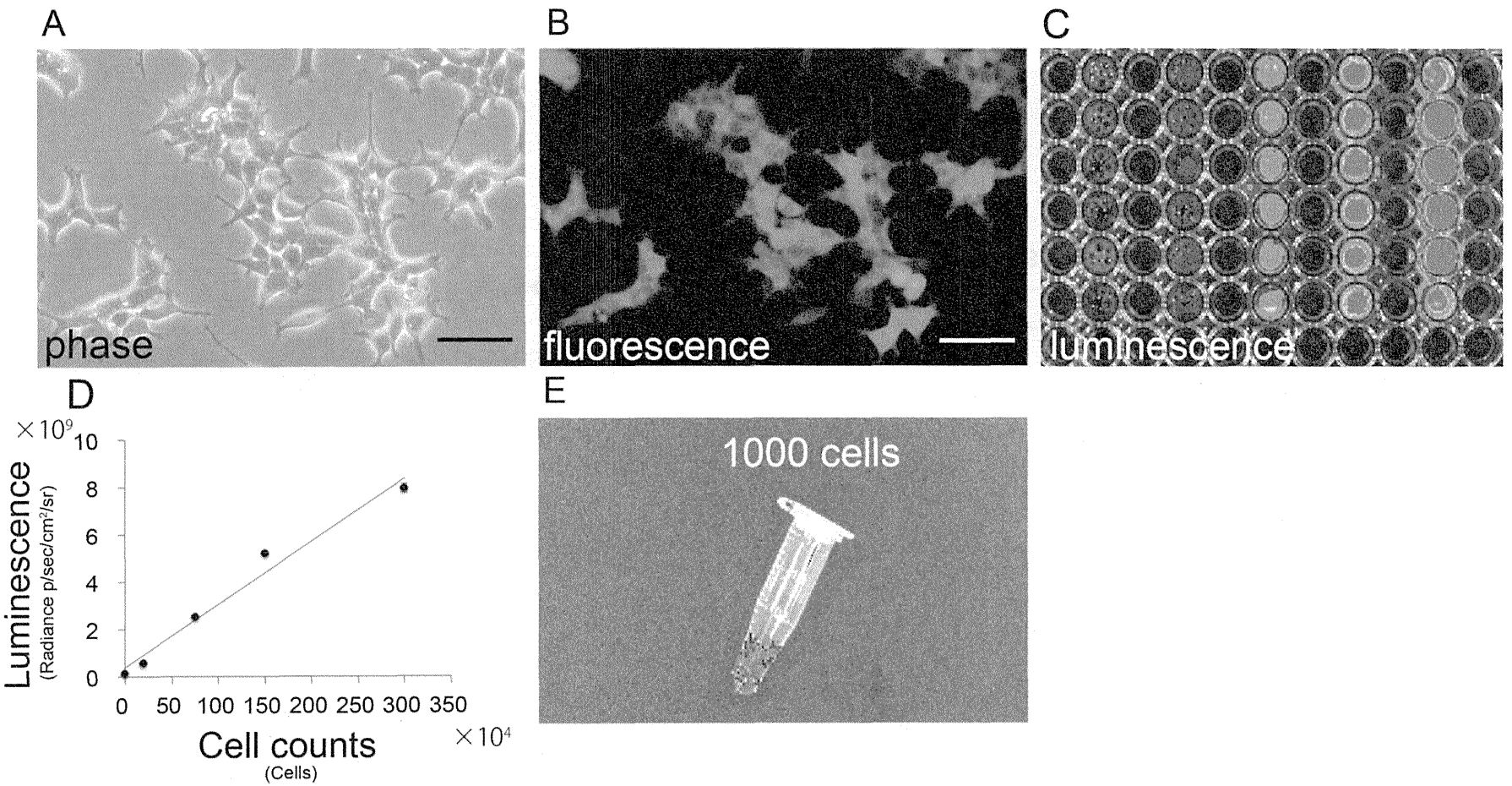

