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—神経の研究へ—

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第6回 iPS細胞を使う
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はじめに

集学的医療の進歩により脊髄損傷患者の平均余命は健常人とかわらなくなってきたが，医療の発達した現代でも損傷された脊髄を治療する有効な手段はいまだ確立されていない．しかし近年の神経科学の進歩に伴い，脊髄損傷に対する細胞移植療法をはじめとするさまざまな治療法に関する基礎研究が報告されるようになった．これらの成果により損傷脊髄でも微小環境が整えば再生することが示され，“中枢神経系は一度損傷を受けると再生しない”という通説はもはや過去のものとなりつつある．

本稿では，われわれがこれまで行ってきた細胞移植研究，特に人工多能性幹細胞（induced pluripotent stem cell：iPS細胞）に関する基礎研究の現状に触れながら，脊髄再生医療における細胞移植療法の現状と今後の展望について概説する．

Ⅰ　脊髄損傷に対する細胞移植療法

われわれは胎児由来神経幹/前駆細胞（neural stem/progenitor cells：NS/PCs）に着目し，ラットNS/PCsをラット損傷脊髄に，さらにヒトNS/PCsをサル損傷脊髄へと移植し，運動機能の良好な回復を報告した[1,2]．しかしNS/PCsを得るためには中絶胎児からの細胞採取が必須であり，倫理的観点からわが国における臨床応用は困難といわざるをえない．また胎児由来NS/PCsを移植治療に用いる際，他家移植であるため免疫拒絶が起こる可能性があり，細胞移植治療における大きな問題の一つとなっている．

一方，McDonaldらは胚性幹細胞（embryonic stem cell：ES細胞）から誘導したNS/PCsをラット損傷脊髄へ移植し，良好な機能回復が得られることを報告した[3]．われわれもES細胞からNS/PCsを誘導するシステムを構築し[4]，マウスES細胞由来NS/PCsをマウス損傷脊髄に移植し，軸索の再生と再髄鞘化および血管新生を認め，良好な下肢運動機能の改善を認めた[5]．しかしヒトへの臨床応用を考えた場合，ES細胞の樹立には不妊治療における余剰胚を用いるため，胎児由来NS/PCsと同様に倫理的な問題，および免疫拒絶の問題は避けられない．

Ⅱ　iPS細胞を用いた脊髄損傷治療

前述した諸問題に解決の糸口を与えたのが，京都大学iPS細胞研究所・山中伸弥教授らにより樹立されたiPS細胞である[6,7]．iPS細胞は患者自身の体細胞から樹立することが可能であり，先に述べた倫理的問題や免疫拒絶の問題を解決できる技術として期待されている．

1．マウスiPS細胞由来NS/PCsを用いた脊髄損傷治療

まず，前述したマウスES細胞の神経幹細胞への誘導培養法をマウスiPS細胞に応用し，マウスiPS細胞由来NS/PCsを作製した．さらにその安全性を確認するために，免疫不全（nonobese diabetic-severe combined immunodeficient：NOD-scid）マウスの大脳の線条体に移植した．その結果，成体組織（tail tip fibrobrast：TTF）由来iPS細胞を分化誘導させた際に残存する未分化細胞の比率が0.01%より高いと腫瘍形成が起こりやすいことが判明した[8]．この結果をふまえて，当研究室のTsujiらはマウスTTF由来のiPS細胞を用いてマウス損傷脊髄への移植実験を行った．TTF由来iPS細胞のうち，安全性が確認できた335D1，腫瘍形成が認

Key words：neural stem/progenitor cell, cell transplantation, spinal cord injury
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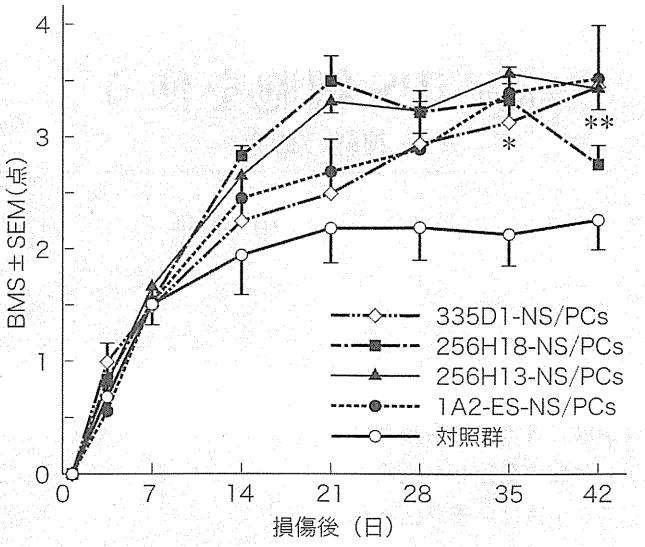
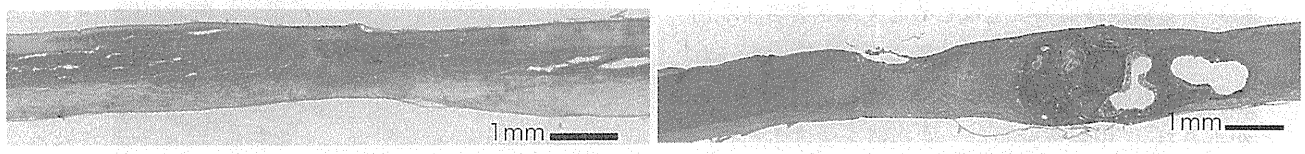


