Tumor and Stem Cell Biology

Cancer Research

MAP Kinase-Interacting Kinase 1 (MNK1) Regulates SMAD2-Dependent TGF-β Signaling Pathway in Human Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is the most common aggressive brain cancer with a median survival of approximately 1 year. In a search for novel molecular targets that could be therapeutically developed, our kinome-focused microarray analysis identified the MAP (mitogen-activated protein) kinase-interacting kinase 1 (MNK1) as an attractive theranostic candidate. MNK1 overexpression was confirmed in both primary GBMs and glioma cell lines. Inhibition of MNK1 activity in GBM cells by the small molecule CGP57380 suppressed eIF4E phosphorylation, proliferation, and colony formation whereas concomitant treatment with CGP57380 and the mTOR inhibitor rapamycin accentuated growth inhibition and cell-cycle arrest. siRNA-mediated knockdown of MNK1 expression reduced proliferation of cells incubated with rapamycin. Conversely, overexpression of full-length MNK1 reduced rapamycin-induced growth inhibition. Analysis of polysomal profiles revealed inhibition of translation in CGP57380 and rapamycin-treated cells. Microarray analysis of total and polysomal RNA from MNK1-depleted GBM cells identified mRNAs involved in regulation of TGF-β pathway. Translation of SMAD2 mRNA as well as TGF-β–induced cell motility and vimentin expression was regulated by MNK1 signaling. Tissue microarray analysis revealed a positive correlation between the immunohistochemical staining of MNK1 and SMAD2. Taken together, our findings offer insights into how MNK1 pathways control translation of cancer-related mRNAs including SMAD2, a key component of the TGF-β signaling pathway. Furthermore, they suggest MNK1-controlled translational pathways in targeted strategies to more effectively treat GBM. *Cancer Res; 71(6); 1–10. ©2011 AACR.*

Introduction

In the last decade, genetic profiling of brain tumors has improved our understanding of gliomagenesis and led to the development of many targeted therapies based on molecular interference with deregulated signaling networks (1–3). Although many screens have characterized and proposed the targeting of deregulated signaling pathways for therapeutic interference, recent reports have identified therapy resistance based on the compensatory activation of alternative signaling pathways. Thus, effective treatment requires combined regimens targeting the glioblastoma kinome (4–7).

The overall rate of protein synthesis is an important determinant of cancer cell metabolism (8). Many previous observations have indicated that deregulated growth pathways in human cancers are involved in the control of translation supporting cell proliferation and survival. In response to nutrients and growth factors, activated AKT/mTOR pathways enhance global protein synthesis. They phosphorylate and inactivate the eukaryotic translation initiation factor 4E-binding protein (eIF4E-BP), a repressor of mRNA translation and activate ribosomal S6 kinase 1 (S6K1) involved in ribosome biogenesis (9). Kinases associated with translation initiation complexes have a potential for regulating translation. MAP (mitogen-activated protein) kinase-interacting kinases (MNK1/2) can bind to translation initiation factor, eIF4G, and phosphorylate the cap-binding protein, the translation initiation factor eIF4E (10). The phosphorylation of eIF4E on Ser209 is increased in cancer cells and eIF4E expression levels are upregulated in many tumors (11–13). Recent findings demonstrate that eIF4E phosphorylation by MNKs is absolutely required for the eIF4E activity that opposes apoptosis and promotes tumorigenesis *in vivo* (14). In addition, MNKs can also phosphorylate RNA-binding protein, hnRNPA1, which binds AU-rich elements of messenger RNA (e.g.,

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TNF-α), thereby regulating its stability and/or translation (15). Thus, MNK signaling appears to play an important role in posttranscriptional regulation of cancer-related gene expression.

Materials and Methods

Antibodies and chemicals

MNK1 (C4C1), p-MNK1 (Thr197/202), SMAD2 (86F7), p-SMAD2 (Ser245/250/255), eIF4E (C46H6), p-eIF4E (Ser209), p-S6 (Ser235/236), p38 (9212), p-p38 (Thr180/Tyr182), ERK1/2 (9102), p-ERK1/2 (Thr202/Tyr204), and p-EGFR (Tyr992) antibodies were from Cell Signaling. Actin (I-19) and EGFR (epidermal growth factor receptor; sc-03) antibodies from Santa Cruz Biotechnology, vimentin (V9) from Thermo Scientific and generated α-tubulin YL1/2 was used as hybridoma supernatant. CGP57380 and rapamycin were from Sigma, RAD001 from Novartis, SB431542 from Tocris, and TGF-β from PeproTech.

Patients

Primary glioma tissues obtained from the operating room were processed as previously described (16) in accordance with the guidelines of the Ethical Committee of the University Hospitals of Basel and Dusseldorf. Tumors were diagnosed and graded according to the current WHO Classification. The patient set is summarized in Supplementary Table 1.

Cell culture and standard techniques

Human glioma cells: BS125, LN18, LN229, BS149, LN319, LN405, LN215, LN71, U343MG, U373, U87MG, Hs683, A175, and M059K were cultured in DMEM supplemented with 10% FCS (fetal calf serum) and antibiotics at 37°C and 5% CO_2. GBM (glioblastoma multiforme)-derived BS287 tumor spheres were cultured as described previously (17). Clonetics normal human astrocytes (NHA) were from Cambrex and cultured according to the manufacturer's recommendations. Transfection, treatments, cellular assays, including proliferation, viability, colony formation, flow cytometry as well as RNA and protein isolation followed by quantitative RT-PCR, microarray hybridization, Western blotting, and immunohistochemistry are described in Supplementary Experimental Procedures.

