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Maternal Embryonic Leucine Zipper Kinase Is a Key Regulator of the Proliferation of Malignant Brain Tumors, Including Brain Tumor Stem Cells

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Emerging evidence suggests that neural stem cells and brain tumors regulate their proliferation via similar pathways. In a previous study, we demonstrated that maternal embryonic leucine zipper kinase (Melk) is highly expressed in murine neural stem cells and regulates their proliferation. Here we describe how *MELK* expression is correlated with pathologic grade of brain tumors, and its expression levels are significantly correlated with shorter survival, particularly in younger glioblastoma patients. In normal human astrocytes, MELK is only faintly expressed, and MELK knockdown does not significantly influence their growth, whereas Ras and Akt overexpressing astrocytes have up-regulated MELK expression, and the effect of MELK knockdown is more prominent in these transformed astrocytes. In primary cultures from human glioblastoma and medulloblastoma, MELK knockdown by siRNA results in inhibition of the proliferation and survival of these tumors. Furthermore, we show that MELK siRNA dramatically inhibits proliferation and, to some extent, survival of stem cells isolated from glioblastoma in vitro. These results demonstrate a critical

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role for MELK in the proliferation of brain tumors, including their stem cells, and suggest that MELK may be a compelling molecular target for treatment of high-grade brain tumors.

Key words: brain tumor; maternal embryonic leucine-zipper kinase; brain tumor stem cell; proliferation

Brain tumors arise from cells of neural tube origin. Such cells could include dedifferentiated mature cells, committed progenitors, or the less committed neural stem cells. These latter cells have the capacity for self-renewal and multipotent differentiation, giving rise to neurons and glia (Gage, 2000). Recent studies have indicated that cells within brain tumors, regardless of cells of origin, have many of the properties of neural stem cells, including self-renewal and multipotent differentiation (Hemmati et al., 2003; Singh et al., 2003, 2004; Galli et al., 2004). In the case of glioblastoma multiforme (GBM; Singh et al., 2003; Galli et al., 2004), medulloblastoma (MB; Singh et al., 2004), and ependymoma (Taylor et al., 2005), these cells can form tumors in experimental animals that recapitulate the phenotypic properties of the parent tumors and are thus called cancer (or brain tumor) stem cells.

Whether or not brain tumors contain cancer stem cells, because of the close ontological relationship between brain tumors and the developing nervous system, it is reasonable to hypothesize that genes that regulate the growth and development of the central nervous system (CNS) will also be critical mediators of brain tumor growth. In fact, key genes and molecular pathways that regulate the proliferation of neural progenitors and stem cells, such as epidermal growth factor (EGF; Reynolds and Weiss, 1992), basic fibroblast growth factor (bFGF; Palmer et al., 1995), and phosphatase and tensin homolog deleted on chromosome ten (PTEN; Groszer et al., 2001) have already been identified as potential targets for therapy of brain tumors.

Maternal embryonic leucine zipper kinase (MELK) is an atypical member of the snf1/AMPK family of serine-threonine kinases (Lizcano et al., 2004). This family is largely associated with cell survival under conditions of environmental challenge, such as nutrient starvation (Kato et al., 2002; Suzuki et al., 2003a,b). In a previous study, however, we demonstrated that Melk regulates neural stem cell self-renewal through control of the cell cycle (Nakano et al., 2005). Likewise, MELK has been identified as a cell cycle modulator in tumor cell lines and was recently identified as an important target for certain somatic tumors, including colorectal, lung, and ovarian cancers (Gray et al., 2005).

Here, we demonstrate that *MELK* is highly expressed in high grades of glioma and that *MELK* expression is negatively correlated with survival of patients (age <50 years) with GBM. Functional assays demonstrated that MELK is a critical regulator of the proliferation and survival of unselected GBM cells and also regulates the proliferation of CD133-positive GBM stem cells in culture. MELK was also necessary for the survival and proliferation of MB cells in vitro. In conclusion, these data suggest that MELK is representative of a class of high-priority targets for potential brain tumor therapy aimed at brain tumor stem cells.

MATERIALS AND METHODS

Cell Cultures

Human fetal neural progenitors (Cambrex Bio Science, Walkersville, MD; lot 4F1565) were thawed and placed in neurosphere media with Neurobasal media (Invitrogen, La Jolla, CA), B27 (Gibco BRL, Grand Island, NY), L-glutamine, penicillin/streptomycin (Gimini Bioproducts), heparin (Sigma-Aldrich, St. Louis, MO), 20 ng/ml basic fibroblast growth factor (bFGF; Peprotech), and 20 ng/ml epidermal growth factor (EGF; Sigma or Preprotech) and passaged every 1–2 weeks by mechanical dissociation and replating in fresh neurosphere medium. Tumor spheres were cultured as described previously (Hemmati et al., 2003). Briefly, cells were dissociated with a fire-polished glass pipette and resuspended at 50,000 cells/ml in Neurobasal medium (Invitrogen) supplemented with B27, bFGF, EGF, penicillin/streptomycin, and heparin. Growth factors were added every 3 days. For differentiation, culture medium was replaced into Neurobasal supplemented with B27 without bFGF onto poly-L-lysine (PLL)-coated dishes and maintained for up to 5 days. For the secondary neurosphere formation assay, the primary spheres were dissociated and plated into 96-well microwell plates in a 0.2-ml volume of growth media at a serial dilution, and the resultant sphere numbers were counted at 7 days. The siRNA experiment using human neural progenitors has been described previously (Nakano et al., 2005). Daoy (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and passaged when confluent. Short-term primary cultures from human GBM, MB, and mouse MB-like cells from Ptc+/–;Pacap+/– mice were cultured in DMEM/F12 with 10% FBS. Genetically defined human astrocytes were generated in the Pieper laboratory (University of California, San Francisco). Normal human astrocytes were infected with retrovirus encoding T-antigen, TERT, Ras, and Akt. Stably expressing cell lines were thus generated for T-antigen + TERT, and T-antigen + TERT + Ras + Akt.

In Situ Hybridization and Immunostaining

In situ hybridization with brain sections from multiple ages was performed as described previously using ^{35}S-labeled riboprobes (Kornblum et al., 1994). Immunocytochemistry of neurospheres was performed as described previously (Kornblum et al., 1998; Geschwind et al., 2001a) using the following antibodies: nestin (1:250; Chemicon, Temecula, CA), TuJ1 (1:500; Berkeley Antibodies), and glial fibrillary acidic protein (GFAP; 1:1,000; Dako Cytomation, Carpinteria, CA). Primary antibodies were visualized with Alexa 568 (red)-, 488 (green)-, and 350 (blue)-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Hoechst 333342 (blue) and propidium iodide (PI; red) were used as fluorescent nuclear counterstains.