a．下肢運動機能評価（$^{*}p<0.05$，$^{**}p<0.01$）



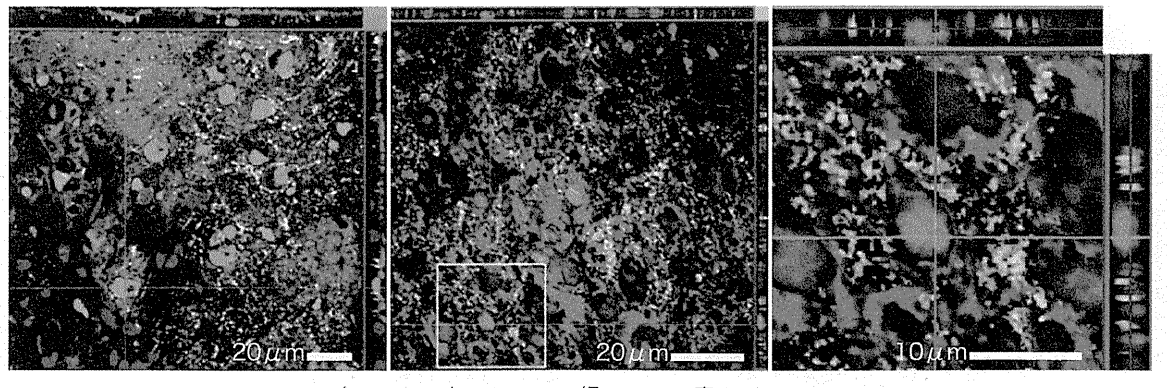
b．335D1-NS/PCs（HE 染色）　　c．256H18-NS/PCs（HE 染色）

図 1．マウス脊髄損傷に対する成体マウス iPS 細胞由来 NS/PCs 移植（文献 9 より引用改変）．“安全な”335D1 由来 NS/PCs 移植後には腫瘍形成は認められず，下肢運動機能の改善が認められる．これに対して，“危険な”256H18 および 256H13 由来 NS/PCs 移植後に奇形腫の形成を認め，一度回復した下肢運動機能が脊髄損傷後 6 週目で低下している．

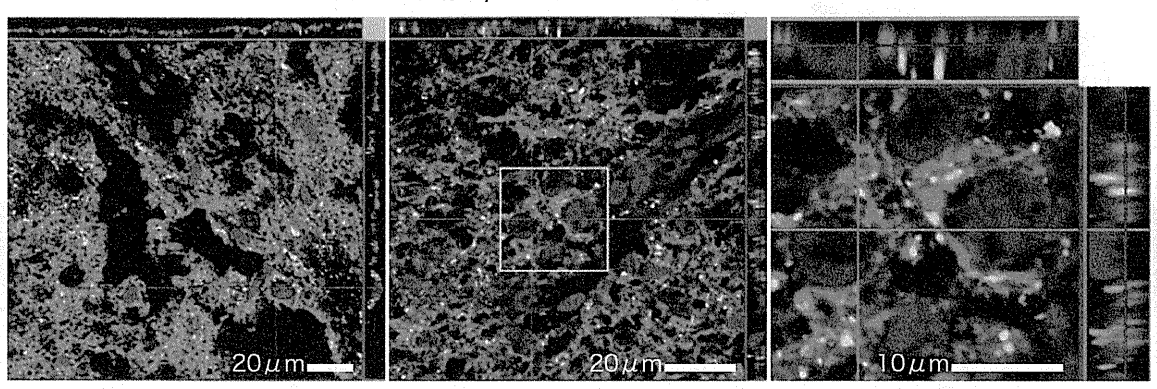
められた“危険な”256H13 と 256H18 の三つのクローンをそれぞれ神経分化誘導し，マウス脊髄損傷モデルに移植を行った．その結果，“危険な”クローン由来の NS/PCs 移植群は脊髄内で巨大な奇形腫を形成し，下肢運動機能の低下が認められた．一方で“安全な”335D1 由来 NS/PCs 移植群では腫瘍形成を認めず，対照群と比較して下肢運動機能の有意な回復がみられた（図 1）．このことより，臨床応用が可能な TTF 由来 iPS 細胞は，厳密にその安全性を検討すれば，脊髄損傷治療への有用な細胞源となることが示唆された[9]．

