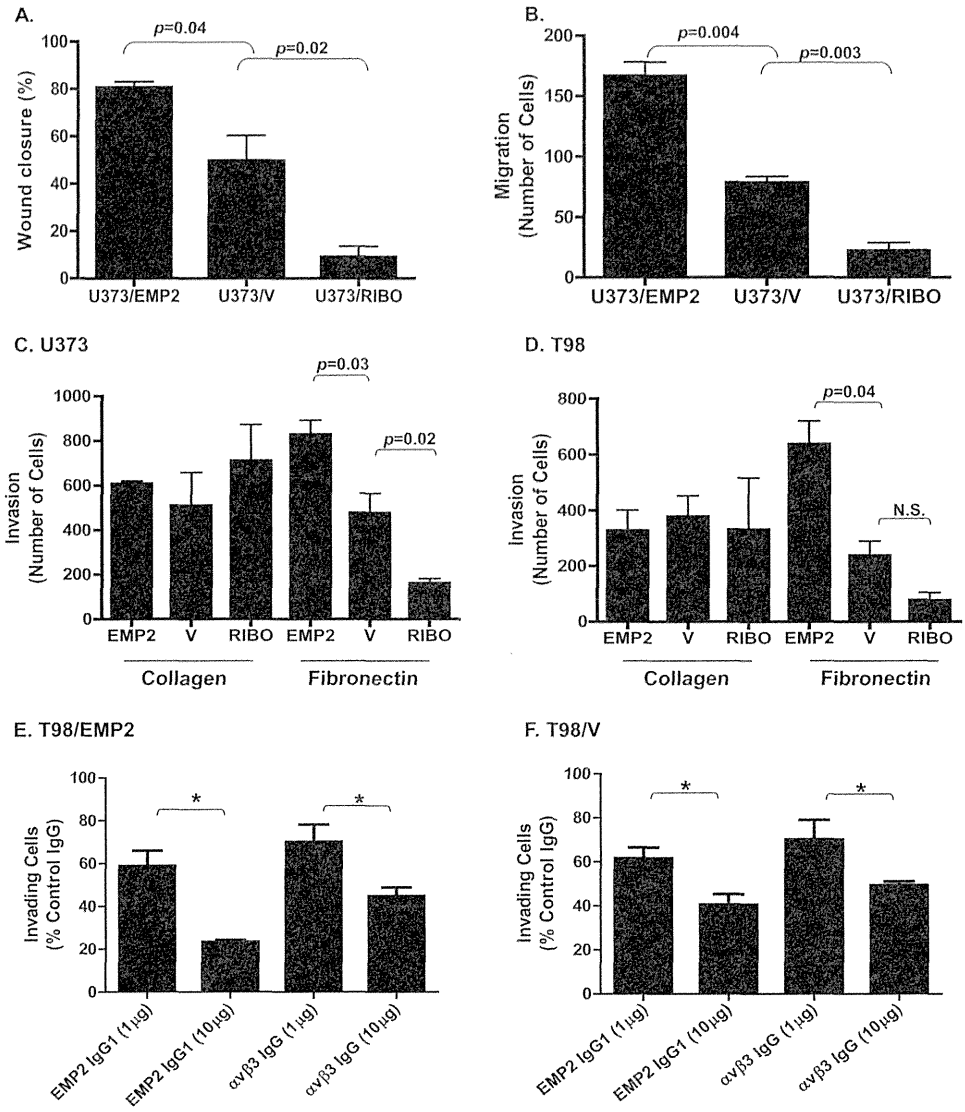


FIGURE 3. **EMP2 promotes GBM invasion.** *A*, U373/EMP2, U373/V, or U373/RIBO cells were grown to form a monolayer. A scratch was then created, and closure of the wound was measured after 24 h. Experiments were performed at least three times, and the results were averaged. *B*, equal numbers of U373/EMP2, U373/V, and U373/RIBO were plated into the top of the transwell. After 6 h, cells that had migrated through the transwell were fixed, stained with crystal violent, and counted. Values are averages of three independent experiments (±S.E.). U373/EMP2, U373/V, or U373/RIBO cells (*C*) or T98/EMP2, T98/V, or T98/RIBO cells (*D*) were added to transwells coated with collagen I or fibronectin. Cells that had invaded through the transwell were determined as above. The experiment was repeated three times, with the data presented as the mean ± S.E. *A–D*, Student's *t* test was used to determine significant differences between groups with specific *p* values indicated in the figure; *N.S.*, not significant. T98/EMP2 (*E*) or T98/V (*F*) cells were preincubated with varying concentrations of an anti-EMP2 IgG1, anti-αvβ3 integrin, or isotype control antibody. Cells were then plated onto a fibronectin-precoated transwell, and percent invasion relative to the isotype control was determined. Results represent averaged results from three independent experiments ± S.E. *, $p < 0.05$.

and fibronectin (32, 33), but it does not have an affinity for collagen. To determine whether EMP2-mediated changes in integrin expression alter the cell's affinity for its ligand, transwells were coated with either fibronectin or collagen. U373 and T98 cells with modified levels of EMP2 were incubated for 6 h and allowed to invade through the matrix. EMP2 up-regulated fibronectin-mediated cell invasion in U373 (Fig. 3*C*) and T98 cells (Fig. 3*D*), but it had no effect on collagen-mediated cell invasion using this assay.

To further confirm the role of EMP2 and αvβ3 integrin in GBM motility, a full-length IgG1 to target EMP2 (12) and commercial antibodies to αvβ3 integrin (34, 35) were tested for their ability to functionally inhibit EMP2-mediated integrin activation. Cells were preincubated with either an EMP2 IgG1 or αvβ3 integrin-specific antibody, and invasion through fibronectin-coated transwells was monitored. EMP2/integrin-mediated cell invasion was significantly impaired by specific antibodies against EMP2 or αvβ3 integrin in a dose-dependent manner in both T98/EMP2 (Fig. 3*E*) and control T98/V cells (Fig. 3*F*). Collectively, these results suggest that EMP-regulated αvβ3 integrin surface expression modulated GBM cell migration and invasion.