Semiquantitative Reverse Transcriptase-PCR

Total RNA was isolated from each sample using TRIzol (Gibco BRL), and 1 μg RNA was converted to cDNA by

reverse transcriptase, following the manufacturer's protocol (Impron). For semiquantitative RT-PCR, cDNA was amplified along with primers for the glyceraldehyde-3-phosphate-dehydrogenase gene (*GAPDH*) as an internal control from 20 to 40 cycles. After correction for *GAPDH* signal for each set, the resultant cDNA was subjected to PCR analysis using gene-specific primers listed in the Supplemental Table. The protocol for the thermal cycler was: denaturation at 94°C for 3 min, followed by cycles of 94°C (30 sec), 60°C (1 min), and 72°C (1 min), with the reaction terminated by a final 10-min incubation at 72°C. Control experiments were done either without RT or without template cDNA to ensure that the results were not due to amplification of genomic or contaminating DNA. Each reaction was visualized following separation on a 2% agarose gel. Direct comparisons were made only between cDNA samples run on the same gel. For semiquantitative PCR, the following sequences were used for the primers: mouse *Melk* sense, CACCGCAGCAGCAGGCAGAC; mouse *Melk* antisense, GGGTTGGTGAGGCGGGTATTTC; human *MELK* sense, ATGTTTGGAGCATGGGCATACTGTTA; human *MELK* antisense, ACTGGTTTCCCCGAGCCTTCTT; *GAPDH* sense, GGGGAGCCAAAAGGGTCATCATCT; *GAPDH* antisense, GACGCCTGCTTCACCACCTTCTTG.

siRNA Synthesis

siRNA was synthesized by using the Silencer siRNA Construction Kit, following the manufacturer's instructions (Ambion, Austin, TX). The sequences are as follows: mouse Melk sense, AATGAGGCGGGTATTTCTCTCCCTGTCTC; mouse Melk antisense, AAGAGAGAAATACCCGCCTCAC CTGTCTC; human MELK sense (CR), AACAAATCAC TCCCTAGTGTGCCTGTCTC; human MELK antisense (CR), AACACACTAGGGAGTGATTTGCCTGTCTC; human MELK sense (5′UTR), AATACGGCGTTCCTAAGAATC CCTGTCTC; human MELK antisense (5′UTR), AAGA TTCTTAGGAACGCCGTACCTGTCTC; human MELK sense (3′UTR), AATACCCACCACATCGGGTTTCCTGTCTC; human MELK antisense (3′UTR), AAAAACCCTATG-TGGTGGGTACCTGTCTC; luciferase-sense, AATCGA-AGTATTCCGCGTACGCCTGTCTC; luciferase-antisense, AACGTACGCGGAATACTTCGACCTGTCTC; EGFP-sense, AACTCGATGTTGTGGCGGATCCCTGTCTC; EGFP-antisense, AAGATCCGCCACAACATCGAGCCTGTCTC; Scramble-sense, AAACGTGACACGTTCGGAGAACCTGTC TC; Scramble-antisense, AATTCTCCGAACGTGTCACGTC CTGTCTC.

Lentiviral Infection of Immortalized Astrocyte Cultures

Equal number of cells were plated in six-well dishes (~60% confluence) and infected with freshly harvested, super concentrated control PGK-GFP lentivirus, lentivirus expressing hMelk shRNA, and lentivirus overexpressing wild-type MELK in triplicate (lentiviral expression constructs were made based on pRRL.SIN18-hPGK-EGFP backbone; Naldini et al., 1996; Zufferey et al., 1998). Cells were harvested in ice-cold RIPA buffer containing protease and phosphatase inhibitors (Sigma; 1:500), separated on precast SDS-PAGE 4–12% gels (Invitrogen), and transferred to nitrocellulose membranes. For determination of MELK protein expression, levels Western blots were performed as described below.

Transient Transfection

Transfections were generally performed as described previously (Nakano et al., 2005). Briefly, cells were transfected using LipofectAMINE 2000 (Invitrogen), following the manufacturer's protocol. For transfection of plasmid vectors, the cells were incubated with reagents for 24 hr. For transfection of the double-stranded siRNA complexes, serial dilutions of siRNA from 5 to 100 nM were tested to determine optimal concentrations resulting specific knockdown of the gene of interest, and either 25 or 50 nM was chosen as the concentration for functional study.

Cell growth assays. GBM1600 and Daoy cells were transfected with 25 nM of the double-stranded siGFP, siScramble, siMELK (CR), or siMELK (3′UTR) complexes for 6 hr in six-well plates and then incubated for 2 days to allow siRNA silencing of expression. For rescue experiment, 25 nM of siGFP or siMELK (3′UTR) was transfected into Daoy cells with 1 μg pCMV-EGFP or pCMV-MELK. To adjust cell number before measurements of cell growth, these cells were trypsinized and removed from the plate; nonviable cells were identified by staining with 0.4% trypan blue (Sigma), and the number of unstained cells was counted with a hemacytometer. For measurements of cell growth, 1×10^2 of the transfected cells were seeded in 96-well plates. The numbers of viable cells were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay (Cell Counting Kit-8; Dojindo Laboratories) as per the manufacturer's protocol. The cell growth rate was determined in triplicate at 1, 2, 3, 4, 5, and 6 days after seeding into 96-well plates.

Western blot analysis. Whole-cell lysates, prepared in NP-40 lysis buffer with a protease-inhibitor cocktail (Sigma), were analyzed by immunoblotting. Protein concentrations were determined by the Bio-Rad protein assay reagent as per the manufacturer's protocol. Equal amounts of proteins in whole-cell extracts from each set of experiments were fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (Millipore, Bedford, MA). The membrane was subjected to Western blot analysis with rabbit anti-MELK antibody (1:1,000; Cell Signaling Technology, Beverly, MA) or rabbit anti-β-actin antibody (1:2,500; Abcam, Cambridge, MA), or mouse anti-*Flag* M2 (1:1,000; Sigma-Aldrich). The signals from the primary antibody were amplified by horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA) and detected with enhanced chemiluminescence (Pierce, Rockford, IL).

Immunoprecipitation of MELK. MELK was immunoprecipitated using either the anti-*Flag* M2 EZview Red Affinity Gel (Sigma-Aldrich) or the anti-MELK (Cell Signaling) antibody. Cells were rinsed in phosphate-buffered saline (PBS) and disrupted with cell lysis buffer [1% Triton X-100 in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and protease inhibitors]. Lysates were precleared by centrifugation at 10,000*g* at 4°C for 10 min. For untagged MELK,

anti-MELK antibody was added to the supernatant and allowed to bind with gentle rotation for 12 hr at 4°C. A mix of protein A/G agarose was added to the antibody-containing lysate, gently mixed for 3 hr at 4°C, centrifuged, and the supernatant ("depleted lysate") removed. For tagged MELK, anti-*Flag* M2 EZ resin was added and allowed to bind with gentle rotation for 12 hr at 4°C. This was centrifuged, and the supernatant was removed. The final resin pellet was washed three times in lysis buffer, and the precipitated proteins were solubilized directly in Western blot sample buffer.

Protein gel. Protein samples were loaded onto a 12% SDS-PAGE gel. After separations, the gel was fixed in 7% acetic acid/10% methanol in water for 30 min and then exposed to Sypro Ruby (Bio-Rad, Hercules, CA) protein stain overnight. Image analysis was performed in PDQuest Gel Imaging software (Bio-Rad). To identify the immunoreactive bands, Western blot film and Sypro Ruby-stained images were merged, and those bands corresponding to immunoreactivity were excised from the Sypro Ruby gel using the Proteome Works Spot Cutter (Bio-Rad) with a 1.5-mm gel punch. Proteins were in-gel digested according to Shevchenko et al. (1996) using sequence-grade trypsin (Promega, Madison, WI). The reduced and alkylated peptides were extracted, dried using a vacuum concentrator, and stored at –80°C until analyzed.

Tandem mass spectrometry. Peptides were analyzed by μLC-MS/MS using either an ion trap mass spectrometer (Thermo-Finnigan LCQ-DECA, San Jose, CA) or a hybrid quadrapole time-of-flight mass spectrometer (Applied Biosystems QSTAR XL Hybrid). Tryptic peptides were injected on to a reverse-phase column (PLRP-S 0.2 × 150 mm; Michrom Biosciences) equilibrated in water/acetonitrile/formic acid (95/5/0.1). Peptides were eluted over 80 min with increasing concentrations of acetonitrile. A coated glass electrospray emitter (New Objective) maintained at 3 kV for ionization was used to introduce the column eluant to the ion trap, which was operated in data-dependent acquisition mode, using a survey scan of 400–1,500 m/z. A stainless steel nanobore emitter (Proxeon Biosystems) with an applied voltage of 1.5 kV was used to introduce the column eluant to the hybid mass spectrometer, which was operated in information-dependent acquisition (IDA) mode with an ion scan of 375–2,000 m/z. MS/MS was performed on ions with charge states of 2–5.