2．ヒト iPS 細胞由来 NS/PCs を用いた脊髄損傷治療

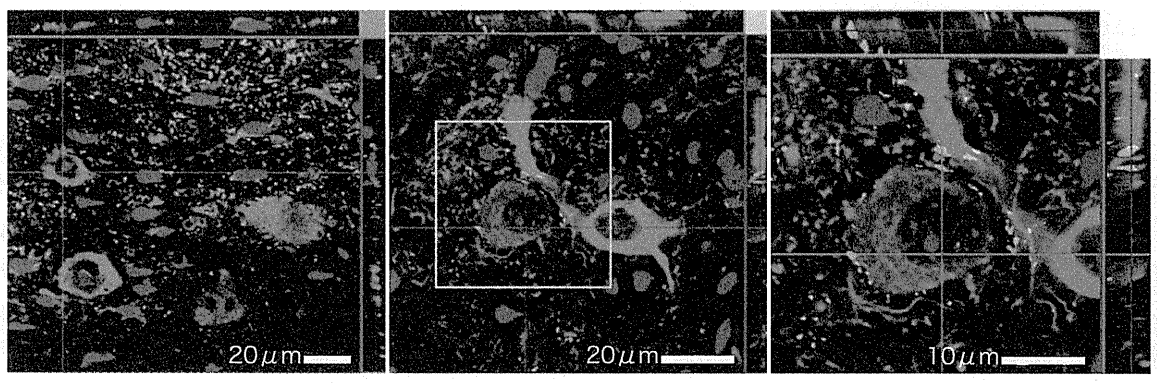
臨床応用へ向けて，当研究室の Nori らはヒト iPS 細胞を用いた脊髄損傷治療への有効性の検討を行った．成人顔面皮膚にレトロウイルスを用いて *Oct3/4*，*Sox2*，*Klf4*，*c-Myc* を導入して作製し，安全性を確認した 201B7 細胞株からマウス iPS 細胞と同様の方法で NS/PCs へ分化誘導した．201B7 由来 NS/PCs の治療効果を確認するため，NOD-scid マウスの損傷脊髄に移植した．移植細胞はマウス脊髄内で生着し，ニューロン，アストロサイト，オリゴデンドロサイトへ分化した．移植細胞の約 50%が β Ⅲtubulin 陽性のニューロンへと分化しており，そのうちの約 70%が GAD67 陽性の GABA 作動性ニューロンへと分化していた．さらに，免疫組織学的解析および電子顕微鏡による解析から，201B7 由来ニューロンとマウスニューロンがシナプスを形成していることが明らかになった（図 2a，b）．運動誘発電位（motor evoked potential：MEP）を計測したところ，201B7 由来 NS/PCs 移植群では MEP の波形が検出されたのに対し，対照群では MEP の波形は検出されなかった（図 2c）．このことより，201B7 由来 NS/PCs がマウス脊髄内で介在ニューロンとして機能して，神経回路の再構築を行っている可能性が示唆された．さらに移植細胞由来アストロサイトによる神経栄養因子の自己分泌，傍分泌作用により血管新生，神経再生，組織保護作用も認められた．これらの結果として，Basso Mouse Scale（BMS）において良好な下肢運動機能の改善が認められた（図 2d）．さらに，長期にわたる 201B7 由来 NS/PCs 移植の安全性を確認するため，脊髄損傷後 112 日まで経過を観察したところ，細胞移植群では良好な下肢運動機



白：Bsn，赤：βIII tub，緑：HNu，青：Hoechst



白：hSyn，赤：βIII tub，緑：HNu，青：Hoechst



緑：hSyn，赤：ChAT，青：Hoechst

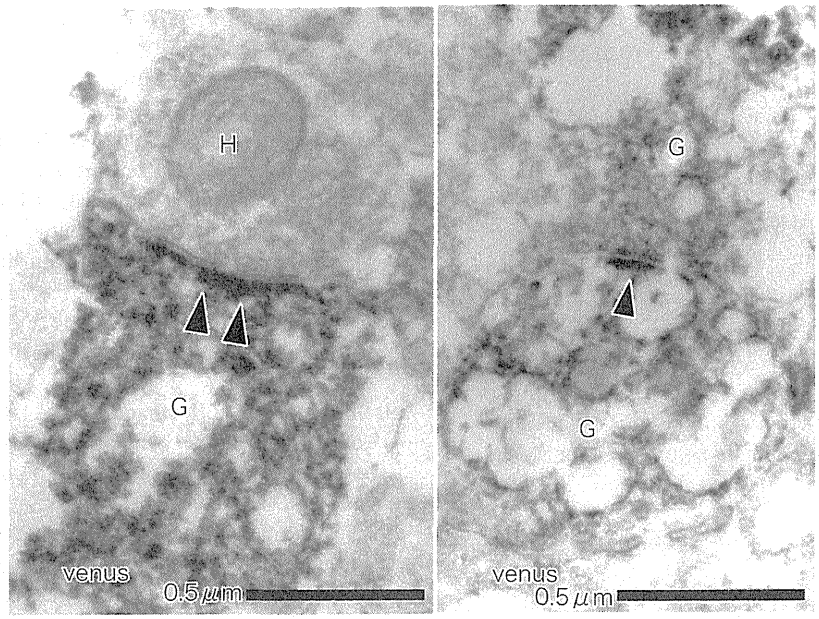
a．免疫組織像．Bsn（Bassoon）：マウス特異的シナプス前蛋白，βIII tub（βIII tubulin），HNu（human nucleus）：ヒト核特異的マーカー，hSyn（human synaptophysin）：ヒト特異的シナプス前蛋白