EMP2 Activates FAK and Src—One consequence of integrin activation is to alter cellular behavior through the recruitment of FAK and Src kinase (36, 37). Panels of U373 and U87MG were plated for 24 h, and levels of activated FAK and Src kinase were measured. EMP2 levels significantly correlated with both FAK and Src activation in these cells (Fig. 4*A*). To confirm these results, EMP2 was overexpressed in GBM lines LN229 and GM97 and compared with U87MG cell lines. Overexpression

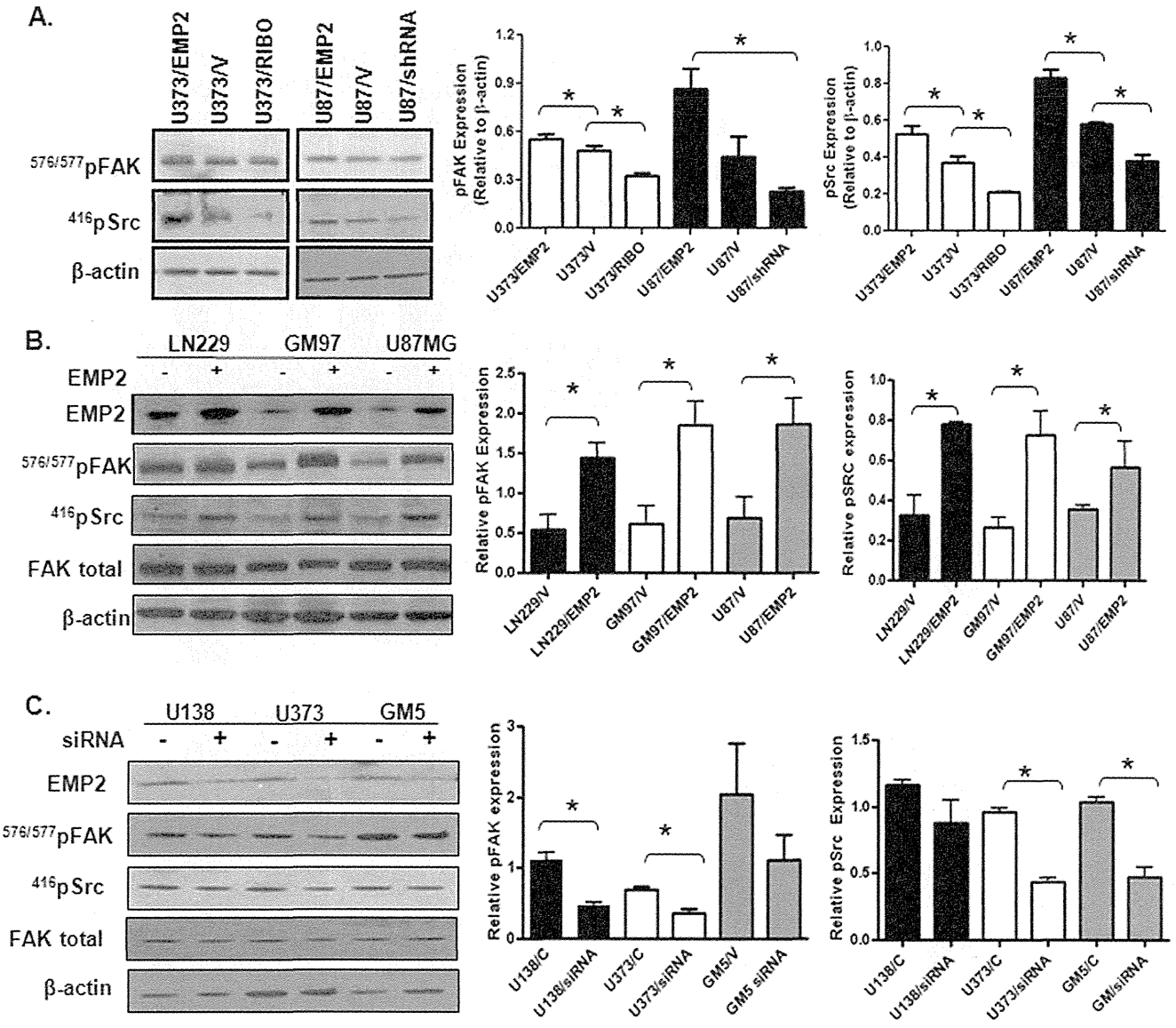


FIGURE 4. **EMP2 expression promotes activation of FAK and Src.** *A*, panels of U373 or U87MG cells were created to overexpress EMP2 or down-regulate its expression through the use of a ribozymes (*RIBO*) or shRNA vector. Cells were plated, harvested, and probed for activated FAK (Tyr-576/577) and activated Src (Tyr-416). *, $p < 0.05$, comparison by Student's *t* test. *B*, vector control LN229, GM97, and U87 or cells which overexpress EMP2 were plated for 24 h, harvested, and then probed for activated FAK and Src, total FAK, and β-actin. *Left*, representative Western blots. *Right*, semi-quantitative analysis of activated FAK and Src levels from three independent experiments; comparison by Student's *t* test, *, $p < 0.05$. *C*, U118, U373, and GM5 cells were transfected with an EMP2 siRNA or control siRNA, and cells were incubated for 48 h, then harvested and probed as above. *Left*, representative Western blots. *Right*, semi-quantitative analysis of pFAK and pSrc after correction for β-actin levels from three independent experiments.

of EMP2 activated FAK and Src by increasing phosphorylation levels at Tyr-576/577 and Tyr-416, respectively, compared with vector control-expressing cells (Fig. 4*B*). This increase was significant in all three cell lines (LN229, U87MG, and GM97), further suggesting that this may be a direct consequence of EMP2 up-regulation. To confirm that a reduction in EMP2 could produce a reciprocal effect, GBM cells with high endogenous levels of EMP2 were transiently transfected with an EMP2 siRNA. Similar to the shRNA knockdown, siRNA vectors to EMP2 decreased FAK and Src phosphorylation in U138, U373, and GM5 cells (Fig. 4*C*) with significant effects observed in U373 and U118 cells. These results collectively suggest that EMP2 promotes activation of the integrin-FAK-Src signaling pathway.