Microarray data analysis

Data mining and visualization of microarray-profiled gliomas were performed using Genedata's Analyst 4.1 package. Median fold ratio values at $P < 0.05$ in the t test were used for analysis. All samples were quintile normalized and median scaled to correct for minor variations in their expression distributions. The data obtained have been deposited in the Gene Expression Omnibus (GEO) database (GSE15824). Polysomal profiles (triplicate experiment for transfection and inhibitor treatments) and RNA extraction was accomplished as previously described (18) and used for microarray hybridization described in Supplementary Material. Data analysis was carried out using R/Bioconductor (19). Signal condensation was performed using the RMA from the Bioconductor Affy package. Differentially expressed genes were identified by the empirical Bayes method (F test) implemented in the LIMMA package and adjusted with the false discovery rate method (20). Visualization was done in R. Probe sets with a $\log_2$ average contrast signal of at least 5, an adjusted P value of < 0.05, and an absolute $\log_2$ fold-change of greater than 0.585 (1.5-fold in linear space) were used.

Cell motility

Forty-eight hours after transfection or 24 hours after treatment, a scratch was made and the migration of cells was monitored using a live-imaging system Widefield TILL5, Long Run, Axiovert 200M (Carl Zeiss). Images were captured every 20 minutes over a period of 22 hours with a CCD camera using MetaMorph software (Molecular Devices) and analyzed using ImageJ software (NIH).

Results

MNK1 overexpression in human glioblastoma

Microarray analysis of 30 brain tumor samples was performed including 15 high-grade gliomas (glioblastomas) and 15 low-grade tumors (8 astrocytomas and 7 oligodendrogliomas). Kinase expression in each tumor was normalized to the expression levels in normal brain and expression values from NHAs were used as an additional control. Apart from kinases known to be associated with gliomagenesis (e.g., EGFR; data not shown), a further protein kinase was found to be upregulated in primary glioblastomas. The transcript level of MNK1 was upregulated more than 2-fold in 12 of 15 glioblastomas and in 5 of 8 astrocytomas ($P < 0.01$ for both groups, compared with expression in normal brain and human normal astrocytes) whereas 3 of 7 patients exhibited elevated MNK1 levels in oligodendroglioma (Fig. 1A). MNK2 kinase expression was not significantly altered in the human gliomas analyzed (data not shown). There was no significant correlation between MNK1 expression and survival in profiled glioma patients. Changes in MNK1 expression were further validated by real-time PCR. In 10 of 11 glioblastoma samples, MNK1 was elevated more than 2-fold (Fig. 1B). Human MNK1 arises from an alternatively spliced transcript giving rise to 2 isoforms, longer MNK1a (50 kDa) and a shorter MNK1b variant (38 kDa) that lacks 89 C-terminal amino acids (21). We determined MNK1a protein levels in GBM tumors and cell lines using an MNK1a-specific monoclonal antibody. An increase in MNK1 kinase was observed in 14 of 20 (70%) primary glioblastomas and in 11 of 13 (84%) glioma cell lines, compared with normal brain and NHAs, respectively (Fig. 1C). Immunohistochemical analysis of tumor sections from 12 GBM patients and tissue arrays containing 34 GBMs and 5 normal brains also demonstrated high MNK1 protein levels, with stronger cytoplasmic signals in GBM cells than in weakly stained normal brain (Fig. 1D). Antibody specificity was tested on whole protein cell lysates (Fig. 1C) and on formalin-fixed BS125 cells (Fig. 1D) transfected with siRNA against the *MNK1* gene or luciferase (control). MNK1 phosphorylation correlated with total level of MNK1 (correlation coefficient = 0.51) and was significantly higher in 7 of 11 (64%) GBM patients than in normal human brain and astrocytes (Fig. 1C and Supplementary Fig. S1). To