Protein identification. Sonar MS/MS software (Genomic Solutions) and Mascot software (Matrix Science) were used to interpret MS/MS data with reference to the nonredundant (nr) database from the National Center Biotechnology Institute. Search parameters for all software analysis included missed cleavages by trypsin, carboxyamidomethylation of cysteines, and oxidized methionines.

Flow Cytometry and Sorting

Flow cytometry and sorting of CD133-positive and -negative cells from GBM spheres was performed according to manufacturer's protocol (Miltenyi Biotec). Clone AC141 of CD133 antibody was used for separation with phycoerythrin using a FACSVantage (Becton Dickinson) with a purification-mode algorithm. Gating parameters were set by side and forward scatter to eliminate dead and aggregated cells.

Image Acquisition

Photomicrographs were obtained using a microscope (model IX50; Olympus) fitted with a bright- and a darkfield condenser using a digital camera (model C2020; Olympus). Digital images were manipulated in Adobe Photoshop 7.0.2 to reflect direct observation accurately.

Cell Death Assay

An assay for apoptosis was performed by using the Apoptosis Detection Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Cell Proliferation Assay

Daoy cells were incubated with bromodeoxyuridine (BrdU; Roche) for 5 hr, 48 hr following transfection, and were fixed with 4% paraformaldehyde; staining was carried out as previously described, using sheep anti-BrdU (Dako; 1:200). To obtain the labeling index, the number of BrdU-labeled cells was expressed as a fraction of the total number of cells in a well for at least three wells per condition.

Statistical Methods

Microarray data were taken from an ongoing project at UCLA and include data from published manuscripts (Freije et al., 2004; Tso et al., 2006) as well as unpublished genome-wide data (S. Nelson). All UCLA microarray experiments analyzed in this work were performed on Affymetrix U133A arrays using the manufacturer's recommended labeling procedures. The total data set consisted of 18 fetal brain samples, 15 adult brain samples, both from autopsies, 63 GBM biopsies, seven astrocytomas grade II biopsies, eight oligodendrogliomas grad II biopsies, nine mixed glioma, grade II–III biopsies, eight astrocytomas grade III biopsies, 11 oligodendrogliomas grade III biopsies, and five MB biopsies. Clinical data were available for all UCLA samples as previously described (Freije et al., 2004) The Kaplan-Meier method was used to estimate survival distributions (Kaplan and Meier, 1958). Log-rank tests were used to test the difference between stratified survival functions. The Cox proportional hazards model (Cox and Oakes, 1990) was used to test the statistical significance of predictors. The proportional hazard assumption was tested using scaled Schoenfeld residuals. Statistical analyses were carried out with the freely available software package R (url: http://cran.r-project.org/). All P values were two sided, and $P < 0.05$ was considered significant. The U133A Affymetrix microarray data were normalized with the perfect match minus mismatch model of dChip (Li and Wong, 2001).

RESULTS

MELK Is Expressed by Brain Tumors and Correlates With Malignancy

Previously, we identified *Melk* in a microarray study comparing proliferating with differentiating neural progenitors (Geschwind et al., 2001; Kornblum and Geschwind, 2001; Easterday et al., 2003) Subsequently,

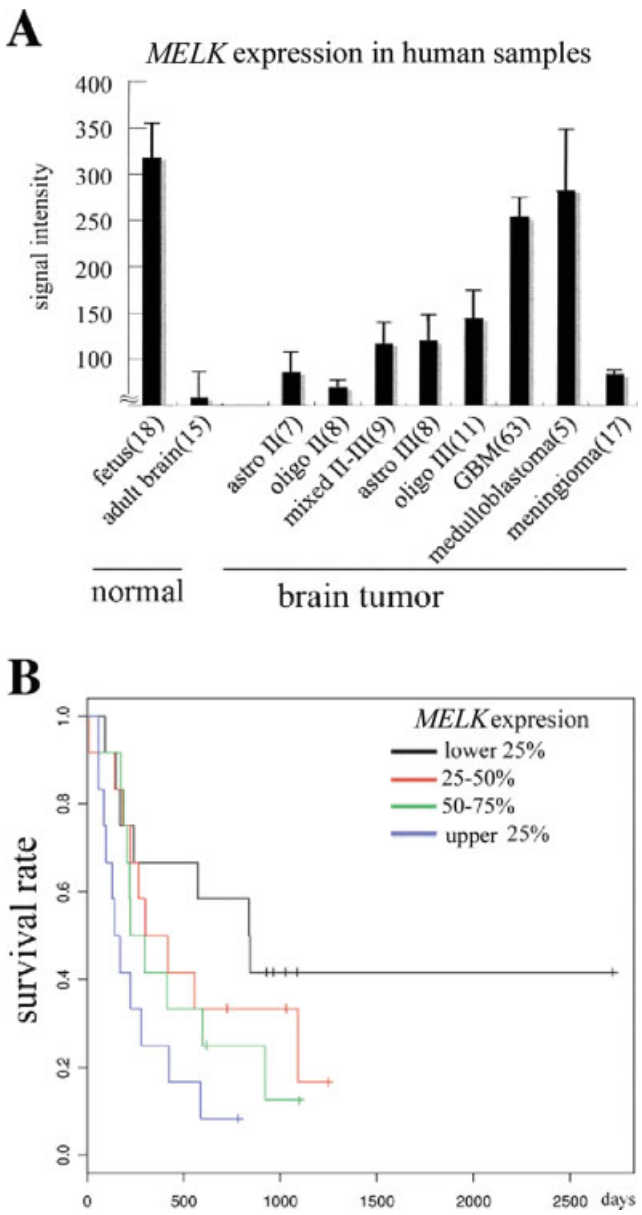


Fig. 1. *MELK* expression and patient survival in human brain tumors. **A:** The graph shows MELK expression levels in 33 normal human samples and 128 brain tumor samples analyzed by cDNA microarray. The number for each condition represents the mean relative *MELK* expression per patient ± SEM. **B:** Analysis of *MELK* expression and patient survival. GBM patients under age 50 years of age were divided into four groups depending on *MELK* expression levels, and their survival rates were plotted using a Kaplan-Meier analysis.

we found Melk to be a key regulator of murine neural progenitor self-renewal. Recently, another group demonstrated that MELK is a regulator of lung, colorectal, and ovarian cancers (Gray et al., 2005). We further focused on MELK expression in brain tumors, using array data previously generated at UCLA (Freije et al., 2004; Tso et al., 2006; Fig. 1A). Analysis of this data set indicated that *MELK* was relatively highly expressed in fetal brain, with much lower expression in adult brain. *MELK* was expressed in brain tumors, and its expression levels roughly correlated with malignancy, with highest levels of expression in GBM and MB. Since the UCLA data set contained relatively few MB patients (n = 5), we also examined *MELK* expression across a cohort of 46 pediatric MBs that were previously analyzed at St. Jude Children's Research Hospital (SJCRH; Thompson et al., 2006). This data set confirmed high level of expression of *MELK* in MB (not shown).