図 2．マウス脊髄損傷に対するヒト iPS 細胞由来 NS/PCs 移植（文献 10 より引用改変）．免疫組織学的および電子顕微鏡による解析より，ヒト iPS 細胞由来ニューロンはマウスニューロンとシナプスを形成している（a，b）．運動誘発電位（MEP）を計測したところ，移植群でのみ波形が検出された（c）．マウス脊髄損傷後の下肢運動機能評価法である BMS において移植群は対照群に比べて有意な改善を認める（d）．

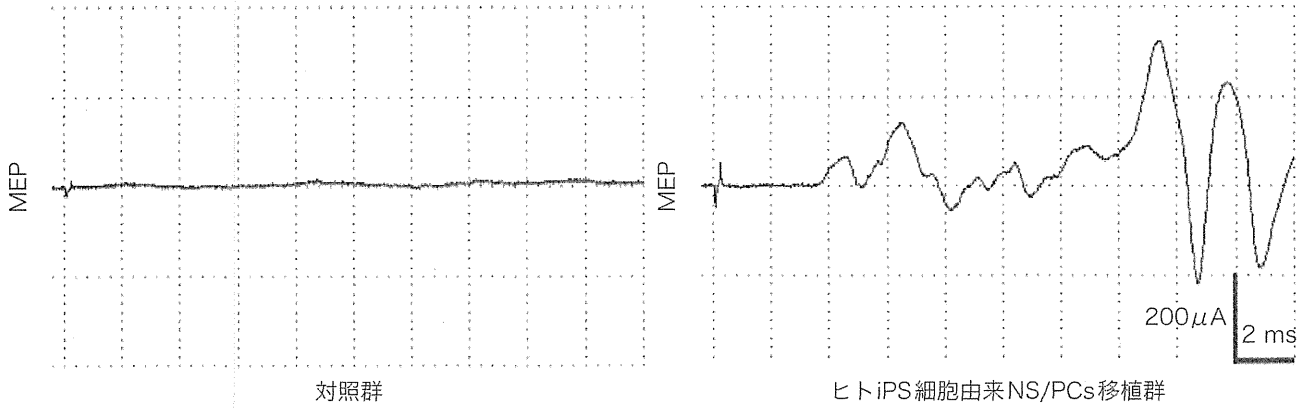
能の回復が継続し，腫瘍形成も認められなかった[10]．

おわりに

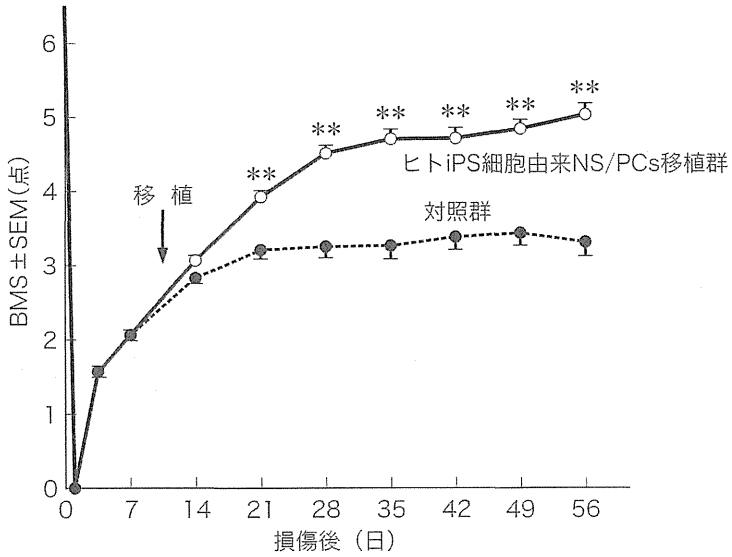
これまでわれわれが報告してきた移植研究には，iPS 細胞樹立時における初期化遺伝子の導入にレトロウイルスを用いてきた．レトロウイルスは遺伝子のプロモータ付近に組み込まれることが多く，近傍の内在性遺伝子の発現状態を変化させ腫瘍化をもたらす危険性がある．この問題を解決するため，ゲノム挿入の起こらないプラスミドベクターを用いた方法[11]，染色体外に挿入しいずれ



b．電子顕微鏡像．G：移植細胞由来ニューロン，H：マウス脊髄内ニューロン，矢頭：post-synaptic density



c．運動誘発電位（MEP）



d．下肢運動機能評価（$^{**}p<0.01$）

図 2（つづき）

消失するエピソーマルベクターの導入[12]などが相次いで報告されている．今後，これらの方法で作製されたintegration free iPS細胞を用いて，①移植前の安全なiPS細胞クローンの選別法，②最終産物であるiPS細胞由来NS/PCsの安全性基準の確立，③GMPに準拠した臨床に使用できるiPS細胞由来NS/PCsの製造を行っていく予定である．

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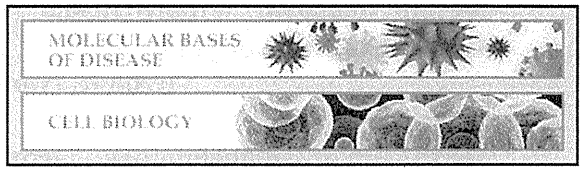
Molecular Bases of Disease:

Epithelial Membrane Protein-2 (EMP2) Activates Src and is a Novel Therapeutic Target for GBM



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Epithelial Membrane Protein-2 (EMP2) Activates Src and is a Novel Therapeutic Target for GBM*

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*Running title: EMP2 promotes GBM tumorigenesis and is a target for therapy

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Keywords: EMP2; GBM; invasion; immunotherapy; tumor marker; tetraspanins

Background: EMP2 is a tetraspan protein linked with aggressive disease.
Results: EMP2 correlates with activated Src in patients with GBM. Using intracranial mouse models, EMP2 promotes tumor cell invasiveness. Antibodies to EMP2 reduce GBM tumor load.
Conclusion: EMP2 is a novel therapeutic target in GBM.
Significance: The clinical outcome for patients with GBM remains poor, and thus new targeted therapies are needed.