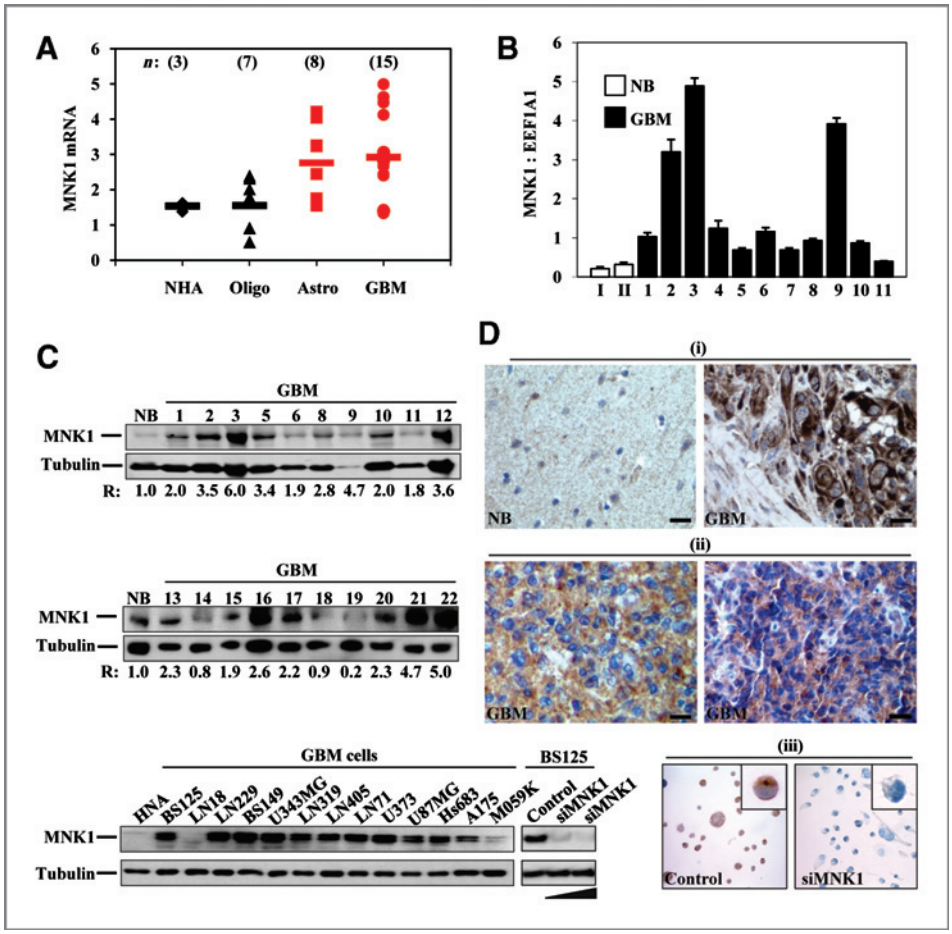

Figure 1. Expression of MNK1 in human gliomas. A, MNK1 transcript levels in primary brain tumors obtained from microarray analysis. NHA, Oligo (oligodendroglioma), Astro (astrocytoma), and GBM. Expression of MNK1 in normal brain (NB) was set to 1. Patient information is given in Supplementary Table 1. B, quantitative RT-PCR analysis of MNK1 (normalized to EEF1A1 expression) on total RNA isolated from glioblastoma and 2 NB: I and II samples. C, Western blot analysis using an MNK1-specific antibody on whole protein lysates obtained from human GBM (lanes 1–22), NB samples as well as from different GBM cell lines, NHA and BS125 cells at 3 days after transfection with increasing concentrations of duplex siRNA against MNK1 or against luciferase (control). Blots were stripped and reprobed with an α-tubulin antibody. Ratios (R) of MNK1-specific signals relative to tubulin expression are shown below. The MNK1/tubulin ratio for NB was set to 1. D, immunohistochemistry (IHC) of MNK1 in normal brain and primary GBM in tissue arrays (i), GBM paraffin sections (ii), and formalin-fixed BS125 cells 72 hours after siRNA transfection (iii); bar = 20 μm.

investigate whether MNK1 expression is regulated by EGFR, which is often amplified and hyperactivated in GBM, the correlation between MNK1 and EGFR was analyzed in glioma patients and in GBM cell lines. Microarray analysis showed no significant correlation between MNK1 and EGFR in human gliomas (correlation coefficient = 0.29). There was also no correlation between MNK1 and EGFR or phopho-EGFR levels in analyzed GBM cells (Fig. 1C and Supplementary Fig. S2). Likewise, treatment with the EGFR inhibitor AEE788 did not affect the MNK1 protein level indicating that MNK1 expression is not regulated by EGFR pathways.

Inhibition of MNK1 reduces GBM cell proliferation and colony formation and acts additively with rapamycin

To investigate MNK1 as a potential therapeutic target, a pharmacologic approach was first taken using an efficient inhibitor of MNK kinases, CGP57380 (22). As MNK1 signaling is involved in the regulation of translation, inhibition of MNK1 was combined with the targeting of mTOR pathways, which also act at the translational level. Treatment with either CGP57380 or rapamycin alone reduced proliferation and their combination had an additive effect on human GBM cell lines BS125, LN319, LN405, and BS287 spheres (Fig. 2A and Supplementary Fig. S3). There was a synergistic effect at the highest concentrations of CGP57380 (10 μmol/L) and rapamycin (10 nmol/L), with combination index values of 0.71 and 0.63 in BS125 and LN319 cells, respectively. Concomitant treatments with CGP57380 and rapamycin over 5 days greatly reduced BS125 and LN319 cell number (data not shown). The observed additive inhibitory effect was confirmed further using CGP57380 together with the rapamycin derivative RAD001 in BS125 cells (Supplementary Fig. S3). The effects of inhibitor treatments were monitored by immunoblotting using phospho-specific antibodies against eIF4E (treatment with CGP57380) and ribosomal protein S6 (treatment with rapamycin; Fig. 2B, right). CGP57380 at 2 μmol/L or rapamycin at 0.5 nmol/L was sufficient to decrease significantly eIF4E and S6 phosphorylation, respectively. Furthermore, the growth of BS125 cells in soft agar at increasing concentrations of CGP57380 and rapamycin was inhibited in a dose-dependent manner (Fig. 2B, left). Concomitant treatment with CGP57380 and rapamycin amplified inhibitory effect; CGP57380 at 2 μmol/L significantly increased the growth inhibition by rapamycin. To analyze the relationship specifically between MNK1 protein level and cell proliferation after rapamycin treatment, RNAi (RNA interference) was used to