Expression of MELK Is Negatively Correlated With Prognoses of Patients With GBM

High *MELK* glioma expression was initially correlated with poor patient survival by Frieje et al. (2004). To determine further the potential significance of *MELK* in GBM, we analyzed survival data on an enlarged sample of 63 GBM patients treated at UCLA. When considering GBM patients of all ages, high *MELK* expression was significantly associated with diminished patient survival (log rank $P = 0.02$, hazard ratio for 1 SD = 1.35, 95% confidence interval 1.04, 1.76). We found that patient age at surgery was an important variable for understanding this relationship further. Patient ages ranged from 18 to 86 years, with a median age of 50 years. When restricting the analysis to patients younger than the median (50 years), the *MELK* expression was even more significantly associated with diminished patient survival ($P = 0.0028$, HR = 1.78, CI = 1.21, 2.62). Figure 1B shows the Kaplan-Meier curves for the four quartiles of *MELK* expression in the younger patient group. There was shorter survival time of patients whose tumors expressed greater levels of *MELK* mRNA. However, *MELK* expression was not significantly ($P = 0.81$) associated with survival in older patients (aged 50 years or older). These results indicate that *MELK* expression is an indicator of survival of GBM patients who are younger than age 50 years.

Transformed Human Astrocytes Express MELK and Are Dependent Upon It for Proliferation

The relative abundance of *MELK* in GBM and MB and its inverse correlation with survival in GBM could indicate that MELK plays an important role in malignant brain tumor growth. Although the distinct cell of origin to form glioma has not been determined, various protooncogenes are overexpressed and/or constitutively activated during the process of gliomagenesis, including Ras and Akt (Newton, 2004; Kesari et al., 2005). We compared MELK expression in TERT-immortalized normal human astrocytes (NHA), which do not give rise to tumors in xenograft models, to those that constitutively overexpress Ras and Akt, alterations that result in the formation of aggressive, GBM-like tumors following xenotransplantation (Sonoda et al., 2001). As shown in Figure 2B, overexpression of Ras

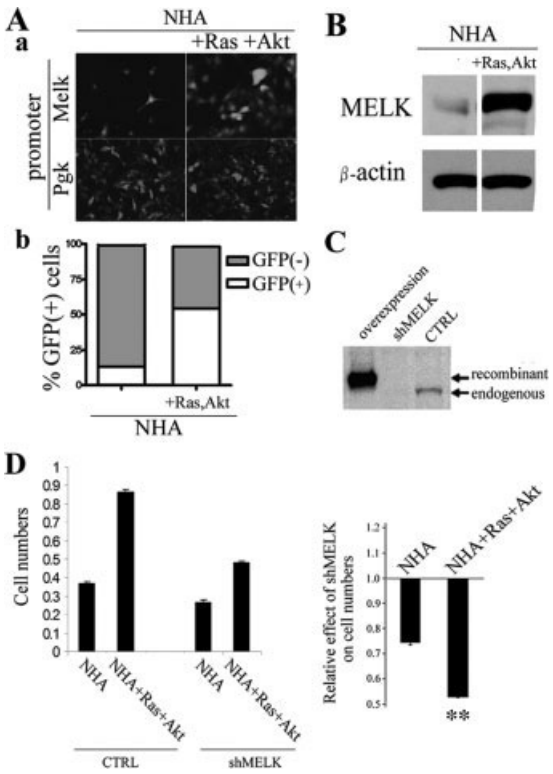


Fig. 2. MELK expression in transformed human astrocytes. **A:** a (top): GFP expression in TERT immortalized and TERT-immortalized, Ras- and Akt-overexpressing human astrocytes following lentiviral infection with the *Melk* promoter-GFP construct. a (bottom): Infection efficiency of the two cultures utilizing DsRed driven by the Pgk promoter. b: The graph shows the proportions of the GFP-positive cells in both cell types. **B:** Western blot of MELK immunoreactivity of both cell types used in A. β-Actin is used as an internal control. **C:** Effect of MELK knockdown, using the shMELK construct on Ras/Akt-overexpressing astrocytes. The left lanes show immunoreactivity following transfection with an MELK-*Flag* construct. **D:** Left: Relative total cell numbers of transformed cells infected with control lentivirus or MELK shRNA lentivirus (shMELK) 5 days following infection. Right: Relative effect of shMELK on total cell numbers in the two astrocyte cell lines. $^{**}P < 0.001$, ANOVA followed by post hoc *t*-test. All graphs show the mean ± SEM.

and Akt results in a dramatically increased number of cells expressing MELK, as indicated by enhanced green fluorescence protein (EGFP) reporter expression using a murine *Melk* promoter construct (Nakano et al., 2005; see Supplementary Fig. 1 for validation of the promoter in human cells). Furthermore, Western blot analysis using an antibody that recognizes endogenous MELK (Supplementary Fig. 2) also demonstrates increased MELK expression in the transformed cells. Knockdown of MELK with a lentivirus encoding MELK shRNA resulted in diminished numbers of cells of both types, but there was a more dramatic effect on the transformed astrocytes. Thus, these data suggest that MELK expression correlates with malignant potential and that MELK regulates either the proliferation or the survival of cells with high levels of MELK expression.

MELK Regulates Survival and Proliferation of GBM and MB in Culture

The data described above suggest that MELK plays an important role in the regulation of transformed glial cells. To assess more directly MELK function in human tumors, we performed RNA interference experiments in cultured GBM and MB cell lines. One cell line depicted in Figure 3 was derived from a UCLA GBM patient (GBM1600). Data from three other patients are documented in Supplementary Figure 1. We also used the MB cell line Daoy, with data from a primary tumor line also documented in Supplementary Figure 1. Transfection of GBM1600 cells with an siRNA directed at the coding region, a strategy we previously utilized for murine cells (Nakano et al., 2005), resulted in diminished *MELK* mRNA expression (Fig. 3Aa) and also nearly abolished protein expression (Fig. 3Ab). This siRNA had little or no significant effect on the proliferation of 293T cells (not shown). We also constructed an siRNA against the 3′UTR of MELK, which was even more efficient at knocking down *MELK* mRNA expression (Fig. 3Aa). Treatment of GBM1600 or Daoy cells with low concentrations (25 nM) of either the coding region or the 3′UTR siRNAs resulted in diminished numbers of cells compared with treatment with a scrambled MELK siRNA (siScramble) or an siRNA directed against EGFP (siGFP) as shown in Figure 3Ba,b. The effect of the 3′UTR siRNA was greater than that of the coding region siRNA, a finding that correlated with the degree of MELK knockdown.

Since off-target effects are generally observed with higher concentrations of siRNA (e.g., 100 nM and above; Cheng et al., 2007), we performed a dose-response experiment in Daoy cells and found that concentrations of the coding region siRNA as low as 10 nM inhibited the expression of *MELK* and also diminished cell number, with concentrations of 50 nM resulting in a nearly complete loss of cells (Fig. 3Ca,b), suggesting that MELK plays an important role in tumor cell survival.

As an additional control for off-target effects, we performed a rescue experiment (Fig. 3D). Transfection of cells with siMELK (3′UTR) and cotransfection with EGFP (as a control) resulted, as expected, in a loss of *MELK* expression (Fig. 3Da) and diminished cell number (Fig. 3Db). However, when siMELK (3′UTR) was cotransfected with MELK cDNA—which should not be recognized by the siMELK (3′UTR)—there was a partial rescue of *MELK* expression and nearly a full rescue of the effects on cell number. Taken together, our RNA interference data strongly support the hypothesis that MELK knockdown produces a dramatic effect on GBM and MB cells, an effect that is not due to off-target effects of the siRNA.