To correlate the *in vitro* data with clinical data, tissue microarrays were probed by IHC for both EMP2 and activated Src

TABLE 1
Correlation of EMP2 and pSRC expression in GBM patient samples
Spearman's rank correlation coefficient is $r = 0.54$, $p < 0.01$.

$n = 87$	EMP2 positive	EMP2 negative	Total
pSRC positive	49	1	50
pSRC negative	20	17	37

(Table 1). Analysis of 87 patients showed a Spearman's rank correlation coefficient of $r = 0.54$, $p < 0.01$ between EMP2 and anti-p-Src (Tyr-416) expression, where 98% (49 out of 50) of tissues positive for p-Src (Tyr-416) showed intense staining for EMP2.

Increased EMP2 Expression Increases GBM Cell Invasion in Vivo—To determine whether EMP2 expression altered GBM tumor growth in the brain, U87/Luc cells with modified EMP2 levels were stereotactically implanted into the right frontal lobe of

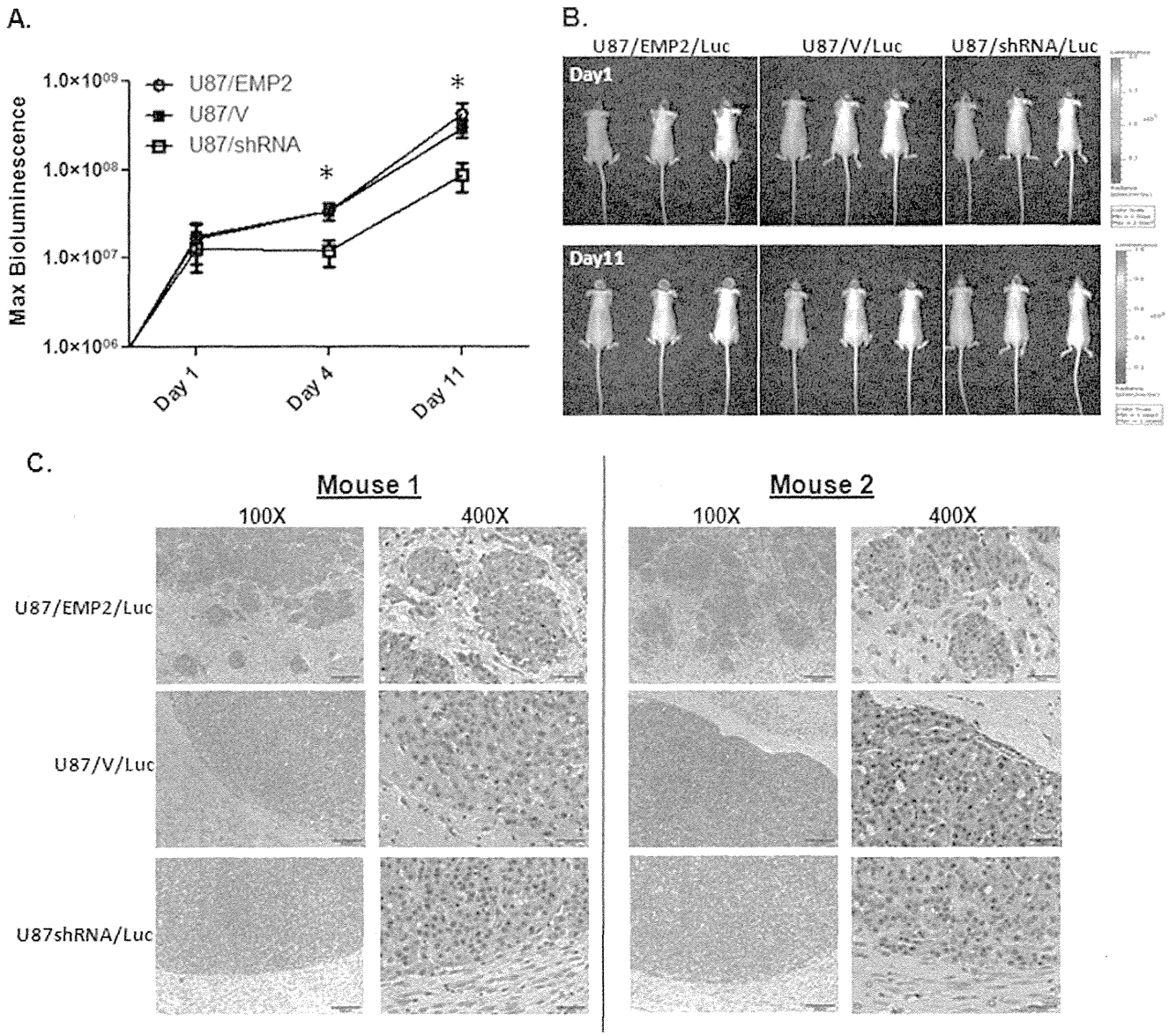
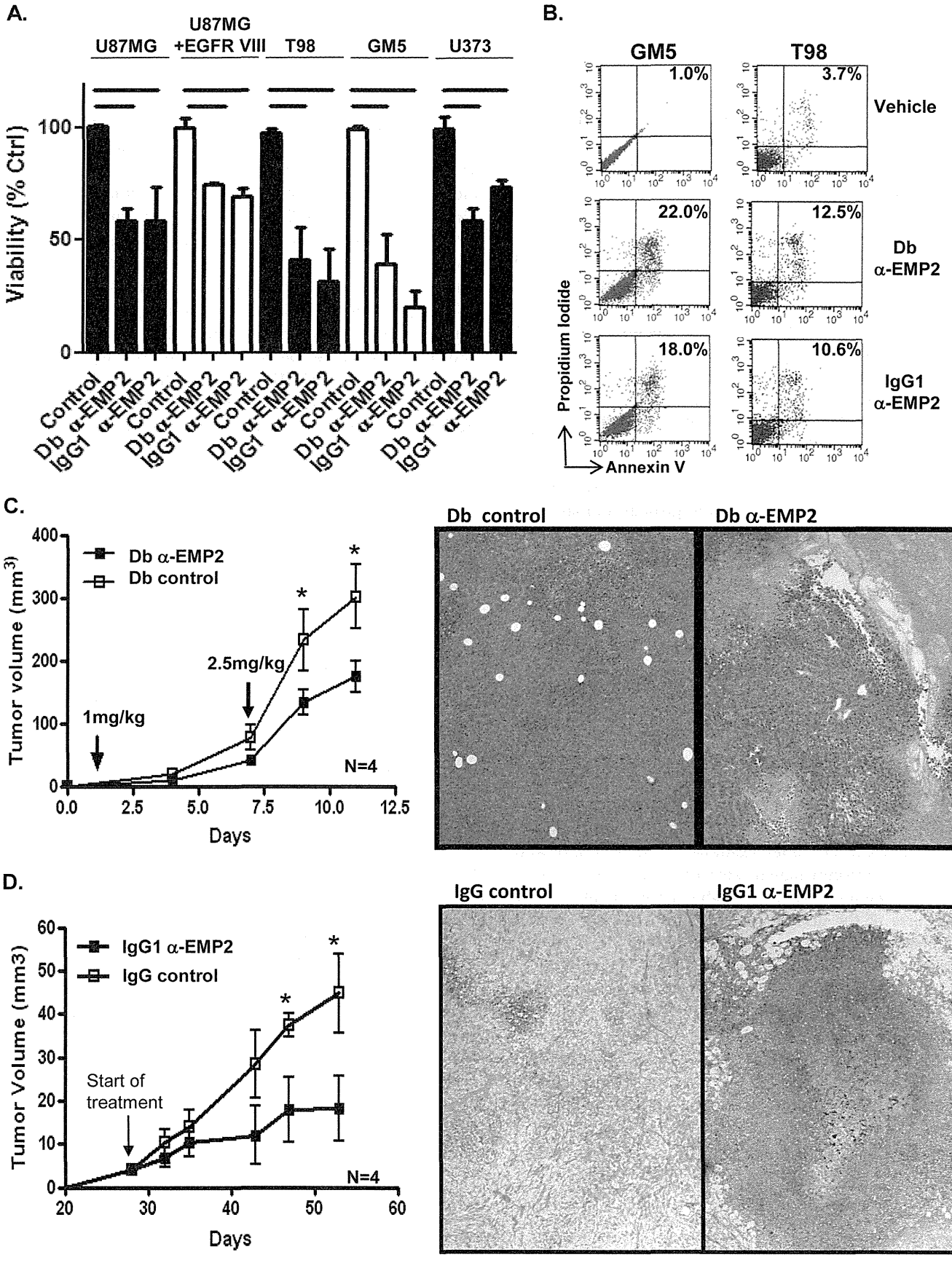