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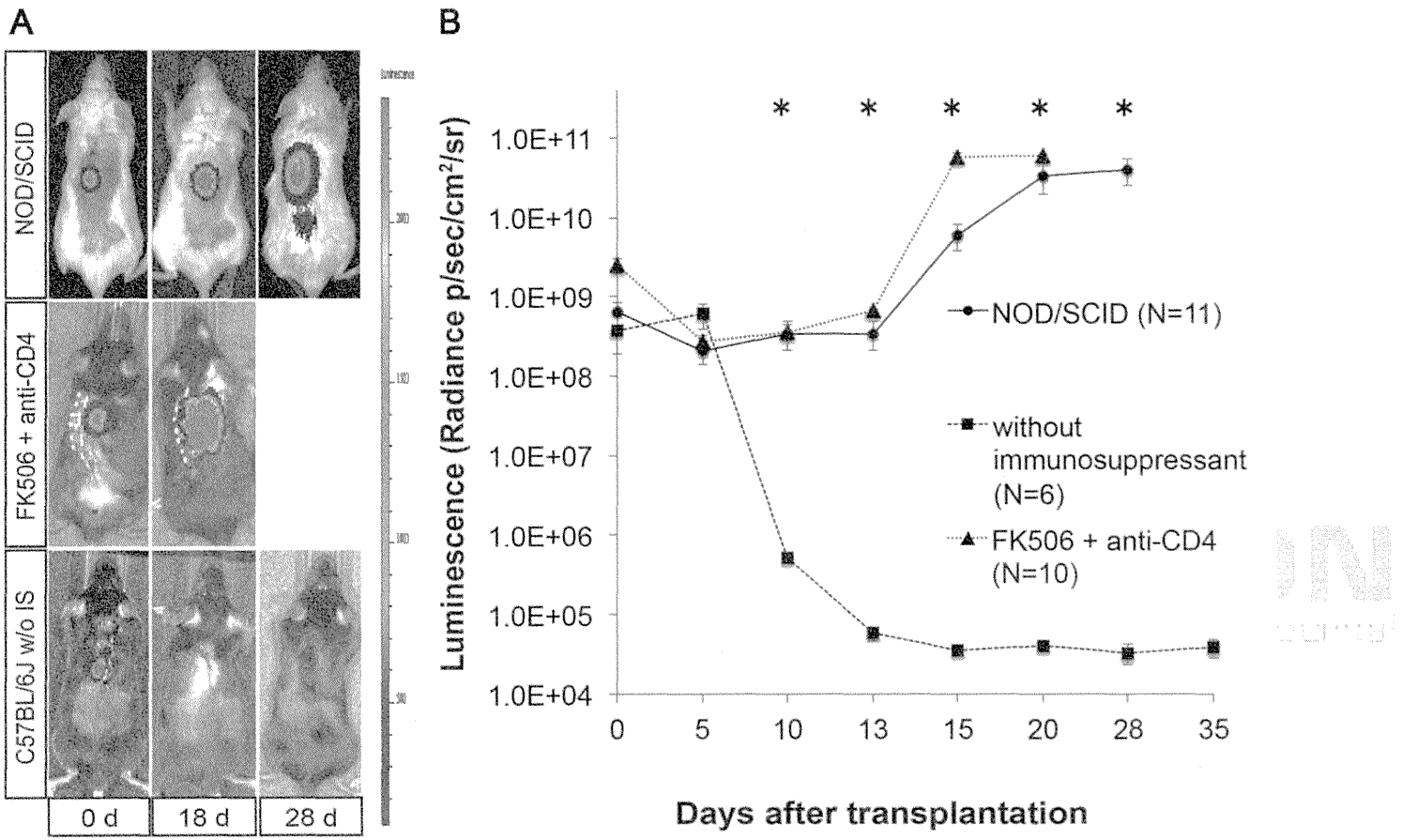