ABSTRACT

Despite recent advances in molecular classification, surgery, radiotherapy, and targeted therapies, the clinical outcome of patients with malignant brain tumors remains extremely poor. In this study, we have identified the tetraspan protein epithelial membrane protein-2 (EMP2) as a potential target for glioblastoma (GBM) killing. EMP2 had low or undetectable expression in normal brain, but was highly expressed in GBM as 95% of patients showed some expression of the protein. In GBM cells, EMP2 enhanced tumor growth *in vivo* in part by upregulating αvβ3 integrin surface expression, activating FAK and Src kinases, and promoting cell migration and invasion. Consistent with these findings, EMP2 expression significantly correlated with activated Src kinase in patient samples and promoted tumor cell invasion using intracranial mouse models. As a proof of principle to determine if EMP2 could serve as a target for therapy, cells were treated using specific anti-EMP2 antibody reagents. These reagents were effective in killing GBM cells *in vitro* and in reducing tumor load in subcutaneous mouse models. These results support the role of EMP2 in the

pathogenesis of GBM and suggest that anti-EMP2 treatment may be a novel therapeutic treatment.

INTRODUCTION

Despite recent advances in molecular classification, surgery and radiotherapy, and targeted therapies, the clinical outcome of patients with malignant brain tumors remains extremely poor. The prognosis for patients with glioblastoma (GBM), the most common and aggressive form of brain tumors, yields only a median survival of 12 months and a five year survival of 5%(1). The rapid and deadly course of the disease is due, in large part, to the highly invasive nature of these malignant cells. In most patients, GBM cells migrate into the surrounding brain parenchyma, thus making complete surgical resection difficult(2).

GBMs are characterized by two spatial and temporal events, uncontrolled proliferation and abnormal cell migration(3). These events are disassociated as tumors cores contain highly proliferative populations which are distinct from more invasive cells at the periphery which show slower proliferation rates(4). With regard to invasion, it is believed that integrins, a family of heterodimeric proteins which link the cytoskeleton to the extracellular matrix, play an important role. Integrin adhesion and invasion activates focal adhesion kinase (FAK), a nonreceptor cytoplasmic tyrosine kinase that is found to be upregulated in both anaplastic astrocytomas and GBM(2,5). FAK phosphorylation activates Src kinase, which is part of the family of kinases that regulate the translation of extracellular signals with intracellular signaling(6). Dysregulated Src signaling has been shown in many cancers, including GBM(7).

A new protein implicated in the activation of FAK is the oncogenic protein EMP2(8,9). EMP2 is a member of the growth arrest specific gene 3/peripheral myelin protein-22 (GAS3/PMP22) group of tetraspan proteins, and its expression is upregulated in ovarian, breast, and endometrial malignancies(10-12). Within these tumors, EMP2 has been shown to be a prognostic indicator as its expression correlates with poor survival and/or advanced disease(12,13).

To date, little is known about the role of EMP2 in the central nervous system. Although there have been no reports of EMP2 in normal brain, a recent Affymetrix study revealed upregulation of its mRNA in GBM (14). Hence, in this study, we generate preliminary evidence as to the protein expression and role of EMP2 in GBM. Specifically, we provide principle data that suggests that EMP2 promotes a more aggressive disease phenotype and that it may ultimately serve as a novel therapeutic target for antibody therapy.

METHODS AND MATERIALS

Cell lines and reagents. Human GBM cell lines U87MG, U138, and U373 (ATCC, Manassas, VA) were cultured in DMEM media supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin and streptomycin, and 1% sodium pyruvate in humidified 5% CO_2 at 37 °C. Primary human cells GM1, GM2, GM3, GM4, GM5, GM6, and GM97 were derived from patient tumors and cultured as described previously (15). U87MG cells which overexpress an in-frame deletion of amino acids 6 to 273 in the EGFR gene termed EGFR VIII (U87/EGFR VIII) have been previously described and were cultured in complete DMEM as above(16,17). Cell lines were used within 2 months after resuscitation of frozen aliquots and were authenticated based on viability, recovery, growth, morphology, and isoenzymology by the supplier. Cells were passaged every 2-4 days. EMP2 expression was stably overexpressed using a retroviral vector encoding both EMP2 and GFP genes

under the control of the CMV promoter, with the GFP gene translated from an internal ribosomal entry sequence or through the use of a human EMP2-GFP fusion (46kD) protein(8). EMP2 expression was reduced using the Mission pLKO.1 puro shRNA lentiviral vector (shRNA; Sigma Aldrich, St. Louis, MO)(18) or through the previously described use of an EMP2 specific ribozyme (RIBO)(19). Vector control cell lines were generated using empty shRNA or control GFP vectors (V). In some experiments, scrambled or EMP2 specific siRNA vectors (Thermo Scientific, Pittsburg, PA) were used to transiently reduce EMP2 expression as previously described (19-21). In order to create tumors for intracranial models, U87/EMP2, U87/V, and U87/shRNA cells were stably infected an HIV-1-based bicistronic lentiviral vector encoding both the firefly luciferase and GFP genes under the control of the CMV promoter (U87/Luc) by the UCLA Vector Core & Shared Resource as previously described(22).