FIGURE 5. **EMP2 expression promotes tumor migration *in vivo*.** *A*, U87/EMP2/Luc, U87/V/Luc, or U87/shRNA/Luc cells were stereoscopically implanted into the right frontal lobe of athymic nude mice. Tumor load was monitored using bioluminescence imaging; $n = 6$ per group. *, $p < 0.05$ by one-way analysis of variance. *B*, representative bioluminescence images of mice from each group on day 1 and day 11. *C*, representative low and high magnification images from two U87/EMP2, U87/V, and U87/shRNA tumors. The high magnification image centers on the brain-tumor margin. Magnification: *left*, ×100; *right*, ×400.

athymic nude mice, and tumor load was monitored using bioluminescence (Fig. 5, *A* and *B*). Although an increase in EMP2 expression did not significantly increase tumor growth compared with control animals, it did increase tumor invasion into the surrounding parenchyma (Fig. 5*C*). In contrast, a reduction of EMP2 through a specific shRNA significantly inhibited tumor growth compared with control mice, suggesting that targeting EMP2 expression may have a therapeutic benefit. Importantly, the rate of growth between the tumor lines is consistent with the U87MG subcutaneous model created above.

Antibodies Targeting EMP2 Inhibit GBM Tumor Growth in Vitro and in Vivo—We have recently shown that anti-EMP2 antibodies are a novel therapeutic option for endometrial, breast, and ovarian cancers in preclinical models using a panel of recombinant immunoglobulin-based reagents (11, 12, 26). These reagents include high affinity diabodies (bivalent scFv dimers, 55 kDa) as well as a full-length IgG1 (150 kDa). The rationale for constructing different sized immunoglobulin reagents was to create a panel of reagents with properties tailored for tumor infiltration and serum half-life (38). In contrast to native IgG1, diabodies biodistribute more quickly, penetrate target tissues efficiently, and clear more rapidly from circulation. Both diabody and native IgG1 reagents bind to the second extracellular loop of EMP2 (26), and these immunoglobulin variants show cross-reactivity for mouse and human EMP2 as detected by flow cytometry and IHC (26). To determine whether the anti-EMP2 diabody or IgG1 could induce cell death, a panel of GBM cells such as U87MG, U87/EGFR VIII, U373, T98, and GM5 were incubated with EMP2-specific immunoglobulin reagents or a vehicle control. Both EMP2-specific reagents significantly reduced viable cell numbers in all GBM cell lines tested (Fig. 6*A*). To confirm that the reduction in


A.
U87MG
U87MG +EGFR VIII
T98
GM5
U373
Viability (% Ctrl)
100
50
0
Control
Db α-EMP2
IgG1 α-EMP2
B.
GM5
T98
1.0%
3.7%
Vehicle
22.0%
12.5%
Db α-EMP2
18.0%
10.6%
IgG1 α-EMP2
Propidium Iodide
Annexin V
C.
Db α-EMP2
Db control
Tumor volume (mm3)
400
300
200
100
0
1mg/kg
2.5mg/kg
N=4
0.0
2.5
5.0
7.5
10.0
12.5
Days
Db control
Db α-EMP2
D.
IgG1 α-EMP2
IgG control
Tumor Volume (mm3)
60
50
40
30
20
10
0
Start of treatment
N=4
20
30
40
50
60
Days
IgG control
IgG1 α-EMP2

cell number translated into an induction of cellular death, T98 and GM5 cells were incubated with EMP2 IgG1, EMP2 diabodies, or a vehicle control, and after 72 h, they were stained with annexin V and propidium iodide. Compared with the control, both EMP2 IgG1 and anti-EMP2 diabodies increased the percentage of cell death (Fig. 6*B*).