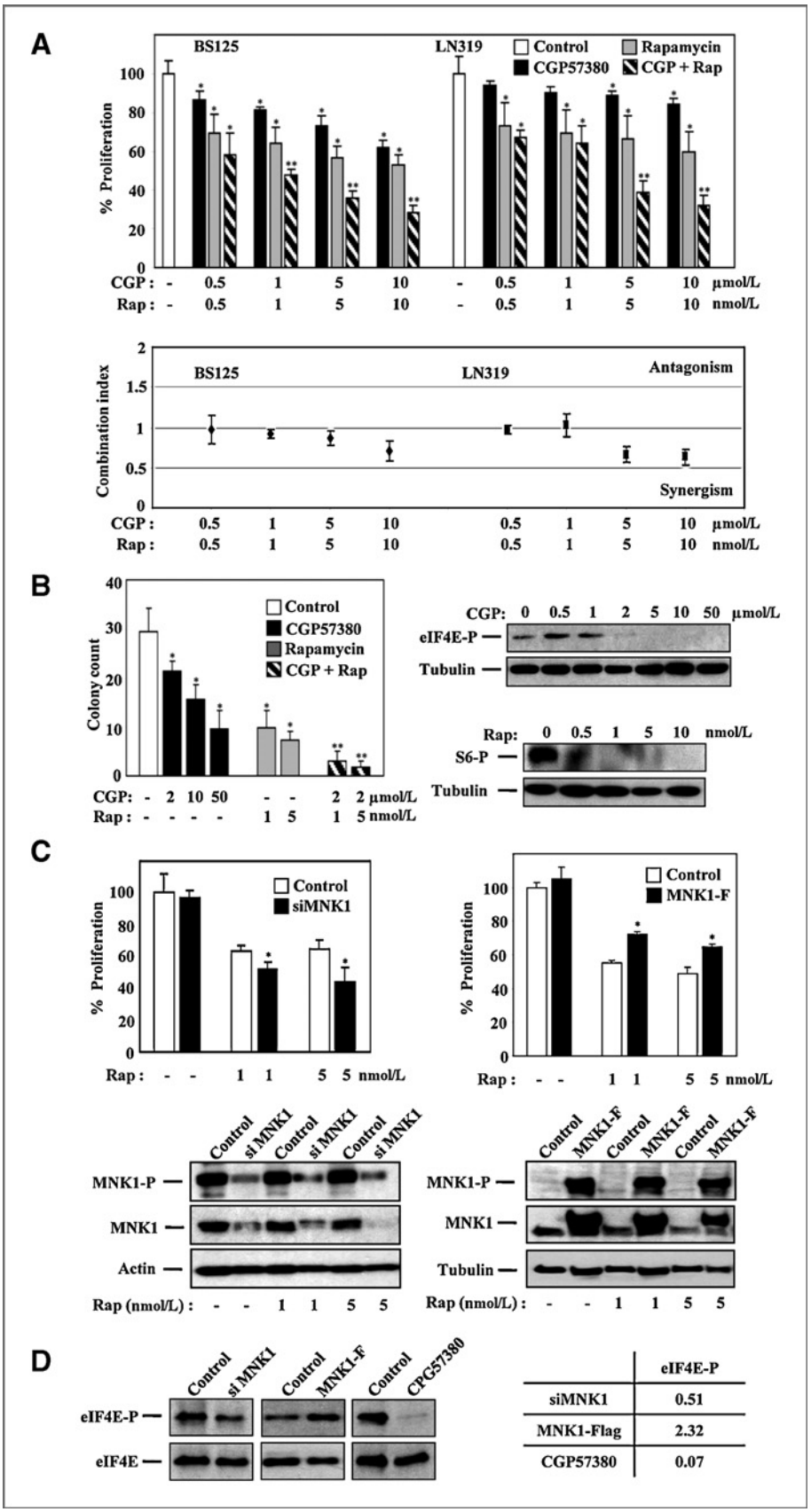

Figure 2. Targeting MNK1 inhibited cell growth and sensitized GBM cells to rapamycin. A, An MTT-based assay of BS125 and LN319 cell proliferation after 3 days of incubation with CGP57380 alone or in combination with rapamycin (Rap). Results were obtained in triplicate and are shown as % proliferation compared with control cells. Below: corresponding combination index (CI) values greater than 1.1 indicate antagonism, between 0.9 and 1.1 additive effects, and lower than 0.9 synergism. B, resuspended BS125 cells in soft agar were incubated at the indicated concentrations of CGP57380 and/or rapamycin. After 16 days, colonies were photographed and analyzed as described in the Material and Methods section. All experiments were carried out in triplicate and the results are shown as mean values ± SD. B, right, phosphorylation of eIF4E (Ser209) or ribosomal protein S6 (Ser235/236) in BS125 cells treated with CGP57380 or rapamycin for 24 hours was monitored by immunoblotting using phospho-specific antibodies. The blots were stripped and reprobed with antitubulin to control for equal loading. C, BS125 cells were transfected with duplex siRNA oligonucleotides against *MNK1* (si) or with a control duplex against luciferase. For the full-length MNK1 overexpression, cells were transfected with a MNK1–Flag construct or an empty control vector. Twenty-four hours after transfection, cells were treated with rapamycin at the concentrations indicated for a further 48 hours and used for MTT-based assay as described above. C, bottom, whole protein lysates isolated from transfected and rapamycin-treated cells were subjected to Western blot analysis using phospho-specific (Thr197/202) and total MNK1 antibodies. Actin- or tubulin-specific antibodies were used as loading controls. D, eIF4E phosphorylation in MNK1-depleted or overexpressing BS125 cells 48 hours after transfection or treatment with 10 µmol/L CGP57380 was analyzed by Western blot using specific antibodies against phosphorylated and total eIF4E. D, right, changes in eIF4E phosphorylation depicted as the ratio of phospho- to total eIF4E-specific signals. Ratios in controls were normalized to 1. *, $P < 0.05$ or **, $P < 0.02$ compared with control or single drug treatment using Student's *t* test.