Previously, we demonstrated that Melk is a critical regulator of murine neural stem cell proliferation, without playing a significant role in survival (Nakano et al., 2005). However, our studies with GBM1600, Daoy, and other tumors (Supplementary Fig. 2) demonstrated a decline in cell numbers following siRNA treatment at 24 hr. Therefore, we determined whether MELK siRNA affected proliferation, survival, or both in Daoy cells. We assayed proliferation by pulse labeling with BrdU (Fig. 3E). MELK siRNA treatment resulted in a marked decline in the labeling index (percentage of total cells labeled with BrdU), suggesting an inhibitory role of MELK knockdown on cell proliferation. To analyze cell death, we treated cells with MELK or control siRNA, and performed flow cytometry-activate cell sorter (FACS) analysis for labeling with annexin V and propidium iodide (PI; Fig. 3F). MELK siRNA treatment resulted in fewer live cells, with greater numbers of cells in early (annexin V positive, PI negative) and late (annexin V positive, PI positive) stages of apoptosis. MELK siRNA-treated cells had over four times more apoptotic cells compared with control siRNA-treated cells (65.0% vs. 16.6%). Taken together, these data suggest that MELK affects the survival and proliferation of tumor cells in cultures.

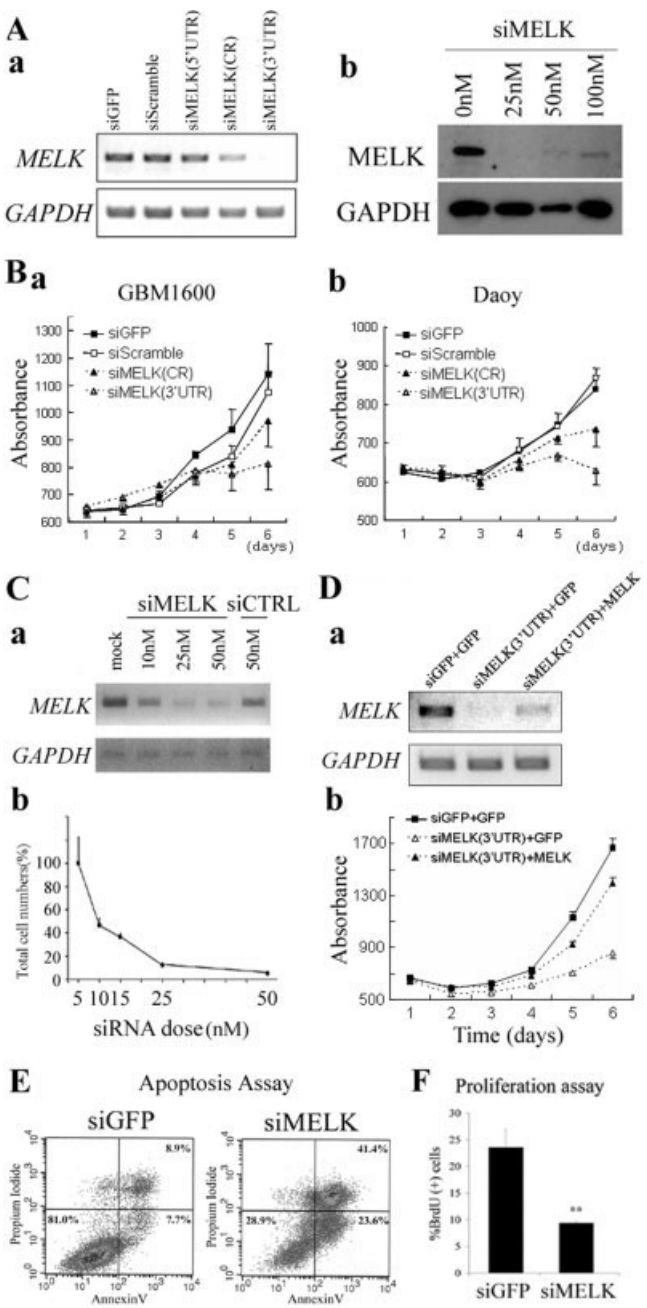


Fig. 3. MELK function in GBM and MB cells in culture. **A:** RT-PCR (a) and Western blot (b) of MELK expression in control siRNA (siGFP and siScramble) or MELK siRNA (5′UTR, CR, 3′UTR)-treated primary GBM (GBM1600) cultures. **B:** Cell viability was determined by WST assay at the indicated times following MELK or control siRNA treatment in GBM1600 and Daoy cells. **C:** RT-PCR for *MELK* expression (a) and the corresponding total cell numbers relative to controls (b) following treatment with different concentrations of MELK siRNA. **D:** RT-PCR for *MELK* expression (a) and the corresponding total cell numbers (b) following transfection with expression vector for EGFP or MELK and siRNA against MELK (3′UTR) or EGFP. **E:** Effect of MELK siRNA on apoptosis of Daoy cells as measured by labeling cells with annexinV and propidium iodide and subsequent FACS, following treatment. The early apoptotic population was identified by single labeling with annexin V, whereas late apoptotic cells were doubly labeled. **F:** BrdU incorporation in Daoy cells following siRNA treatment. The graphs represent the labeling index (percentage BrdU-positive cells). Asterisk denotes different from controls: $^{**}P < 0.001$, ANOVA, followed by post hoc *t*-test. All graphs show the mean ± SEM. The results were obtained from three independent experiments.

Melk Regulates the Growth of Primary Cultures Derived From Murine Medulloblastoma

We also examined the expression and function of Melk in a murine model of MB. Mice heterozygous for a mutation in the gene encoding the sonic hedgehog (Shh) receptor patched 1 (Ptc1), known spontaneously to develop MB (Goodrich et al., 1997), were bred with mice heterozygous for an inactivating mutation in the pituitary adenylyl cyclase-activating peptide (PACAP) gene (Colwell et al., 2004). The latter is a neuropeptide shown to regulate the in vitro proliferation of cerebellar granule progenitors (Nicot et al., 2002). As described previously (Nakano et al., 2005), and shown in Figure 4A, *Melk* is expressed by the proliferating external granule cells of the cerebellum, the putative cell of origin of MB, but not by postmitotic, adult granule cells. *Melk* mRNA was also highly expressed in tumors arising in Ptc+/–, Pacap+/– mice (Fig. 4A). Overexpression of full-length Melk cDNA in mouse MB resulted in moderately increased numbers of these tumor cells in culture (Fig. 4B). Interestingly, the effect of Melk overexpression was more prominent in cells treated with retinoic acid, a treatment that causes granule cell precursors to