To determine whether recombinant anti-EMP2 antibodies could be effective in treating GBM tumors *in vivo*, U87/EGFR VIII cells were inoculated subcutaneously to the flanks of athymic nude mice. When tumors reached 4 mm^3, mice were treated intratumor injections of either the anti-EMP2 or control diabody. Mice were injected with 1 mg/kg twice in the 1st week followed by 2.5 mg/kg twice in the week. Tumor growth was reduced when treated with anti-EMP2 diabody (Fig. 6*C*, *left panel*), and the residual tumors showed marked necrosis compared with the control diabody (Fig. 6*C*, *right panel*), suggesting that the anti-EMP2 diabody retarded tumor growth by inducing tumor cell death.

Similar results were observed using systemic treatment of anti-EMP2 IgG1 (Fig. 6*D*). U373 xenografts were implanted subcutaneously into the shoulder of athymic nude mice. When the tumors reached 4 mm^3, mice were treated intraperitoneally with full-length anti-EMP2 or control IgG1 weekly at 3 mg/kg. Anti-EMP2 IgG1 retarded U373 tumor growth compared with control IgG-treated mice (Fig. 6*D*, *left panel*), with tumors exhibiting significant necrosis throughout the tumor (Fig. 6*D*, *right panel*).

DISCUSSION

The current repertoire of chemotherapy, surgical options, and targeted therapies has not significantly enhanced the survival profile for patients with GBM, and it remains the most common and aggressive form of brain tumors with a median survival time of 12 months (39). In this study, we identify a membrane protein EMP2 as an important contributor to GBM tumorigenicity as well as a novel target for GBM killing. Several properties and characteristics of EMP2 make it a potentially attractive therapeutic target. First, EMP2 is expressed in most GBM tumors and cell lines examined to date. Importantly, EMP2 is low in nonmalignant adjacent brain tissue. Second, EMP2 has prognostic value as higher levels suggest a more rapid course of the disease for GBM, and this effect can be reproduced using human xenograft models. Third, we have developed a therapeutic approach to target GBM cells *in vitro* and *in vivo* using specific anti-EMP2 antibody reagents. These reagents are effective in killing GBM cells *in vitro* and in reducing tumor load in mouse model systems.

How does EMP2 contribute to GBM tumorigenicity? It appears that in GBM EMP2 enhances tumor growth in part or exclusively by modulating $\alpha v\beta 3$ integrin surface expression. The importance of $\alpha v\beta 3$ integrin in glioma has been well documented, and it is thought to play a variety of roles in tumorigenesis (2, 40, 41). One role is the involvement of $\alpha v\beta 3$ integrin in cellular migration and invasion. Recruitment of $\alpha v\beta 3$ to focal adhesions within the leading edge of the migratory tumor cells has been observed using patient samples (42, 43). In this study, we have shown that the increase in $\alpha v\beta 3$ integrin expression correlates with increased activation of FAK and Src kinases and an increase in cell migration and invasion *in vitro*. *In vivo*, increased EMP2 promotes tumor cell invasion using intracranial models, and EMP2 and activated Src are correlated in patient samples. In concert, these results suggest that regulation of the integrin-FAK-Src nexus is at least one of the pathways by which EMP2 significantly contributes to pathogenicity.

How does this regulation of $\alpha v\beta 3$ integrin expression occur? Although the mechanism of integrin regulation by EMP2 is not known in glioma, previous studies suggest that integrin expression is downstream of EMP2 (9, 19, 44). Notably, in endometrial cancer, EMP2 promotes $\beta 3$ integrin transcription and helps traffic this integrin pair to the plasma membrane (19). Additional experiments will be needed to decipher its regulation in GBM as well as determine whether $\alpha v\beta 3$ integrin expression can cross-regulate EMP2 expression.

Notably, both EMP2 diabodies and IgG1 induced tumor cell death *in vitro* and *in vivo*. Although modulation of EMP2 levels did not affect GBM cell proliferation *in vitro*, several possibilities exist to explain how EMP2 may be affecting cell survival. First, anti-EMP2 antibodies may down-regulate $\alpha v\beta 3$ integrin. As $\alpha v\beta 3$ integrin is thought to be important for GBM progression, invasion, and survival, inactivation or suppression of this integrin may be sufficient to induce cell death (45). Consistent with the observed cytotoxicity of anti-EMP2 antibodies, down-regulation of $\alpha v\beta 3$ integrin by tumistatin or RGD peptides has been shown to induce apoptosis (46, 47). In the case of tumistatin, apoptosis is induced via dampening of AKT signaling, and whether anti-EMP2 therapy has a similar effect on AKT signaling is currently being studied by our group. Another possibility for how anti-EMP2 antibodies induce cell death is that they may modulate the tumor microenvironment and alter tumor angiogenesis. GBM is known for being highly hemorrhagic, and gliomas express important pro-angiogenic molecules such as vascular endothelial growth factor (VEGF) at elevated levels (48). Recent studies from our laboratory suggest that EMP2 may be important for regulating VEGF expression in endometrial cancer, and it is possible that a similar effect is observed in GBM (18). Hence, anti-EMP2 antibodies may inhibit VEGF levels in the tumor and thus indirectly suppress tumor growth *in vivo*.

An important premise in oncology is that cancers can be classified and treated according to their molecular phenotype.