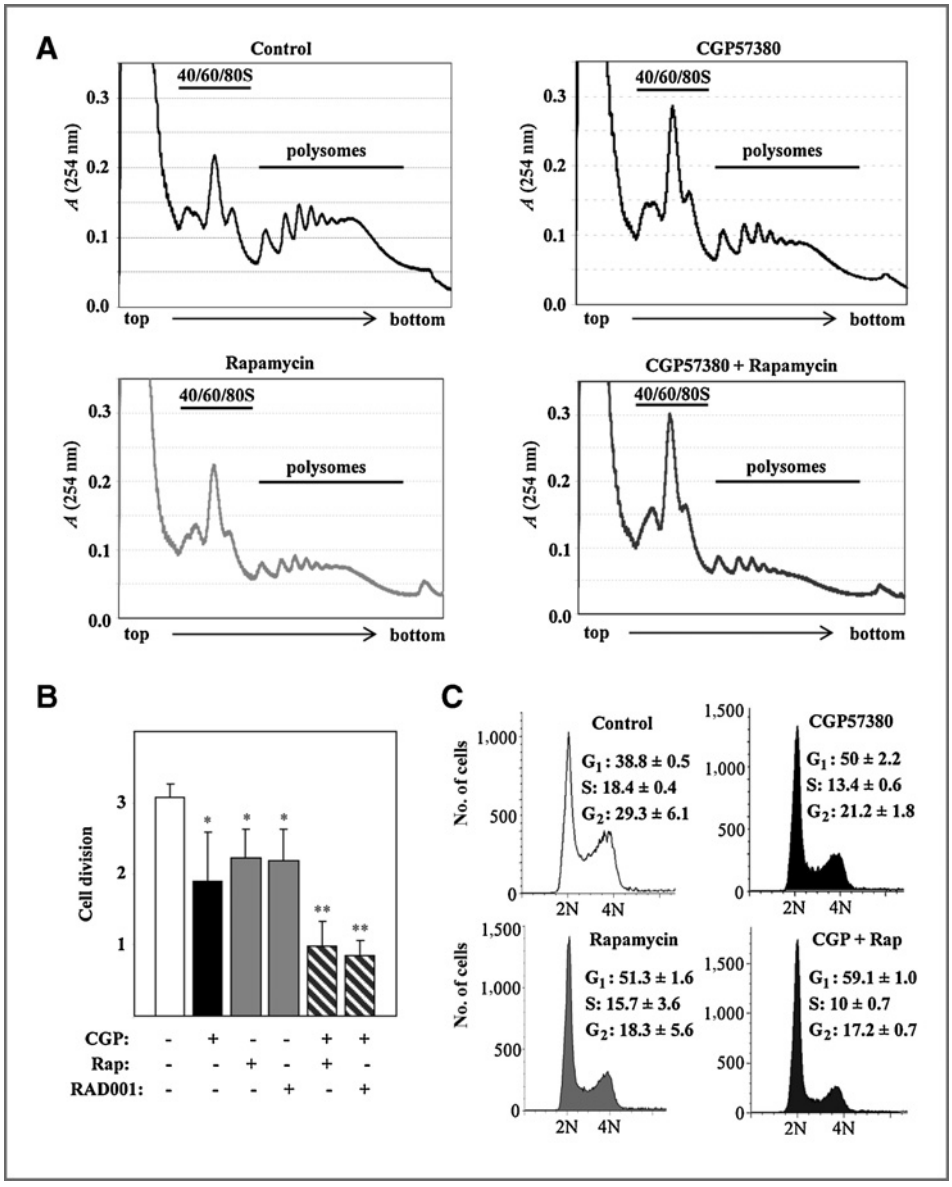

Figure 3. Inhibition of MNK1 by CGP57380 reduced global translation and induced cell-cycle arrest; combined treatment with rapamycin increased the inhibitory effect. A, polysome profiles from BS125 cells treated with DMSO (dimethyl sulfoxide; control), 10 μmol/L CGP57380, and/or 10 nmol/L rapamycin for 24 hours. The A_{254} peaks corresponding to ribosomal subunits and polysomes are indicated. B, cell division was determined 72 hours after treatment (as described above) using the CFSE (carboxyfluorescein succinimidyl ester) cell-labeling assay followed by FACS (fluorescence-activated cell-sorting) analysis. The rapamycin analogue RAD001 was used at 10 nmol/L. *, $P < 0.05$ or **, $P < 0.01$ versus control or single treatment. C, cell-cycle analysis of BS125 cells treated for 24 hours as indicated above. Results are means ± SD from 3 independent incubations.

inhibit MNK1 expression. For complete depletion of MNK1, duplex siRNA were used specific for splice variants *MNK1a* and *MNK1b*, which encode identical catalytic domains. BS125 cells transfected with an MNK1-specific siRNA duplex showed significantly reduced endogenous expression of both phospho- and total MNK1 protein and enhanced sensitivity to rapamycin (Fig. 2C). To investigate this further, a full-length MNK1–Flag fusion protein was overexpressed in BS125 cells. Ectopically expressed MNK1 protein was phosphorylated and this reduced the inhibition of BS125 cell proliferation by rapamycin (Fig. 2C, right). MNK1 overexpression increased phosphorylation of the MNK1 substrate eIF4E more than 2-fold, indicating that the MNK1-Flag–tagged kinase was fully functional whereas MNK1-specific knockdown or CGP57380 treatment reduced eIF4E phosphorylation to 51% and 7%, respectively, compared with control-treated cells (Fig. 2D). Finally, the MNK1 protein level correlated positively (correlation coefficient = 0.7) with resistance to mTOR inhibition after 3 days treatment with 5 nmol/L rapamycin in 6 GBM cell lines: BS125, LN319, LN18, LN405, U343MG, and BS145 (Fig. 2A, Supplementary Fig. S3, and data not shown).