Immunohistochemistry. A GBM array containing 0.6mm cores (two tumors, one normal) from 110 patients has been previously described(14). The array was stained with human EMP2 antisera or a preimmune control. Briefly, for antigen retrieval, sections were incubated at 95°C for 20 minutes in 0.1 M citrate, pH 6.0. EMP2 was detected using rabbit human EMP2 antisera at a dilution of 1:400 as previously described (13) followed by visualization using the Vector ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. DAB or deNOVO Red was used for visualization, and staining was quantified by 2 independent pathologists (P.M.) and (A.I.) on a 0-3 histological score.

Preparation of Xenografts. *Ethical Treatment of Animals Statement:* This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Research Committee at the University of California, Los Angeles. All efforts were made to minimize animal suffering.

Four to six-week-old nude BALB/c female mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the vivarium at the University of California, Los Angeles. Animals were inoculated subcutaneously with 1 x 10^6 U373/EMP2, U373/V, or U373/RIBO, or 5 x 10^5 U87/EMP2, U87/V, or U87/shRNA cells. The number of mice used per group is indicated in the figure legends. Tumors were measured twice a week, and tumor volumes were calculated by the formula $\pi/6$ x larger diameter x smaller diameter^2 (23). Data are expressed as mean ± SEM. Student's t-test was used to evaluate overall differences in the means between groups versus the control at a given time, and significance was defined as $p<0.05$.

To create an intracranial model for GBM, 1 x 10^5 U87/EMP2/Luc, U87/V/Luc, and U87/shRNA/Luc were stereotactically implanted into the right frontal lobe of mouse brain of 6-8-week-old female BALB/c nude mice. (24,25). Animal health was evaluated for the development of behavioral and neurological signs and weight loss. Mice were euthanized if weight loss exceeded 10%. Tumor loads were monitored by bioluminescence imaging. Briefly, mice received an intraperitoneal (i.p.) injection of 100µl D-luciferin (30mg/ml), 30 minutes after injection, mice were anesthetized with ketamine/xylazine (100 and 10mg/kg) and placed on the imaging stage. The bioluminescence signals were captured using an IVIS-200 (Xenogen

Corp., Alameda, CA, USA). The data were analyzed using maximum photon flux emission (photons/second) in the regions of interest. A one way ANOVA analysis was used to evaluate differences between the different experimental groups, with significance defined as $p<0.05$.

To determine the therapeutic potential for EMP2 antibodies in GBM, U87/EGFR VIII or U373 tumors were created subcutaneously on the shoulder of Balb/c nude mice. Anti-EMP2 diabodies and control diabodies have been detailed previously (11,26), and the variable regions were recently cloned to produce a fully human IgG1 (12). Both cell lines were also tested for murine pathogens including mycoplasma by the Division of Laboratory Animal Medicine at the University of California, Los Angeles prior to injection. When tumors approached 4 mm^3, they were injected twice a week with intratumoral injections of the anti-EMP2 diabodies at 1mg/kg during week 1 and then 2mg/kg during week 2. In order to test the full length EMP2 IgG1, tumors were created using the wild type U373 cell line, and mice were treated weekly through i.p. injections using 3 mg/kg anti-EMP2 IgG1 or control antibodies. Tumors were measured twice a week. Following treatment, tumors were excised, fixed in formalin, and then processed for hematoxylin and eosin staining by the Tissue Procurement Laboratory at UCLA.

Proliferation Assays. Cellular Proliferation was monitored using a BrdU Cell Proliferation Assay (EMD Chemicals, Gibbstown, NJ) as per the manufacturer's instructions. Briefly, 10^4 cells were cultured in a 96-well plate. Triplicate wells were used for each condition. Cells were incubated in DMEM + 0.5% FCS overnight to arrest the cells, then were released in complete media containing BrdU for 2 or 24 hrs. Cells were fixed, permeabilized and the DNA was denatured. A detector anti-BrdU monoclonal antibody was added, and ultimately detected using a horseradish peroxidase (HRP)-conjugated goat anti-mouse. To determine the amount of incorporated BrdU, a fluorogenic substrate was added and the absorbance was quantified at dual wavelengths of 450 and 595 nm.

Wound healing. 10^5 GBM cells with modulated EMP2 expression were plated on 35-mm tissue culture dishes. When cells were confluent, a "wound" was created using a 100-µl pipette tip as described (9,27). Wound healing was monitored over 48 hours with a 10x phase contrast objective, and images were collected using a Power Shot S80 camera (Canon, Lake Success, NY). Quantification of the wound healing was determined by measuring the remaining wound diameter. Wound healing was calculated as a percent of the closed wound divided by the original scratch area. Three independent experiments were performed, and the results averaged.