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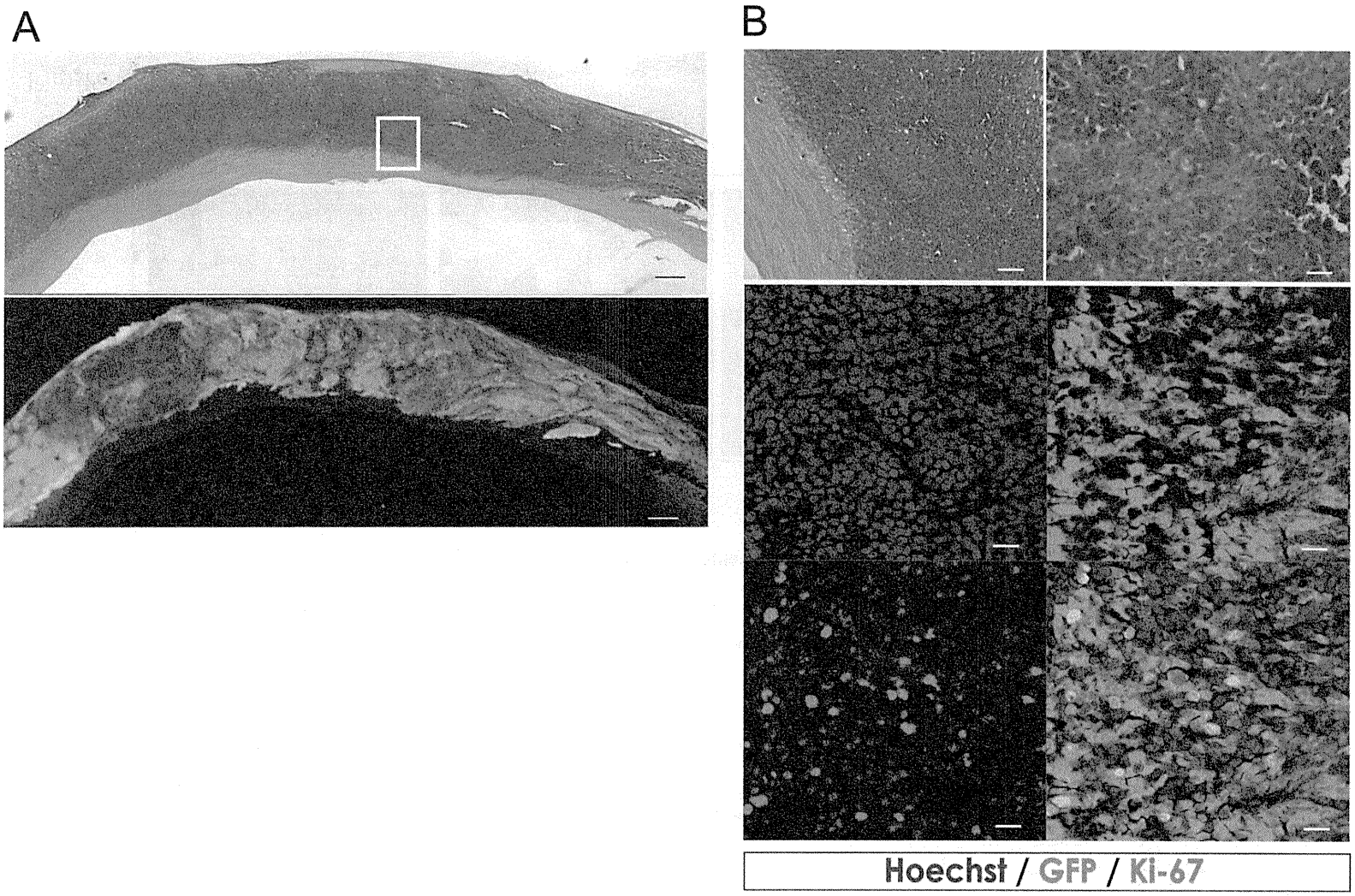