FIGURE 6. **EMP2 antibodies reduced cellular viability and tumor load.** *A*, 2×10^5 GM5, U87MG, U87/EGFR VIII, T98, and U373 cells were incubated for 72 h with a vehicle control (PBS) or molar equivalents of the anti-EMP2 diabody or anti-EMP2 IgG1. Cellular viability was enumerated using trypan blue exclusion. Data represent viability as a percentage of control from three independent experiments. *B*, GM5 and T98 cells were treated as above, with cellular viability determined using annexin V and propidium iodide staining. The experiment was repeated three times, and a representative graph is shown. *C*, U87/EGFR VIII cells were inoculated subcutaneously in BALB/c nude mice. When tumors reached 4 mm^3, they were treated twice weekly (1st week, 1.0 mg/kg; 2nd week, 2.5 mg/kg), with anti-EMP2 diabody or control diabody. *Left*, tumor size (*arrow* indicates start of treatment). *Right*, tumor histology after treatment. Magnification, ×100. $n = 6$. *, $p < 0.05$ as determined by Student's *t* test. *D*, U373 cells were subcutaneously injected into athymic nude mice. When tumors reached 4 mm^3, mice were treated systemically (intraperitoneally) with anti-EMP2 IgG1 or control IgG (3 mg/kg). *Left*, tumor growth. *Right*, tumor histology at day 65. Magnification, ×100. $n = 6$. *, $p < 0.05$ as determined by Student's *t* test.

In addition to identifying targeting reagents, a particular challenge in GBM is delivery because many molecules, such as antibodies, fail to cross the blood-brain barrier (1, 49). Multiple methods have been developed to enhance antibody delivery to the central nervous system, including direct injection, mechanical or biochemical disruption of the blood-brain barrier, and more recently, stem cell-mediated antibody delivery (49). As an initial proof of principle, we have evaluated diabody and IgG1 forms of anti-EMP2 antibody as each offers distinct advantages for *in vivo* therapy. Although clearance through the blood-brain barrier may be an issue for both immunoglobulin reagents, we predict that diabodies may have a distinct advantage for brain tumors as their small size allows them to access tissues that are poorly accessible by intact antibodies (49). In contrast, intact IgG1 antibodies can elicit antibody-dependent cellular cytotoxicity, which may improve their *in vivo* efficacy (50, 51), and preliminary data suggest that the anti-EMP2 IgG1 is able to elicit such an effect (12). However, additional experiments will be needed to fully elucidate the desired molecular format for GBM as well the optimized delivery strategy.

Our results suggest that EMP2 may be a promising molecular therapeutic target in GBM, and additional work will be needed to determine whether anti-EMP2 antibodies can be combined with standard chemotherapy or molecularly targeted treatments. Although it may be difficult to overcome the permeability issues in the brain using an antibody or antibody fragments, studies here indicate that EMP2 has an important role in GBM tumorigenesis and tumor progression. Moreover, a reduction of EMP2 in GBM cells was sufficient to inhibit tumor growth *in vivo*, lending support to the idea that anti-EMP2 treatment may be therapeutically beneficial.