Targeting signaling pathways regulating translation reduces global translation and induces cell-cycle arrest

Polysome profiles obtained by sucrose gradient centrifugation revealed a substantial increase in the inhibition of global translation in BS125 cells treated simultaneously with CGP57380 and rapamycin (Fig. 3A). Comparison analysis of the areas occupied under the curves in polysomal profiles (data not shown) indicates that CGP57380 increased the relative abundance of free ribosomes (1.35 ± 0.3-fold) and decreased polysomes (0.65 ± 0.05) whereas rapamycin alone

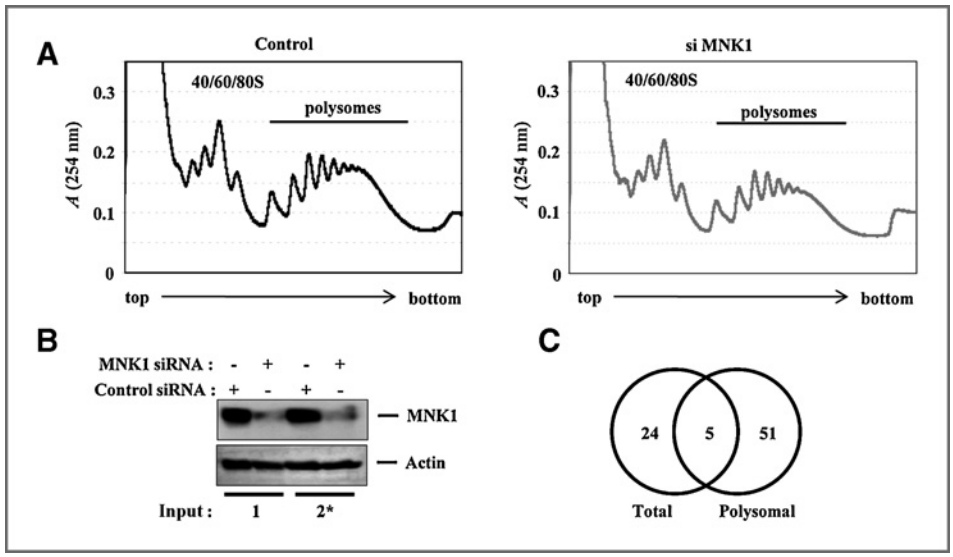

Figure 4. MNK1 knockdown had no major impact on global translation but regulated the expression of a subset of mRNAs. A, polysome profiles from BS125 cell lysates prepared 48 hours after transfection with duplex siRNA against *MNK1* gene (si) or with a control duplex against luciferase. The A_{254} peaks corresponding to ribosomal subunits and polysomes are indicated. B, the same protein lysates were subjected to Western blot analysis with MNK1- and tubulin-specific antibodies. *, profiles in A were obtained from the indicated lysates. C, results from expression profiling of total and polysome-associated mRNAs from MNK1-depleted and control-transfected BS125 cells shown as a Venn diagram representing the numbers of significantly affected RNAs.

significantly reduced the abundance of polysomes (0.43 ± 0.04) compared with control. Cotreatment with CGP57380 and rapamycin both increased free ribosomes (1.48 ± 0.23) and reduced polysomes abundance (0.31 ± 0.05). Furthermore, as shown in Figure 3B and C, the inhibition of global translation was accompanied by G_1 cell-cycle arrest. This demonstrates a major cytostatic effect in BS125 cells treated with CGP57380 together with rapamycin or RAD001.

Identification of MNK1-regulated targets

Sucrose gradient separation revealed no major differences in polysome profiles between BS125 cells transfected with siRNA against *MNK1* and the control duplex (Fig. 4A and B), suggesting that MNK1 is involved rather in the regulation of a small subset of mRNAs. To investigate this further, total and polysome-associated RNAs isolated from the same transfected cells were compared using microarray hybridization. In addition to reduced MNK1 expression, we identified 56 differentially expressed mRNAs ($P < 0.05$) using polysomal RNA and 29 using total RNA (Fig. 4C, Supplementary Tables S2 and S3). Of these, 5 targets were common to both polysomal and total RNAs. The identified MNK1 targets have been subjected to gene ontology and pathway analysis using the DAVID web-based bioinformatics tool (23, 24). These analyses indicated that MNK1 regulates a group of mRNAs encoding proteins involved in TGF-β signaling, with the highest enrichment value of 8.3 whereas mRNAs associated with signal transduction and cell communication showed 3.1- and 2.6-fold enrichment, respectively (Table 1). Of note, the SMAD2 transcript was present in all 3 identified gene groups, suggesting that SMAD2 expression plays an important role in the MNK1-regulated phenotype.

MNK1 regulates SMAD2-dependent TGF-β pathways in human GBM

The microarray data demonstrating decreased SMAD2 mRNA association with polysomes (Fig. 5A) were further validated by RT-PCR analysis of polysomal and total RNA (Fig. 5B) or at the protein level in MNK1-depleted BS125 and LN319 cells (Fig. 5C). SMAD2 protein was also reduced at the presence of RNA polymerase II inhibitor, actinomycin D, indicating posttranscriptional regulation. In addition, the SMAD2 protein level was found to be higher in MNK1-overexpressing BS125 stable clones than in control-transfected cells (Fig. 5C, bottom). To further validate the *in vitro* data, tissue arrays were used to analyze MNK1 and SMAD2 expression at the protein level in 34 GBM tumors and 5 normal brains, as described above. Strong MNK1 and SMAD2 immunostaining was recorded in comparable areas of analyzed tumors, whereas normal brain tissue showed significantly weaker expression (Fig. 5D). Comparative bioinformatics analysis of MNK1 and SMAD2 expression gave a correlation coefficient of 0.6, supporting our *in vitro* observation that MNK1 regulates SMAD2 protein levels in human GBM.