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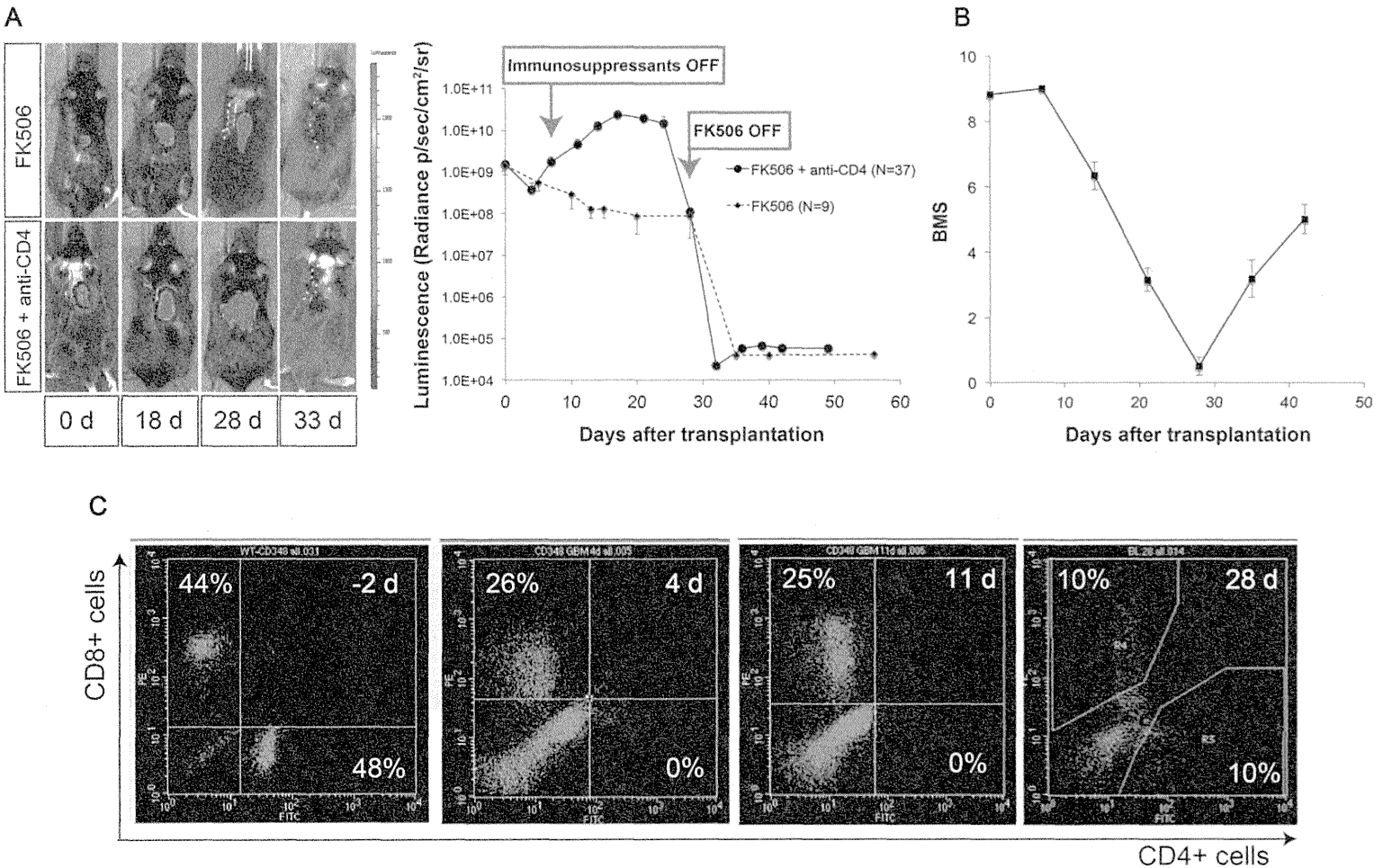