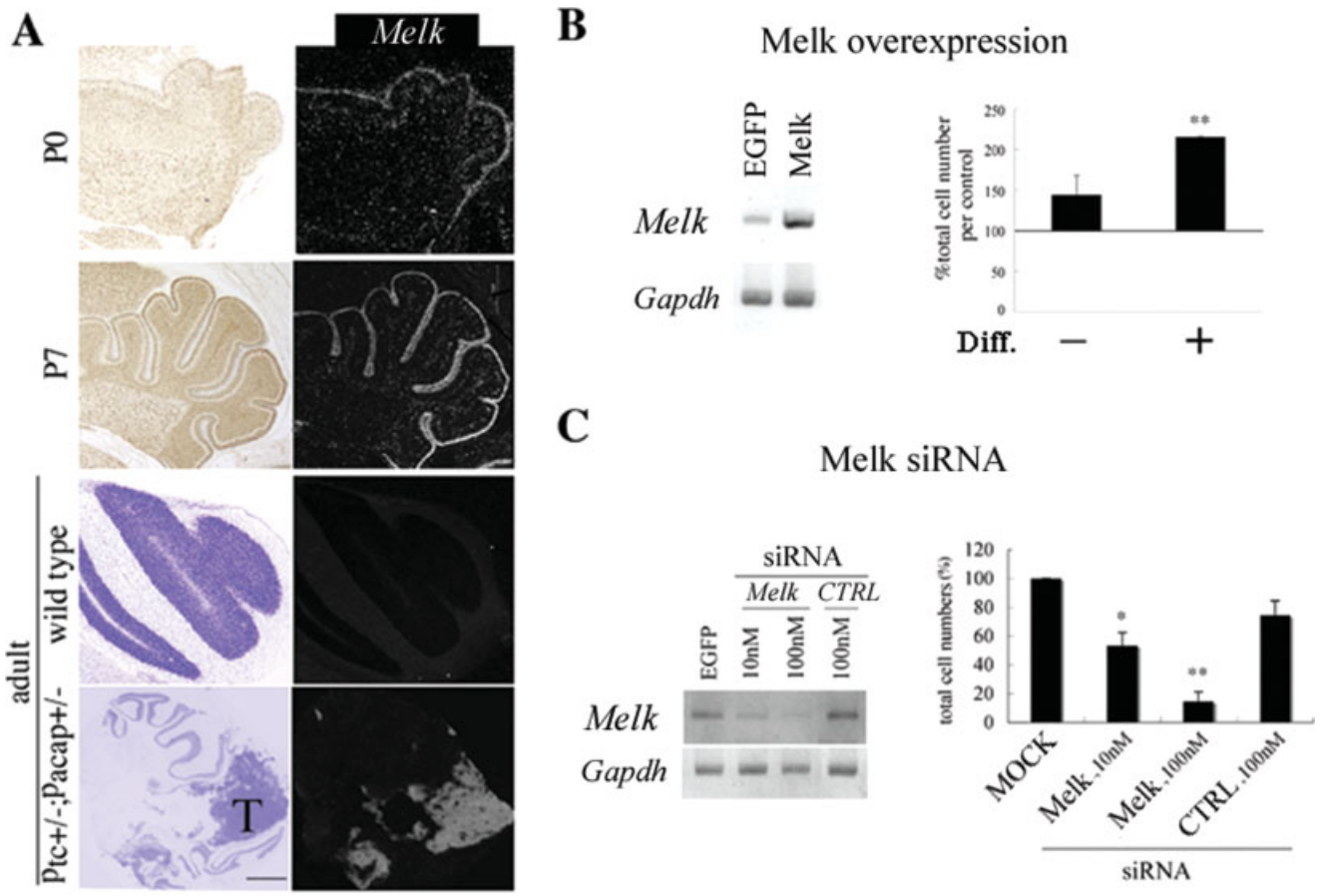


Fig. 4. Melk function in mouse medulloblastoma (MB). **A:** Right column: In situ hybridization of *Melk* during mouse cerebellar development as well as an MB spontaneously formed in the cerebellum of Ptc+/–, Pacap+/– mouse. Left column: The top two images show TuJ1 immunohistochemistry at P0 and P7 to delineate cellular layers and general architecture. The bottom two images show Nissl staining of adult cerebellum of wild-type mice and of Ptc+/–, Pacap+/– mice bearing mouse MB (T). **B:** Effect of overexpression of Melk on mouse MB cells in culture. *Melk* expression was examined by RT-PCR in tumor cells after transfection of EGFP or Melk overexpression vectors. The graph on the right shows the influence of Melk overexpression on cell growth, expressed as a percentage of EGFP-transfected control in the presence or absence of retinoic acid, which was used to induce differentiation (diff). **C:** The effect of Melk knockdown on mouse MB cells. RT-PCR for *Melk* expression is shown following treatment with Melk or control siRNA at the concentrations shown. The graph shows the influence of Melk knockdown on cell numbers. Asterisk denotes different from controls: $\star P < 0.05$, $\star\star P < 0.001$, ANOVA, followed by post hoc *t*-test. All graphs show the mean ± SEM from three independent experiments. FBS; fetal bovine serum, RA; all-trans retinoic acid, T; mouse MB.

cease proliferation and begin differentiation into neurons (Liu et al., 2000). This observation suggests that the pro-proliferative signaling of Melk could override the differentiation/antiproliferative signaling of retinoic acid or that Melk rescues dividing cells that undergo apoptosis in response to retinoic acid. Similar to the effects observed in human GBM cells, Melk knockdown with siRNA inhibited growth of these cells (Fig. 4C).

MELK Is Highly Expressed in Normal and Brain Tumor Stem/Progenitor Cells and Regulates Their Proliferation

Previously, we identified that Melk is highly expressed in mouse neural stem cells and plays a role in their proliferation (Nakano et al., 2005). In addition, we found that MELK is highly expressed in cultures containing brain tumor stem cells derived from GBM (Hemmati et al., 2003). To determine the relevance of our mouse results to human neural progenitors and putative cancer stem cells, we used fetal human neural progenitors and floating neurospheres isolated from primary GBM. The GBM patients' characteristics are described in Table I. Under proliferation conditions, both normal and GBM progenitor cells grow as floating neurospheres (Fig. 5Aa,e). Cells in these neurospheres were positive for the neural progenitor marker nestin (Fig. 5Ab,f). After withdrawal of EGF and bFGF, normal neural progenitors in clonally derived neurospheres were capable of differentiating into TuJ1-positive neurons and GFAP-positive astrocytes (Fig. 5Ac,d). As expected, withdrawal of mitogens and plating on adherent substrate resulted in

TABLE I. Patient Characteristics*

Patient	Age	Gender	Histology	Location
GBM1600	34 Years	Male	GBM	Right frontotemporal
GNS1	60 Years	Male	GBM	Right temporal
GNS2	59 Years	Male	GBM	Left temporal
GNS3	63 Years	Male	GBM	Right frontal
GNS4	62 Years	Male	GBM	Right frontoparietal
GBM2345	37 Years	Female	GBM	Left frontotemporal
GBM97	74 Years	Male	GBM	Left temporoparietal
GBM2313	42 Years	Male	GBM	Right temporal
MB1	10 Months	Male	MB	Midcerebellum

*The patients whose tumors were used for the experiments displayed in Figure 3 and 5 are shown here.

morphological differentiation of the GBM sphere cultures (Fig. 5Ag). Similar to the normal neurospheres, GBM cells, grown as clonal neurospheres, were capable of differentiating into cells expressing markers of the neuronal (TuJ1-positive) lineage as well as glial (GFAP-positive) lineage (Fig. 5Ah). Because GBM stem cells have been reported to express CD133 (Singh et al., 2003, 2004; Hemmati et al., 2003), we examined CD133 expression in spheres grown from GBM by FACS. The percentage of cells within spheres that were CD133 positive ranged from 95% to 99% (data not shown). Withdrawal of mitogen or culture with serum resulted in a dramatic loss in the number of CD133-positive cells, as expected (Lee et al., 2006). For example, in the cultures shown in Figure 5A, the proportion of CD133-positive cells went from 95% to 52% at 3 days following growth factor withdrawal. RT-PCR showed that *MELK* is highly expressed in proliferating progenitors compared with the differentiating sister cultures (Fig. 5B), suggesting that MELK is enriched in normal and GBM stem cells. Then, we examined MELK function in progenitors both in normal and GBM cells. In both cell types, MELK siRNA treatment resulted in a drop in *MELK* mRNA levels compared with untreated siRNA-treated controls (data not shown). This treatment resulted in a dramatic loss of clonal neurosphere forming capacity of all the tested samples (data not shown). This effect was observed following treatment with either the coding region siRNA (Fig. 5C,D) or the 3′UTR siRNA (data not shown).

Two methods have been identified to enrich brain tumor stem cells, neurosphere cultures and cell sorting with a cell surface marker, CD133 (Hemmati et al., 2003; Singh et al., 2004; Lee et al., 2006; Phillips et al., 2006). Thus far, CD133 is the only marker that can enrich live brain tumor stem cells. Therefore, we directly tested the effect of MELK knockdown on CD133-positive tumor cells (Fig. 5D). Neurospheres derived from the GBM sample (GNS4) contained 98.9% CD133-positive cells. MELK siRNA treatment resulted in a reduction of the total cell numbers without affecting the fraction of the CD133-positive cells in neurospheres. These data indicate that MELK regulates growth of CD133-positive tumor progenitors as well as the CD133-negative cells, as would be expected based on our data with cultures grown in serum.