Invasion. Transwell inserts of 24-well plates were coated with fibronectin or collagen I (BD Biosciences) for the *in vitro* cell invasion assays. Equivalent numbers ($5x10^3$ cells) of GBM cells with modified EMP2 levels were added to the top chamber of the transwell, and complete DMEM medium was added to the bottom of the well. Cells were allowed to invade for 6 hours at 37°C. The filters were then fixed and stained with 0.1% crystal violet in 20% methanol. The invasive cells were visualized using bright-field microscopy. Cells were enumerated by counting 4 random fields per transwell. The experiment was repeated three times, with the data averaged. In some experiments, cells were pretreated with anti-EMP2 IgG1 or anti-$\alpha v\beta 3$ integrin antibodies for 2 hours at 4°C.

SDS-PAGE/Western blotting analysis. Cells were resuspended in Laemmli sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromphenol blue, 2% β-mercaptoethanol). As EMP2 contains multiple glycosylation sites, *N*-linked glycans were cleaved using peptide *N*-glycanase (PNGase; New England Biolabs, Beverly, MA) as previously described(28). Lysates were treated as per the manufacturer's instructions at 37 °C for 2 h. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (AmersAmersham Biosciences), and stained with Ponceau S (Sigma-Aldrich, St. Louis, MO) to determine transfer efficiency. Membranes were blocked with 10% low fat milk in TBS containing 0.1% Tween 20 and probed with EMP2 antisera (1:1000), anti-$^{576/577}$p-FAK (Santa Cruz Biotechnology), anti- total FAK (BD Biosciences), anti-^{416} p-Src (Cell Signaling, Danvers, MA), anti-total Src (Cell Signaling) or β-actin (Sigma-Aldrich). Protein bands were visualized using HRP-conjugated secondary antibodies (BD Biosciences; Southern Biotechnology Associates, Birmingham, AL) followed by chemiluminescence (ECL; Amersham Biosciences). Band intensities were quantified using Image J(29). At least three independent experiments were performed and the results were evaluated for statistical significance using Student's t-test (unpaired, two-tail).

Cellular viability. $2x10^5$ U87MG, U87/EGFR VIII, U373, T98, and GM5 cells were plated in triplicate. Cells were incubated with a vehicle control (PBS) or molar equivalents of the anti-EMP2 antibodies (20ug/ml anti-EMP2 diabody or 60ug/ml anti-EMP2 IgG1). After 72 hours, cells were enumerated using the trypan blue exclusion assay. In some experiments, to validate changes in viability, T98 or GM5 cells were treated as above, harvested and stained with an Annexin V-Propidium Iodide detection kit as per manufacturer's instructions (BD Biosciences). Flow cytometry analysis was performed with a Becton Dickinson FACScan Analytic Flow Cytometer (Becton Dickinson) at the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility.

Statistical Analysis. All values in the text were mean + SEM. Differences between means were evaluated using a two-tailed Student's *t*-test or ANOVA as indicated. Significant differences were taken at the $p < 0.05$ level.

RESULTS

EMP2 is expressed in most GBMs.

In a previously published Affymetrix gene chip dataset(14), we were intrigued to find that EMP2 expression was significantly increased in 50 human GBM compared to 24 normal brain samples (Figure 1A). In that study, when EMP2 mRNA was dichotomized into high or low expression levels, high EMP2 mRNA expression predicted early death and thus was an independent prognostic indicator (Figure 1B). To translate this expression, we tested EMP2 levels by western blot analysis in a small panel of GBM tumors and normal brain as well as in a panel of primary and established GBM cell lines (Figure 1C). Using whole tumor or normal brain homogenates, EMP2 expression was elevated in tumor lysates. Concordantly, EMP2 expression was detectable at various levels in all primary and established GBM cell lines. As EGFR gene amplification and mutation are a particularly striking feature of GBM (17), we tested if EMP2 levels were altered in U87MG cells harboring an intragenic rearrangement termed EGFR

VIII(30). However, this mutation did not detectably alter EMP2 protein levels.

To extend upon the expression of EMP2 in GBM on a population basis, a tissue microarray (TMA) consisting of 329 cores from 110 patients was stained using anti-EMP2 antisera as described in Materials and Methods. Preimmune sera was used as an isotype control. Using the EMP2 antisera, significant EMP2 expression was observed in tumors compared to non-malignant brain tissue (Figure 1D). The staining pattern observed was similar to what has previously been reported in secretory endometrium with EMP2 localization on both the membrane and within the cytoplasm of cells. When the expression was quantitated, the vast majority of tumors from GBM patients (95%) expressed some EMP2 compared to adjacent non-malignant brain tissues (Figure 1E), with 53% of tumors expressing high levels (histological score ≥ 2) of EMP2. Although the mean survival period for most individuals with GBM is relatively short, EMP2 was a negative prognostic indicator as higher levels of EMP2 (≥ 2) predicted a poor outcome compared to tumors with lower levels of EMP2 (Figure 1F), and notably this was concordant with the prior association between EMP2 mRNA and survival (Figure 1B).

EMP2 accelerates GBM tumor growth.