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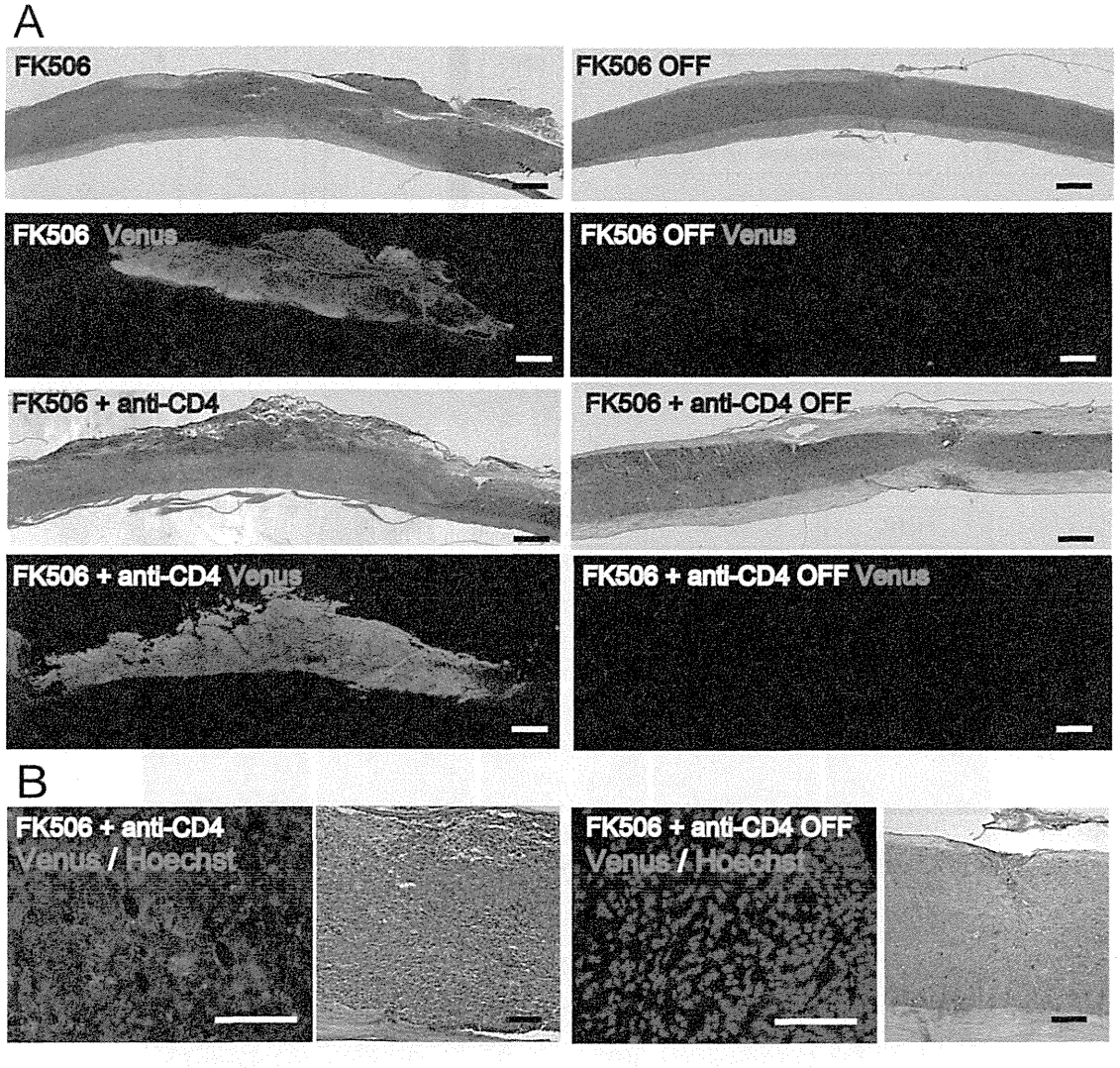
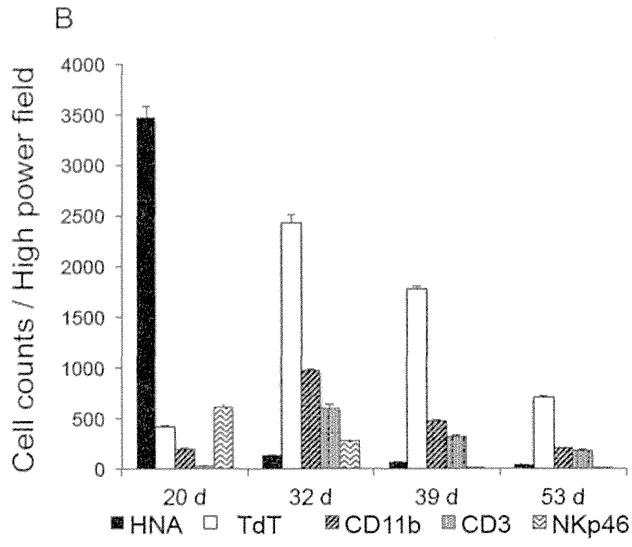
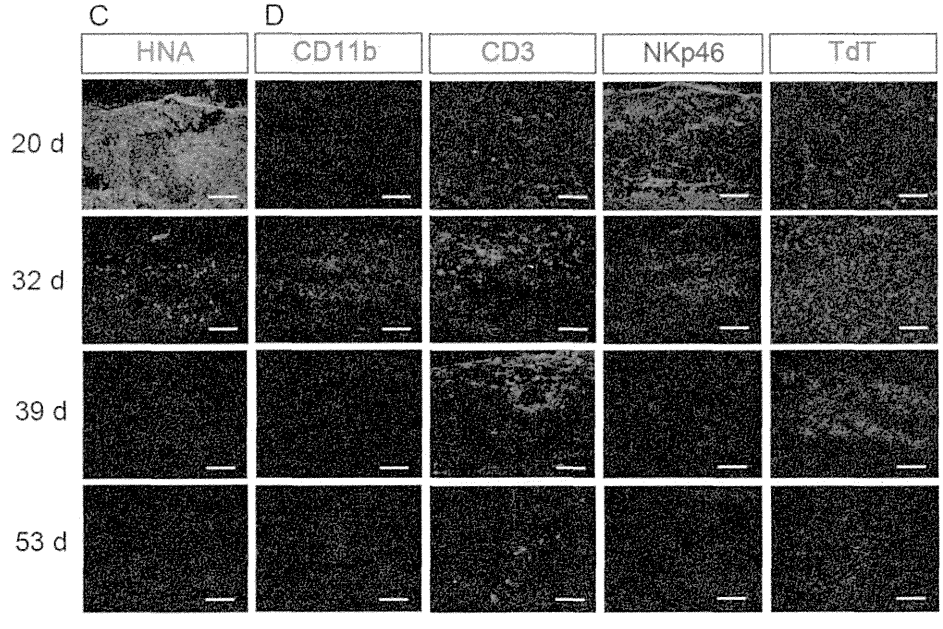