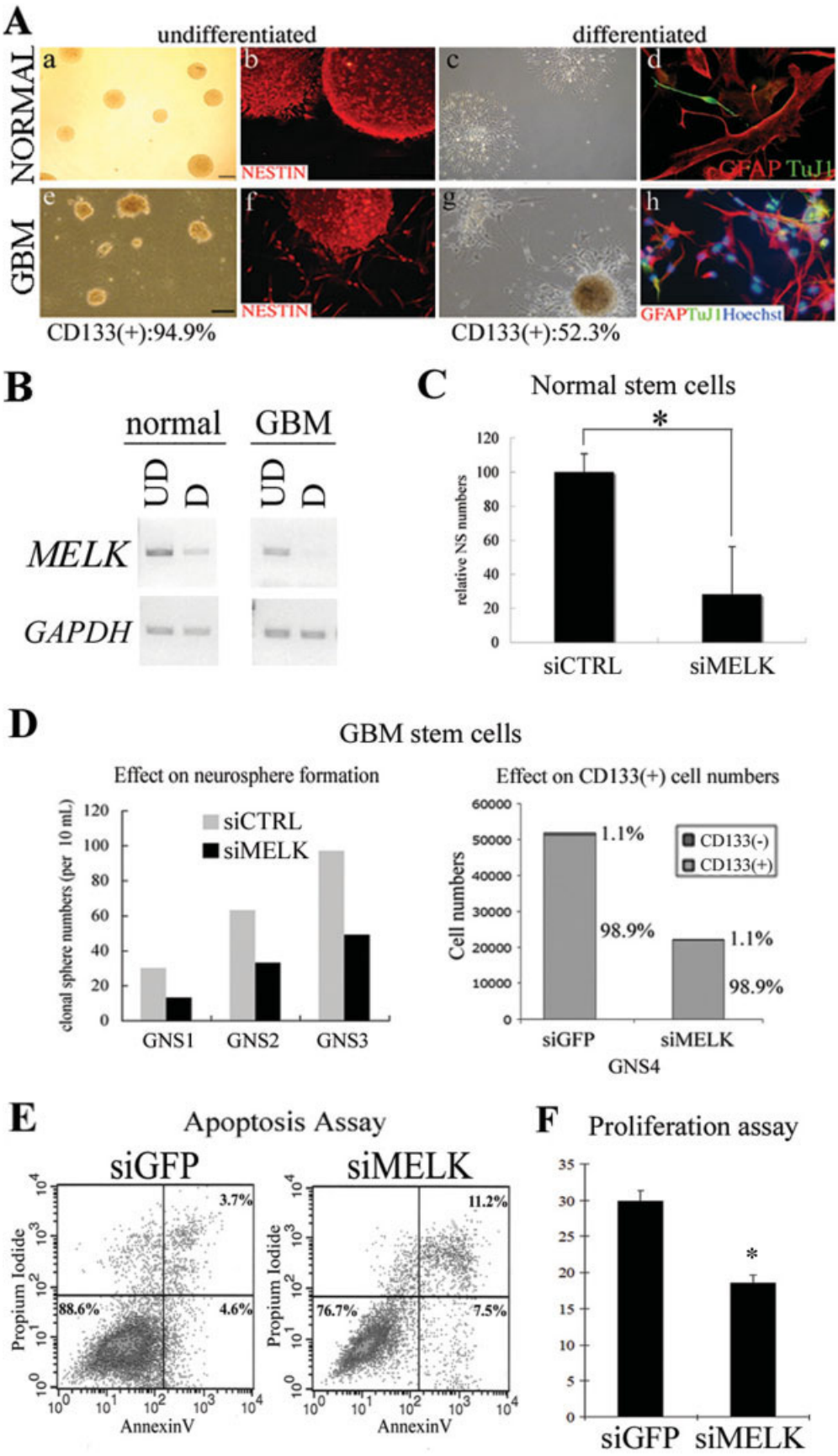
In contrast to the significant apoptotic effect of MELK siRNA on serum-derived cultures, the effect of MELK siRNA on neurospheres derived from GBM sample (GNS4) was not as great (18.7% in MELK siRNA treated cells vs. 8.3% in control siRNA treated cells; Fig. 5E). On the other hand, the effect of MELK siRNA on BrdU incorporation was as dramatic in GBM neurospheres compared with serum-derived cells (Fig. 5F).

DISCUSSION

We hypothesized that genes associated with neural stem cell self-renewal are critical regulators of brain tumor proliferation. MELK was a target of high priority based on several observations: its high level of expression in neural progenitors, its function in neural stem cell self-renewal, and its expression levels in malignant brain tumors. Therefore, we tested its function in GBM and MB cells in vitro. The results described here demonstrate that MELK is an important regulator of proliferation and survival in these cells.

MELK expression across brain tumors was associated with increased histological grade, the highest levels

Fig. 5. (Figure appears on preceding page.) MELK function in normal neural stem cells and GBM stem cells. **A:** Upper panels: Proliferating neurospheres (a,b), isolated from human fetal brain tissue, are nestin positive (b) and are capable of differentiating (c) into TuJ-1-positive neurons (green in d), as well as GFAP-positive astrocytes (red in d). Lower panels: Examples of GBM-derived neurospheres in undifferentiated (e,f) and differentiated (g,h) states. Immunofluorescence shows nestin expression in the undifferentiated cells (f) and GFAP and TuJ1 staining in differentiated cells (h). Proportion of CD133-positive cells by flow cytometry is shown in e,g. **B:** RT-PCR of MELK expression in normal and GBM progenitors under proliferating conditions (UD) and 3 days following growth factor withdrawal (D). **C:** Treatment of human neural progenitors with MELK siRNA. The graphs show the numbers of clonal neurospheres (mean percentage control per well) following treatment of control siRNA or MELK siRNA. The graphs are the means ± SEM based on the results from three independent experiments. Asterisk denotes different from controls: $*P < 0.05$ ANOVA, followed by post hoc *t*-test. **D:** Effects of MELK siRNA treatment on clonal sphere formation in brain tumor progenitors derived from GBM. Results are expressed as the number of spheres per 10 ml growth medium. The graph at right shows the effects of EGFP siRNA (siGFP) or MELK siRNA (siMELK) on CD133-positive and -negative cells in the GBM progenitor cultures in cells derived from samples derived from GNS4. **E:** FACS analysis of control (siGFP) and MELK siRNA treated GBM progenitors (GNS4). **F:** BrdU incorporation in brain tumor progenitors derived from GBM following siRNA treatment. The graphs represent the labeling index (percentage BrdU-positive cells). Scale bars = 55 μm in a (applies to a–c); 8 μm for d; 55 μm in e (applies to e–g); 8 μm for h. Asterisk denotes different from controls. $*P < 0.05$, ANOVA, followed by post hoc *t*-test. All graphs show the mean ± SEM from three independent experiments.


A
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NESTIN
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CD133(+):94.9%
CD133(+):52.3%
B
normal
GBM
UD
D
UD
D
MELK
GAPDH
C
Normal stem cells
*
relative NS numbers
120
100
80
60
40
20
0
siCTRL
siMELK
D
GBM stem cells
Effect on neurosphere formation
clonal sphere numbers (per 10 mL)
siCTRL
siMELK
GNS1
GNS2
GNS3
Effect on CD133(+) cell numbers
Cell numbers
CD133(-)
CD133(+)
1.1%
98.9%
1.1%
98.9%
siGFP
siMELK
GNS4
E
Apoptosis Assay
siGFP
siMELK
Propium Iodide
AnnexinV
3.7%
88.6%
4.6%
11.2%
76.7%
7.5%
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Proliferation assay
*
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Figure 5.

of expression being in the most malignant tumors, GBM and MB. A priori, then, one might expect to find a significant, inverse association of MELK with brain tumor patient survival, in general. High *MELK* expression was correlated with poor patient survival by Freije et al. (2004), which has been supported by data from Phillips et al. (2006). Interestingly, we found that this correlation was strongest and statistically significant in patients younger than 50 years of age. This latter observation is consistent with previous reports demonstrating differences in tumor biology and gene expression in GBMs derived from younger patients (Freije et al., 2004). The reasons for this are unknown, but it will be interesting to determine the relative stem cell content and activity of GBM from younger vs. older patients.