REFERENCES

1. Rolle, C. E., Sengupta, S., and Lesniak, M. S. (2010) Challenges in clinical design of immunotherapy trials for malignant glioma. *Neurosurg. Clin. N. Am.* **21,** 201–214
2. Teodorczyk, M., and Martin-Villalba, A. (2010) Sensing invasion: cell surface receptors driving spreading of glioblastoma. *J. Cell. Physiol.* **222,** 1–10
3. Wang, S. D., Rath, P., Lal, B., Richard, J. P., Li, Y., Goodwin, C. R., Laterra, J., and Xia, S. (2012) EphB2 receptor controls proliferation/migration dichotomy of glioblastoma by interacting with focal adhesion kinase. *Oncogene* **31,** 5132–5143
4. Giese, A., Loo, M. A., Tran, N., Haskett, D., Coons, S. W., and Berens, M. E. (1996) Dichotomy of astrocytoma migration and proliferation. *Int. J. Cancer* **67,** 275–282
5. Zagzag, D., Lukyanov, Y., Lan, L., Ali, M. A., Esencay, M., Mendez, O., Yee, H., Voura, E. B., and Newcomb, E. W. (2006) Hypoxia-inducible factor 1 and VEGF upregulate CXCR4 in glioblastoma: implications for angiogenesis and glioma cell invasion. *Lab. Invest.* **86,** 1221–1232
6. de Groot, J., and Milano, V. (2009) Improving the prognosis for patients with glioblastoma: the rationale for targeting Src. *J. Neurooncol.* **95,** 151–163
7. Wick, W., Weller, M., Weiler, M., Batchelor, T., Yung, A. W., and Platten, M. (2011) Pathway inhibition: emerging molecular targets for treating glioblastoma. *Neuro-Oncology* **13,** 566–579
8. Fu, M., Rao, R., Sudhakar, D., Hogue, C. P., Rutta, Z., Morales, S., Gordon, L. K., Braun, J., Goodglick, L., and Wadehra, M. (2011) Epithelial membrane protein-2 promotes endometrial tumor formation through activation of FAK and Src. *PLoS One* **6,** e19945
9. Morales, S. A., Mareninov, S., Wadehra, M., Zhang, L., Goodglick, L., Braun, J., and Gordon, L. K. (2009) FAK activation and the role of epithelial membrane protein 2 (EMP2) in collagen gel contraction. *Invest. Ophthalmol. Vis. Sci.* **50,** 462–469
10. Habeeb, O., Goodglick, L., Soslow, R. A., Rao, R. G., Gordon, L. K., Schirripa, O., Horvath, S., Braun, J., Seligson, D. B., and Wadehra, M. (2010) Epithelial membrane protein-2 expression is an early predictor of endometrial cancer development. *Cancer* **116,** 4718–4726
11. Fu, M., Maresh, E. L., Soslow, R. A., Alavi, M., Mah, V., Zhou, Q., Iasonos, A., Goodglick, L., Gordon, L. K., Braun, J., and Wadehra, M. (2010) Epithelial membrane protein-2 is a novel therapeutic target in ovarian cancer. *Clin. Cancer Res.* **16,** 3954–3963
12. Fu, M., Maresh, E. L., Helguera, G., Kiyohara, M., Qin, Y., Ashki, N., Daniels-Wells, T. R., Aziz, N., Gordon, L. K., Braun, J., Elshimali, Y., Soslow, R. A., Penichet, M. L., Goodglick, L., and Wadehra, M. (2014) Rationale and preclinical efficacy of a novel anti-EMP2 antibody for the treatment of invasive breast cancer. *Mol. Cancer Ther.,* in press 10.1158/1535–7163.MCT-13-0199
13. Wadehra, M., Natarajan, S., Seligson, D. B., Williams, C. J., Hummer, A. J., Hedvat, C., Braun, J., and Soslow, R. A. (2006) Expression of epithelial membrane protein-2 is associated with endometrial adenocarcinoma of unfavorable outcome. *Cancer* **107,** 90–98
14. Freije, W. A., Castro-Vargas, F. E., Fang, Z., Horvath, S., Cloughesy, T., Liau, L. M., Mischel, P. S., and Nelson, S. F. (2004) Gene expression profiling of gliomas strongly predicts survival. *Cancer Res.* **64,** 6503–6510
15. Wang, Y., Zhu, S., Cloughesy, T. F., Liau, L. M., and Mischel, P. S. (2004) p53 disruption profoundly alters the response of human glioblastoma cells to DNA topoisomerase I inhibition. *Oncogene* **23,** 1283–1290
16. Wang, M. Y., Lu, K. V., Zhu, S., Dia, E. Q., Vivanco, I., Shackleford, G. M., Cavenee, W. K., Mellinghoff, I. K., Cloughesy, T. F., Sawyers, C. L., and Mischel, P. S. (2006) Mammalian target of rapamycin inhibition promotes response to epidermal growth factor receptor kinase inhibitors in PTEN-deficient and PTEN-intact glioblastoma cells. *Cancer Res.* **66,** 7864–7869
17. Kim, K., Brush, J. M., Watson, P. A., Cacalano, N. A., Iwamoto, K. S., and McBride, W. H. (2008) Epidermal growth factor receptor vIII expression in U87 glioblastoma cells alters their proteasome composition, function, and response to irradiation. *Mol. Cancer Res.* **6,** 426–434
18. Gordon, L. K., Kiyohara, M., Fu, M., Braun, J., Dhawan, P., Chan, A., Goodglick, L., and Wadehra, M. (2013) EMP2 regulates angiogenesis in endometrial cancer cells through induction of VEGF. *Oncogene* **32,** 5369–5376
19. Wadehra, M., Forbes, A., Pushkarna, N., Goodglick, L., Gordon, L. K., Williams, C. J., and Braun, J. (2005) Epithelial membrane protein-2 regulates surface expression of $\alpha v\beta 3$ integrin in the endometrium. *Dev. Biol.* **287,** 336–345
20. Wadehra, M., Dayal, M., Mainigi, M., Ord, T., Iyer, R., Braun, J., and Williams, C. J. (2006) Knockdown of the tetraspan protein epithelial membrane protein-2 inhibits implantation in the mouse. *Dev. Biol.* **292,** 430–441
21. Mellinghoff, I. K., Wang, M. Y., Vivanco, I., Haas-Kogan, D. A., Zhu, S., Dia, E. Q., Lu, K. V., Yoshimoto, K., Huang, J. H., Chute, D. J., Riggs, B. L., Horvath, S., Liau, L. M., Cavenee, W. K., Rao, P. N., Beroukhim, R., Peck, T. C., Lee, J. C., Sellers, W. R., Stokoe, D., Prados, M., Cloughesy, T. F., Sawyers, C. L., and Mischel, P. S. (2005) Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N. Engl. J. Med.* **353,** 2012–2024
22. Cronin, M., Akin, A. R., Collins, S. A., Meganck, J., Kim, J.-B., Baban, C. K., Joyce, S. A., van Dam, G. M., Zhang, N., van Sinderen, D., O'Sullivan, G. C., Kasahara, N., Gahan, C. G., Francis, K. P., and Tangney, M. (2012) High resolution *in vivo* bioluminescent imaging for the study of bacterial tumour targeting. *PLoS One* **7,** e30940
23. Agus, D. B., Scher, H. I., Higgins, B., Fox, W. D., Heller, G., Fazzari, M., Cordon-Cardo, C., and Golde, D. W. (1999) Response of prostate cancer to anti-Her-2/neu antibody in androgen-dependent and -independent human xenograft models. *Cancer Res.* **59,** 4761–4764
24. Wang, W., Zhu, N. L., Chua, J., Swenson, S., Costa, F. K., Schmitmeier, S., Sosnowski, B. A., Shichinohe, T., Kasahara, N., and Chen, T. C. (2005) Retargeting of adenoviral vector using basic fibroblast growth factor ligand for malignant glioma gene therapy. *J. Neurosurg.* **103,** 1058–1066
25. Huang, T. T., Hlavaty, J., Ostertag, D., Espinoza, F. L., Martin, B., Petznek, H., Rodriguez-Aguirre, M., Ibañez, C. E., Kasahara, N., Gunzburg, W., Gruber, H. E., Pertschuk, D., Jolly, D. J., and Robbins, J. M. (2013) Toca 511