Exploring how far MNK1 may be involved in the regulation of TGF-β/SMAD–dependent phenotype, we found that TGF-β at 5 nmol/L is sufficient to increase cell motility of BS125 and Hs683 cells compared with untreated cells (data not shown). Furthermore, siRNA knockdown of MNK1 or inhibition by CGP57380 reduced total as well as TGF-β–induced phosphorylated SMAD2 levels and led to a pronounced inhibition of cellular motility (Fig. 6A). Bioinformatics analysis of microarray data from GBM patients revealed a significant correlation between MNK1 expression and TGF-β–regulated genes. For this analysis, we chose hallmarks of the epithelial to

Table 1. Bioinformatics analysis of MNK1-regulated genes identified by microarray hybridization. Enrichment was scored for terms with $P < 0.05$ for a group of at least 3 genes.

Functional annotations for MNK1-regulated genes	Enrichment
TGF-β signaling pathway (pathway: hsa04350, $P = 0.04$); *SMAD2, BMP8, DP1*	8.3
Regulation of signal transduction (GO:0009966, $P = 0.01$); regulation of cell communication (GO:0010646, $P = 0.02$); *CTGLF3, CTGLF5, GPR89B, SMAD2, TGIF, RAC1, SNX13, TLR4, TNFSF15*	3.1; 2.6

mesenchymal transition (EMT) that plays a crucial role in malignant cancer cell migration and motility (25). Expression of vimentin and fibronectin positively correlated with MNK1 expression levels (correlation coefficients = 0.74 and 0.70, respectively), whereas, E-cadherin and tight junction protein 1 showed negative correlations of −0.64 and −0.65, respectively (Fig. 6B). Decreased vimentin expression was further confirmed at the protein level in MNK1-depleted BS125 GBM cells (Fig. 6B) whereas E-cadherin expression was not detected by Western blotting (data not shown). The lack of vimentin and other TGF-β–regulated EMT markers among the MNK1-regulated mRNAs (Supplementary Tables S2 and S3) may be explained by the fact that microarray analyses were performed 48 hours after transfection, which was sufficient for MNK1 knockdown at the protein level, whereas a significant decrease in SMAD2 protein was only observed after 72 hours (Fig. 5C). As only a marginal decrease in SMAD2 protein expression was reached 48 hours after transfection, we concluded that a significant decrease in SMAD2 protein synthesis begins after that time point resulting in alterations in TGF-β/SMAD2 signaling pathways.

In this study, we showed that SMAD2 protein synthesis depends on MNK1 and that targeting MNK1 sensitizes GBM cells to rapamycin. Therefore, we asked whether inhibition of the TGF-β/SMAD2 pathway, instead of MNK1, also has an additive effect. For this, we made use of the SB431542 compound known to inhibit SMAD2 phosphorylation (26). As demonstrated in Supplementary Figure S4, concomitant treatment with SB431542 and rapamycin (or RAD001) for 5 days had an inhibitory effect on GBM cell growth (27%–30% of control) similar to that in cells treated with CGP57380, rapamycin or RAD001 (20%–23%). Targeting all 3 pathways (mTOR, SMAD2, and MNK1) did not increase the inhibitory effect (20%–22%) and concomitant treatment with CGP57380 and SB431542 was not additive. Finally, as shown in Figure 6C, TGF-β treatment induced activation of MNK1 upstream kinases p38 and ERK1/2, and increased MNK1 phosphorylation, thus supporting our model for MNK1 and TGF-β/SMAD2 pathways convergence (Fig. 6D).

Discussion

The biological function of eIF4E phosphorylation at Ser209 by MNKs has long been controversial. Some studies have reported that eIF4E phosphorylation enhances translation in general (27), whereas others concluded that it has no effect or even reduces translation (22, 28). Our analysis of polysome profiles from MNK-depleted cells revealed that growth inhibition occurs as a result of reduced translation, arguing for positive regulation of translation via the MNK/eIF4E pathway. Although eIF4E is known to be a general translation factor, it can also preferentially enhance the translation of carcinogenesis-associated mRNAs, including regulators of the cell cycle, apoptosis, angiogenesis and invasion (29–32). Therefore, it is very likely that CGP57380-mediated reduction of translation and G_1 cell-cycle arrest results from inhibition of the selective translation of MNK-regulated, growth-promoting transcripts. Furthermore, analysis of polysomal profiles prepared from MNK1-depleted glioblastoma cells indicated that MNK1 signaling is not required for global translation. As previously reported, mice lacking both Mnk1 and Mnk2 develop normally and eIF4E phosphorylation at Ser209 does not occur, even when MNK upstream kinases are activated (33). These results demonstrate that MNK signaling is not crucial for normal growth; although, MNK activity may be necessary for growth and survival under certain conditions, for example particular stresses or cancer states. Indeed, in the same model, loss of MNK function sensitized mouse fibroblasts to apoptosis induced by serum withdrawal suggesting a function in adaptive responses to stress (34). Similarly, arsenic-induced apoptosis was enhanced in cells with targeted disruption of *MNK* genes (35). Thus, our results together with the previously published data, promote elevated MNK activity in human glioblastoma as an attractive therapeutic target for 2 reasons. First, MNK signaling is not required for normal cell growth or development. Second, its inhibition may neutralize the cellular stress responses that aid cancer cell survival and are triggered by many therapies.