Figure 5;

A

Days post transplantation	graft status
20 d	grafted
32 d	rejected
39 d	1 week post rejection
53 d	3 weeks post rejection





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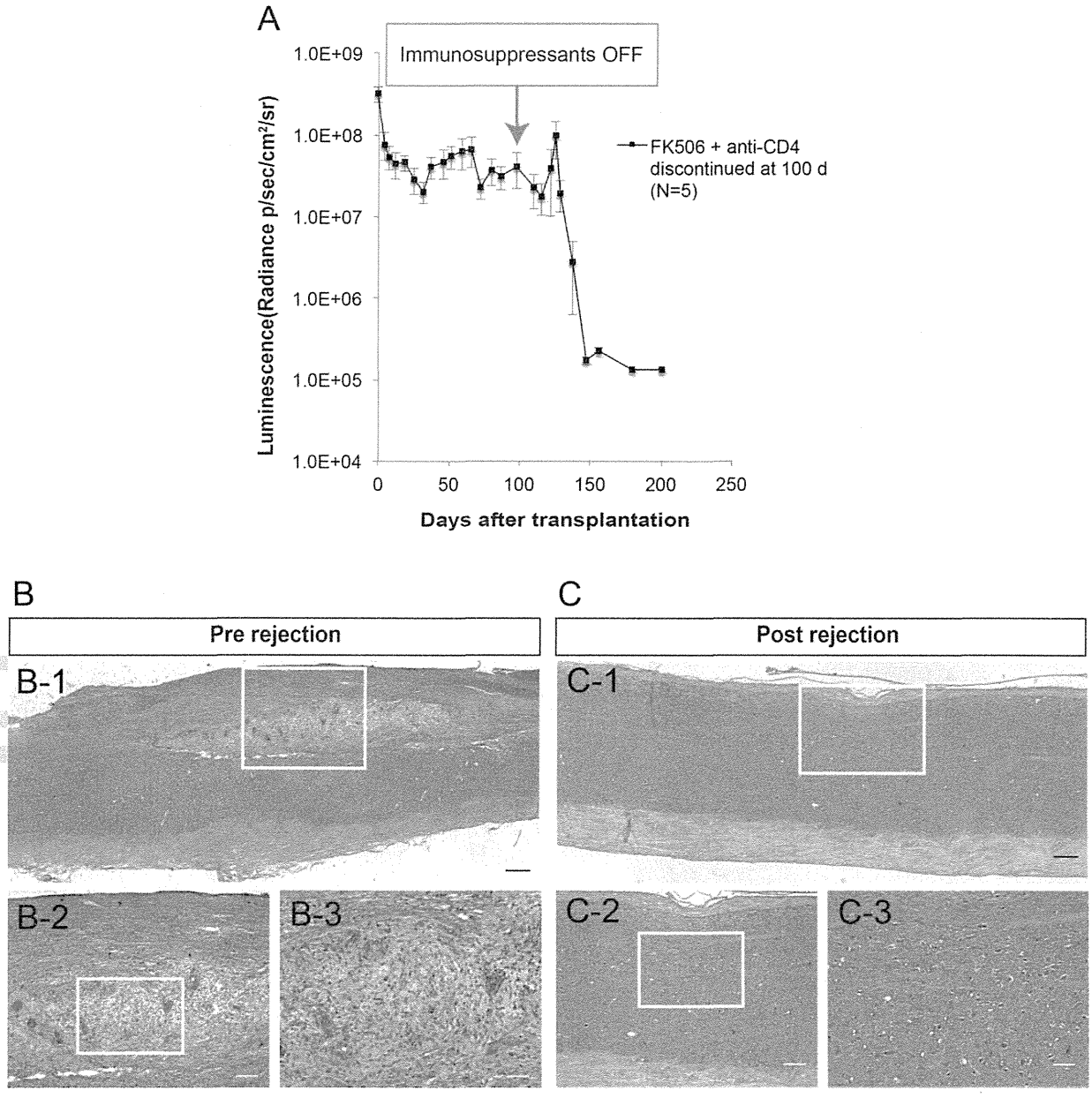