In vitro, we also found that MELK expression correlates with malignancy in established cell lines. TERT-immortalized human astrocytes proliferate but have a low tumorigenic potential. However, when these cells overexpress the oncogenes Akt and Ras, they form tumors with the appearance of GBM (Sonoda et al., 2001). MELK expression was greater in these highly malignant cells, and these cells were more dependent on MELK for their proliferation.

Our results, like those of Phillips et al. (2006), highlight the close relationship between brain tumors and neural stem cells. MELK is highly expressed in both malignant brain tumors and neural stem and/or progenitor cells. Brain tumors are derived from the neural tube, as are neural stem cells. It is tempting to speculate that commonalities of gene expression, such as for MELK, are based on a common cell of origin, the neural stem cell. One class of brain tumors, ependymomas, has recently been shown to have strong genetic and cellular similarities to radial glia, cells with the key characteristics of neural stem cells; self-renewal and the capacity to give rise to neurons astrocytes and oligodendrocytes (Taylor et al., 2005). *MELK* is highly expressed in these tumors, particularly those derived from the cerebral hemispheres (Taylor et al., 2005). MBs, on the other hand, are less likely to be derived from neural stem cells and, instead, are likely to be derived from granule cell precursors, another self-renewing progenitor in the CNS (Pomeroy et al., 2002). The cell of origin of GBM is not at all clear. Although GBMs are often located at sites distant from the lateral ventricles, the location of neural stem cells, their cells are highly migratory, so it is possible that a transformed stem cell could migrate away from the ventricle and initiate a tumor. Alternatively, GBMs may arise from the mutations in other progenitors, including restricted glial progenitors, that then take on more stem cell-like characteristics as a result of the mutations.

Despite these arguments regarding the cell of origin, there are fundamental phenotypic similarities among rapidly self-renewing neural stem cells, GBM, and MBs that may explain commonalities of gene expression, including the expression of MELK. One such similarity is simply the degree to which they are proliferating. It may be that dysregulated proliferation uses similar pathways in cells of neural origin, or, indeed in different kinds of cells. Another potential explanation for similarities lies in the existence of brain tumor stem cells. Like neural stem cells, brain tumor stem cells possess the capacity to divide and form clonally derived spherical clusters in the presence of EGF and bFGF and undergo multipotent differentiation (Hemmati et al., 2003; Singh et al., 2003). Thus, it is possible that the similar ''tasks'' required for cancer stem cells and neural stem cells are accomplished by similar patterns of gene expression.

Our previous study (Hemmati et al., 2003) and a more recent one (Liu et al., 2006) suggest that MELK is highly expressed in GBM stem cells. Our current data indicate that MELK regulates the proliferation of cancer stem cells derived from GBM. as with human neural stem cells, many tumor stem cells express the antigen CD133 and produce spheres that can differentiate into cells with markers characteristic of neuronal and glial lineage (Singh et al., 2003, 2004; Taylor et al., 2005). Xenotransplantation studies demonstrate that these cells are tumorigenic, and the tumors produced from them recapitulate the phenotype of the parent tumor (Galli et al., 2004; Singh et al., 2004). It is theorized that only therapies that prevent the proliferation of tumor stem cells will prevent recurrence of the malignant brain tumors that bear them. Here, we show that culturing of GBM cells as spheres results in the majority of cells expressing CD133, whereas placing them under differentiating conditions diminishes the numbers of CD133-positive cells. This is an indication that the number of brain tumor stem cells is reduced under differentiating conditions, although it is not clear what proportion of the CD133-positive cells within the cultures are stem cells and what proportion are their derivatives. When MELK expression is reduced by siRNA, both the numbers of CD133-positive cells and the numbers of secondary spheres are dramatically reduced, suggesting that, as is the case of human neural stem cells, MELK regulates their self-renewal. MELK, however, does not appear to regulate the cancer stem cells selectively, insofar as both CD133-negative and CD133-positive cells are inhibited by MELK knockdown, a finding consistent with our studies in nonselected, serum-treated cells.

MELK also regulates the proliferation of brain tumor cells cultured under conditions that are not as conducive to tumor stem cell propagation. Culturing primary GBM in serum results in a loss of cancer stem cell activity (Lee et al., 2006). Thus, our findings suggest that MELK regulates both tumor stem cell and nonstem cell populations within a tumor. MELK knockdown did not have significant effects on fibroblast cultures and had limited effects on immortalized astrocytes, suggesting that MELK is not a universal cell cycle regulatory protein but has more selective effects.

In the current study, we found that MELK is critical not only for proliferation but also for cell survival of unselected cultures, with knockdown resulting in a dramatic increase of apoptotic cells. These findings suggest that MELK takes on a role in brain tumors that is more like

other members of the AMPK family, which largely mediate cell survival under stressful conditions (Stapleton et al., 1996; Kato et al., 2002). This additional role for MELK in tumor cell survival will be advantageous if MELK is developed as a therapeutic target. On the other hand, MELK appears to have a more limited effect on the survival of cancer stem cells derived from GBM, although some effects were observed, in contrast to our previous findings with murine neural stem cells. It is likely that stem cells, in general, express proteins that protect them from cell death induced under a variety of circumstances, such as through the expression of transporters that cause the efflux of toxic compounds. It is interesting to note that cancer stem cells are similar to neural stem cells in this respect. Other studies, using xenograft models, have shown that putative GBM stem cells are radioresistent relative to other cells in the tumor population (Bao et al., 2006) and that GBM stem cells are more resistant to chemotherapeutic toxicity (Clement et al., 2007).

The mechanism of MELK function is poorly understood. Previous studies have demonstrated that MELK has kinase activity, as predicted (Beullens et al., 2005), but can also play a role in RNA processing. This function is independent of the kinase domain and is mediated by the expression of NIPP1 (Vulsteke et al., 2003). A growing body of evidence supports the hypothesis that MELK exerts its function by regulating the mitotic (M) phase of the cell cycle. Previously, we reported that genes coregulated with Melk were highly expressed in M phase, suggesting a role for Melk during this phase of the cell cycle (Nakano et al., 2005). A previous study in colon and other cancer cell lines (Gray et al., 2005) came to a similar conclusion; MELK knockdown resulted in the accumulation of cells in the G2-M phase of the cell cycle. The precise molecular mechanism by which MELK might regulate these processes is unknown. MELK has previously been shown to influence the expression and function of B-MYB, a protooncogene that had previously been thought to regulate G1-S transitions, although recent studies indicate a role for this protein in G2-M progression (Zhu et al., 2004). MELK, known also as *pEg3*, has been shown to phosphorylate CDC25b, which also regulates the G2-M transition (Davezac et al., 2002). Thus, several lines of evidence support a role for MELK in the control of mitotic entry in cancer and other cells.

In conclusion, our findings suggest that MELK is a potential target for brain tumor therapy. MELK is highly activated in states of increased malignancy and appears to be a key regulator of cancer cell proliferation and survival. Additionally, MELK influences the proliferation of brain tumor stem cells. Future studies will be needed to address the mechanisms of MELK action and to discover specific inhibitors of MELK.

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