gene transfer and 5-fluorocytosine in combination with temozolomide demonstrates synergistic therapeutic efficacy in a temozolomide-sensitive glioblastoma model. *Cancer Gene Ther.* **20,** 544–551
26. Shimazaki, K., Lepin, E. J., Wei, B., Nagy, A. K., Coulam, C. P., Mareninov, S., Fu, M., Wu, A. M., Marks, J. D., Braun, J., Gordon, L. K., and Wadehra, M. (2008) Diabodies targeting epithelial membrane protein 2 reduce tumorigenicity of human endometrial cancer cell lines. *Clin. Cancer Res.* **14,** 7367–7377
27. Acconcia, F., Barnes, C. J., and Kumar, R. (2006) Estrogen and tamoxifen induce cytoskeletal remodeling and migration in endometrial cancer cells. *Endocrinology* **147,** 1203–1212
28. Wadehra, M., Sulur, G. G., Braun, J., Gordon, L. K., and Goodglick, L. (2003) Epithelial membrane protein-2 is expressed in discrete anatomical regions of the eye. *Exp. Mol. Pathol.* **74,** 106–112
29. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Image processing with ImageJ. *Biophotonics Int.* **11,** 36–42
30. Fan, Q.-W., Cheng, C. K., Gustafson, W. C., Charron, E., Zipper, P., Wong, R. A., Chen, J., Lau, J., Knobbe-Thomsen, C., Weller, M., Jura, N., Reifenberger, G., Shokat, K. M., and Weiss, W. A. (2013) EGFR phosphorylates tumor-derived EGFRvIII driving STAT3/5 and progression in glioblastoma. *Cancer Cell* **24,** 438–449
31. Hood, J. D., and Cheresh, D. A. (2002) Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* **2,** 91–100
32. Charo, I. F., Nannizzi, L., Smith, J. W., and Cheresh, D. A. (1990) The vitronectin receptor $\alpha v\beta 3$ binds fibronectin and acts in concert with $\alpha 5\beta 1$ in promoting cellular attachment and spreading on fibronectin. *J. Cell Biol.* **111,** 2795–2800
33. Jiao, Y., Feng, X., Zhan, Y., Wang, R., Zheng, S., Liu, W., and Zeng, X. (2012) Matrix metalloproteinase-2 promotes $\alpha v\beta 3$ integrin-mediated adhesion and migration of human melanoma cells by cleaving fibronectin. *PLoS One* **7,** e41591
34. Kerr, J. S., Slee, A. M., and Mousa, S. A. (2000) Small molecule αv integrin antagonists: novel anticancer agents. *Expert Opin. Investig. Drugs* **9,** 1271–1279
35. Rooprai, H. K., Vanmeter, T., Panou, C., Schnüll, S., Trillo-Pazos, G., Davies, D., and Pilkington, G. J. (1999) The role of integrin receptors in aspects of glioma invasion *in vitro*. *Int. J. Dev. Neurosci.* **17,** 613–623
36. Mitra, S. K., and Schlaepfer, D. D. (2006) Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr. Opin. Cell Biol.* **18,** 516–523
37. Hehlgans, S., Haase, M., and Cordes, N. (2007) Signalling via integrins: implications for cell survival and anticancer strategies. *Biochim. Biophys. Acta* **1775,** 163–180
38. Beckman, R. A., Weiner, L. M., and Davis, H. M. (2007) Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors. *Cancer* **109,** 170–179
39. Buonerba, C., Di Lorenzo, G., Marinelli, A., Federico, P., Palmieri, G., Imbimbo, M., Conti, P., Peluso, G., De Placido, S., and Sampson, J. H. (2011) A comprehensive outlook on intracerebral therapy of malignant gliomas. *Crit. Rev. Oncol. Hematol.* **80,** 54–68
40. Petrás, M., Hutóczki, G., Varga, I., Vereb, G., Szöllosi, J., Bognár, L., Ruszthi, P., Kenyeres, A., Tóth, J., Hanzély, Z., Scholtz, B., and Klekner, A. (2009) Expression pattern of invasion-related molecules in brain tumors of different origin. *Magy. Onkol.* **53,** 253–258
41. Kanamori, M., Kawaguchi, T., Berger, M. S., and Pieper, R. O. (2006) Intracranial microenvironment reveals independent opposing functions of host $\alpha V\beta 3$ expression on glioma growth and angiogenesis. *J. Biol. Chem.* **281,** 37256–37264
42. D'Abaco, G. M., and Kaye, A. H. (2007) Integrins: molecular determinants of glioma invasion. *J. Clin. Neurosci.* **14,** 1041–1048
43. Gladson, C. L., and Cheresh, D. A. (1991) Glioblastoma expression of vitronectin and the $\alpha v\beta 3$ integrin. Adhesion mechanism for transformed glial cells. *J. Clin. Invest.* **88,** 1924–1932
44. Wadehra, M., Iyer, R., Goodglick, L., and Braun, J. (2002) The tetraspan protein epithelial membrane protein-2 interacts with $\beta 1$ integrins and regulates adhesion. *J. Biol. Chem.* **277,** 41094–41100
45. Lorger, M., and Felding-Habermann, B. (2012) in *Integrin Signaling in Angiogenesis and Metastatic Cancer Progression in the Brain Signaling Pathways and Molecular Mediators in Metastasis* (Fatatis, A., ed) pp. 311–329, Springer, Netherlands
46. Kawaguchi, T., Yamashita, Y., Kanamori, M., Endersby, R., Bankiewicz, K. S., Baker, S. J., Bergers, G., and Pieper, R. O. (2006) The PTEN/Akt pathway dictates the direct $\alpha V\beta 3$-dependent growth-inhibitory action of an active fragment of tumstatin in glioma cells *in vitro* and *in vivo*. *Cancer Res.* **66,** 11331–11340
47. Chang, M.-W., Lo, J.-M., Juan, H.-F., Chang, H.-Y., and Chuang, C.-Y. (2012) Combination of RGD compound and low-dose paclitaxel induces apoptosis in human glioblastoma cells. *PLoS One* **7,** e37935
48. Kerbel, R. S. (2008) Tumor angiogenesis. *N. Engl. J. Med.* **358,** 2039–2049
49. Frank, R. T., Aboody, K. S., and Najbauer, J. (2011) Strategies for enhancing antibody delivery to the brain. *Biochim. Biophys. Acta* **1816,** 191–198
50. McEarchern, J. A., Oflazoglu, E., Francisco, L., McDonagh, C. F., Gordon, K. A., Stone, I., Klussman, K., Turcott, E., van Rooijen, N., Carter, P., Grewal, I. S., Wahl, A. F., and Law, C.-L. (2007) Engineered anti-CD70 antibody with multiple effector functions exhibits *in vitro* and *in vivo* antitumor activities. *Blood* **109,** 1185–1192
51. Fukai, J., Nishio, K., Itakura, T., and Koizumi, F. (2008) Antitumor activity of cetuximab against malignant glioma cells overexpressing EGFR deletion mutant variant III. *Cancer Sci.* **99,** 2062–2069

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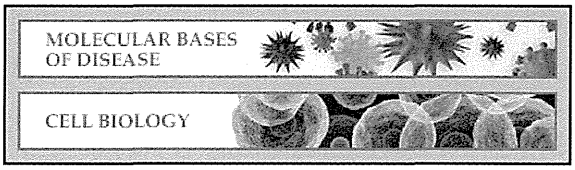

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