Figure 7:

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 20, pp. 13974–13985, May 16, 2014

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

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Yu Qin[‡1], Maoyong Fu[§1], Masamichi Takahashi[¶], Akio Iwanami[§], Daisuke Kuga[§], Rajiv G. Rao[§], Deepthi Sudhakar[§], Tiffany Huang[§], Meagan Kiyohara[§], Kathleen Torres[§], Christen Dillard[§], Akihito Inagaki[¶], Noriyuki Kasahara[¶], Lee Goodglick[§‖], Jonathan Braun[§‖], Paul S. Mischel[‡‡], Lynn K. Gordon[‡], and Madhuri Wadehra[§‖2]**

From the Departments of [‡]Ophthalmology and [§]Pathology and Laboratory Medicine, [‖]Jonsson Comprehensive Cancer Center, and [¶]Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California 90095 and the [**]Ludwig Institute for Cancer Research, [‡‡]Department of Pathology, University of California at San Diego, La Jolla, California 92093

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.

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 up-regulating $\alpha v\beta 3$ integrin surface expression, activating focal adhesion kinase 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 whether 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.

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),[3] the most common and aggressive form of brain tumors, yields only a median survival of 12 months and a 5-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 tumor cores contain highly proliferative populations that 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 that link the cytoskeleton to the extracellular matrix, play an important role. Integrin adhesion and invasion activate focal adhesion kinase (FAK), a nonreceptor cytoplasmic tyrosine kinase that is found to be up-regulated 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 up-regulated 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 up-regulation 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 principal data that suggest that EMP2 promotes a more aggressive disease phenotype and that it may ultimately serve as a novel therapeutic target for antibody therapy.

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[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Dept. of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Box 951732, 675 Charles Young Dr. South, Los Angeles, CA 90095. Tel.: 310-825-1590; Fax: 310-825-5674; E-mail: mwadehra@mednet.ucla.edu.
[3] The abbreviations used are: GBM, glioblastoma; FAK, focal adhesion kinase; EMP2, epithelial membrane protein-2; EGFR, epithelial growth factor receptor; RIBO, ribozyme; IHC, immunohistochemistry; V, vector.

MATERIALS AND METHODS

Cell Lines and Reagents

Human GBM cell lines U87MG, U138, and U373 (ATCC, Manassas, VA) were cultured in DMEM 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 that overexpress an in-frame deletion of amino acids 6–273 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 (46 kDa) protein (8). EMP2 expression was reduced using the Mission pLKO.1 puro shRNA lentiviral vector (shRNA; Sigma) (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, Pittsburgh, PA) were used to transiently reduce EMP2 expression as described previously (19–21). To create tumors for intracranial models, U87/EMP2, U87/V, and U87/shRNA cells were stably infected with an HIV-1-based lentiviral vector containing the firefly luciferase gene under the control of the CMV promoter (U87/Luc) by the UCLA Vector Core and Shared Resource as described previously (22).