In order to characterize the effects of EMP2 in GBM, we initially determined if EMP2 expression was necessary for tumorigenicity. Equivalent numbers of U373 and U87MG GBM cells with modified EMP2 levels were inoculated into athymic nude mice subcutaneously. Tumor growth kinetics of EMP2 over-expressing U373 and U87MG GBM xenografts were accelerated in athymic nude mice, compared to control vector-expressing U373 and U87MG xenografts. Furthermore, reduction of EMP2 through either a shRNA or ribozyme in U87MG and U373 respectively, significantly decreased tumor size (Figure 2A & B). These results suggested that EMP2 was an oncogenic protein in GBM and promoted tumorigenesis.

EMP2 increases αvβ3 integrin surface expression.

Previously we have shown that EMP2 upregulates αvβ3 integrin surface expression in endometrial cancer cells(19). To determine if 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 (Figure 2C). 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), and hence we first examined whether EMP2 levels altered GBM proliferation. BrdU incorporation assays over 24 hours revealed that cell proliferation was unaffected by over-expression 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 over-expression of EMP2 increased wound healing (Figure 3A) and cell migration (Figure 3B) compared to 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 and fibronectin(32,33), but it does not have an affinity for collagen. To determine if EMP2-mediated changes in integrin expression alters 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 hours and allowed to invade through the matrix. EMP2 upregulated fibronectin-mediated cell invasion in U373 (Figure 3C) and T98 cells (Figure 3D), but had no effects 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 a 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 (Figure 3E) and control T98/V cells (Figure 3F). 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 focal adhesion kinase (FAK) and Src kinase(36,37). Panels of U373 and U87MG were plated for 24 hours, and levels of activated FAK and Src kinase were measured. EMP2 levels significantly correlated with both FAK and Src activation in these cells (Figure 4A). To confirm these results, EMP2 was overexpressed in GBM lines LN229 and GM97 and compared to U87MG cell lines. Overexpression of EMP2 activated FAK and Src by increasing phosphorylation levels at $^{576/577}$Y and ^{416}Y, respectively compared to vector control-expressing cells (Figure 4B). This increase was significant in all three cell lines (LN229, U87MG, GM97) further suggesting that this may be a direct consequence of EMP2 upregulation. 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 (Figure 4C) 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). Analysis of 87 patients showed a Spearman's rank correlation coefficient of $r = 0.54$, $p < 0.01$ between EMP2 and ^{416}pSrc expression where ninety-eight percent (49 out of 50) of tissues were positive for ^{416}pSrc showed intense staining for EMP2.

Increased EMP2 expression increases GBM cell invasion *in vivo*. To determine if 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 athymic nude mice, and tumor load was monitored using bioluminescence (Figure 5A, 5B). While an increase in EMP2 expression did not significantly increase tumor growth compared to control animals, it did increase tumor invasion into the surrounding parenchyma (Figure 5C). In contrast, a reduction of EMP2 through a specific shRNA significantly inhibited tumor growth compared to 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). Compared to native IgG1, diabodies biodistribute more quickly, penetrate target tissues efficiently, but more rapidly clear from circulation compared to intact antibodies. 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). In order to determine if 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 (Figure 6A). To confirm that the reduction in 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 hours, they were stained with Annexin V and propidium iodide. Compared to the control, both EMP2 IgG1 and anti-EMP2 diabodies increased the percentage of cell death (Figure 6B).

To determine if 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 $4mm^3$, mice were treated intratumor with anti-EMP2 or control diabody at 1mg/kg twice a week followed by 2.5mg/kg twice a week. Tumor growth was reduced when treated with anti-EMP2 diabody (Figure 6C, left panel), and the residual tumors showed marked necrosis compared to the control diabody (Figure 6C, 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 (Figure 6D). U373 xenografts were implanted subcutaneously into the shoulder of athymic nude mice. When the tumors reached $4mm^3$, mice were treated i.p. with full-length anti-EMP2 or control IgG1 weekly at 3mg/kg. Anti-EMP2 IgG1 retarded U373 tumor growth compared to control IgG-treated mice (Figure 6D, left panel), with tumors exhibiting significant necrosis throughout the tumor (Figure 6D, 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 non-malignant 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 αvβ3 integrin surface expression. The importance of αvβ3 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 αvβ3 integrin in cellular migration and invasion. Recruitment of αvβ3 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 αvβ3 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 αvβ3 integrin expression occur? While 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 β3 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 if αvβ3 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 downregulate αvβ3 integrin. As αvβ3 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, downregulation of αvβ3 integrin by tumistatin or RGD peptides have 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 lab 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. In addition to identifying targeting reagents, a

particular challenge in GBM is delivery as many small and large molecules like 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. While 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 ADCC which may improve their *in vivo* efficacy(50,51), and preliminary data suggests 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 if anti-EMP2 antibodies can be combined with standard chemotherapy or molecular-targeted treatments. While 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.

Abbreviations

GBM, glioblastoma; ctrl, control; Db, diabody; FAK, focal adhesion kinase; EMP2, epithelial membrane protein-2; TMA, tissue microarray; EGFR, epithelial growth factor receptor; IgG1, Immunoglobulin G isotype 1; DAB, diaminobenzidine; SEM, standard error of mean.

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