Immunohistochemistry

A GBM array containing 0.6-mm 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 min in 0.1 M citrate, pH 6.0. EMP2 was detected using rabbit human EMP2 antisera at a dilution of 1:400 as described previously (13) followed by visualization using the Vector ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Diaminobenzidine or deNOVO Red was used for visualization, and staining was quantified by two 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 by the National Institutes of Health. The protocol was approved by the Animal Research Committee at UCLA. All efforts were made to minimize animal suffering.

Four-6-week-old nude BALB/c female mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the vivarium at UCLA. Animals were inoculated subcutaneously with 1×10^6 U373/EMP2, U373/V, or U373/RIBO or 5×10^5 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 (length $\times$ width^2)/2 (23). Data are expressed as mean $\pm$ S.E. 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, 1×10^5 U87/EMP2/Luc, U87/V/Luc, and U87/shRNA/Luc were stereotactically implanted into the right frontal lobe 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 injection of 100 μl of D-luciferin (30 mg/ml), and 30 min after injection, mice were anesthetized with ketamine/xylazine (100 and 10 mg/kg) and placed on the imaging stage. The bioluminescence signals were captured using an IVIS-200 (Xenogen Corp., Alameda, CA). The data were analyzed using maximum photon flux emission (photons/s) in the regions of interest. A one-way analysis of variance 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 UCLA prior to injection. When tumors approached 4 mm^3, they were injected twice a week with intratumoral injections of the anti-EMP2 diabodies at 1 mg/kg during week 1 and then 2 mg/kg during week 2. To test the full-length EMP2 IgG1, tumors were created using the wild type U373 cell line, and mice were treated weekly through intraperitoneal 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 and then were released in complete media containing BrdU for 2 or 24 h. Cells were fixed and 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 h with a ×10 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 were 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 (5×10^3 cells) of GBM cells with modified EMP2 levels were added to the top chamber of the transwell, and complete DMEM was added to the bottom of the well. Cells were allowed to invade for 6 h 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 four 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-αvβ3 integrin antibodies for 2 h 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 (New England Biolabs, Beverly, MA) as described previously (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 (Amersham Biosciences), and stained with Ponceau S (Sigma) 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-p-FAK (Tyr-576/577) (Santa Cruz Biotechnology), anti-total FAK (BD Biosciences), anti-p-Src (Tyr-416) (Cell Signaling, Danvers, MA), anti-total Src (Cell Signaling), or β-actin (Sigma). Protein bands were visualized using HRP-conjugated secondary antibodies (BD Biosciences and Southern Biotechnology Associates, Birmingham, AL) followed by chemiluminescence (ECL; Amersham Biosciences). Band intensities were quantified using ImageJ (29). At least three independent experiments were performed, and the results were evaluated for statistical significance using Student's *t* test (unpaired, two-tailed test).

Cellular Viability—2×10^5 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 (20 μg/ml anti-EMP2 diabody or 60 μg/ml anti-EMP2 IgG1). After 72 h, 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 FACScan Analytic Flow Cytometer (BD Biosciences) at the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility.

Statistical Analysis—All values in the text were mean ± S.E. Differences between means were evaluated using a two-tailed Student's *t* test or analysis of variance 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 with 24 normal brain samples (Fig. 1*A*). 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 (Fig. 1*B*). 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 (Fig. 1*C*). 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 whether 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 the expression of EMP2 in GBM on a population basis, a tissue microarray consisting of 329 cores from 110 patients was stained using anti-EMP2 antisera as described under "Materials and Methods." Preimmune serum was used as isotype control. Using the EMP2 antisera, significant EMP2 expression was observed in tumors compared with nonmalignant brain tissue (Fig. 1*D*). 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 with adjacent nonmalignant brain tissues (Fig. 1*E*), 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 with tumors with lower levels of EMP2 (Fig. 1*F*), and notably this was concordant with the prior association between EMP2 mRNA and survival (Fig. 1*B*).

EMP2 Accelerates GBM Tumor Growth—To characterize the effects of EMP2 in GBM, we initially determined whether 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 overexpressing U373 and U87MG GBM xenografts were accelerated in athymic nude mice, compared with control vector-expressing U373 and U87MG xenografts. Furthermore, reduction of EMP2 through either an shRNA or ribozyme in U87MG and U373, respectively, significantly decreased tumor size (Fig. 2, *A* and *B*). These results suggested that EMP2 was an oncogenic protein in GBM and promoted tumorigenesis.