The different degree of translation reduction in CGP57380-treated cells compared with MNK1-specific knockdown (Figs. 3 and 4) may be explained by the fact that CGP57380 targets all human MNKs and inhibits eIF4E phosphorylation almost completely whereas MNK1 knockdown reduces the phosphorylated form of eIF4E by 50%, presumably due to an MNK2 compensatory function. In our study, similar effects were also observed in proliferation assays, where CGP57380 had a more dramatic effect on cell phenotype than a genetic approach using MNK1-specific knockdown. We found MNK1, but not MNK2, to be overexpressed in human gliomas. Although the 2 kinases share eIF4E as a substrate, they exhibit 70% identity in their catalytic domains (36), potentially indicating regulation of different downstream pathways. An MNK1-specific role that is not compensated by MNK2 has already been proposed from the results of experiments in which reintroduction of wild-type Mnk1, but not Mnk2,

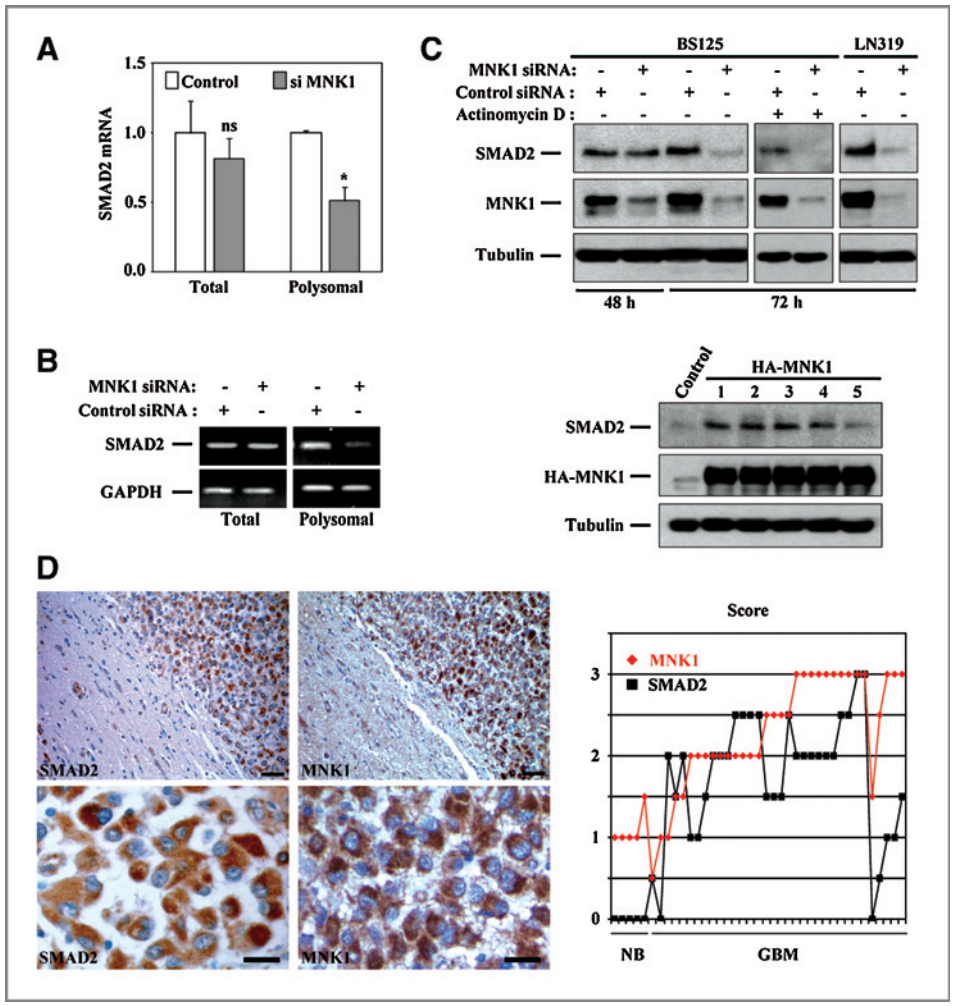

Figure 5. MNK1 regulated SMAD2 translation in human glioblastoma. A, SMAD2 transcript levels obtained by microarray analysis of total and polysomal RNA from MNK1-depleted and control-transfected BS125 cells. Expression of SMAD2 in controls was set to 1. *, $P < 0.05$ or nonsignificant (ns) versus controls. B, microarray data validation by semi–quantitative RT-PCR analysis using SMAD2 and GAPDH (control) specific primers. C, BS125 and LN319 whole cell protein lysates prepared 48 or 72 hours after transfection with duplex siRNA against MNK1 or with a control duplex against luciferase were subjected to Western blot analysis using antibodies specific to total SMAD2, MNK1, and tubulin, as an equal loading control. In addition, 48 hours after transfection, BS125 cells were further incubated with 1 μmol/L of actinomycin D for the next 24 hours and the protein lysates were analyzed as described above. C, bottom, SMAD2 and MNK1 expression analyzed by Western blotting in stable BS125 cell clones overexpressing the full-length HA-MNK1 fusion protein and control cells transfected with an empty vector. D, examples of immunostaining for MNK1 and SMAD2 in GBM tumors. Slides were stained with MNK1- or SMAD2-specific monoclonal antibodies (brown) and counterstained with hematoxylin (blue); bars 50 and 20 μm. D, right, expression of MNK1 and SMAD2 obtained by tissue array analysis in 34 GBM patients and 5 normal brains (NB).

rescued the starvation-induced apoptotic phenotype of mouse embryonic fibroblasts from *mnk1/2* double-knockout animals (34). Furthermore, in the most recent study, MNK1-specific knockdown in U87MG cells was sufficient to reduce tumor formation in mouse xenograft model (37) suggesting that MNK1 plays an essential role in gliomagenesis. Therefore, to investigate the role of MNK1 in regulation of gene expression, we used a genetic approach for specifically knocking down MNK1 expression. Our microarray analysis detected 80 MNK1-dependent mRNAs, with 56 of them using polysomal RNA, indicating that MNK1-dependent gene expression is regulated mainly at the translational level. In a previous study, translation of the antiapoptotic protein Mcl-1 was found to be regulated by eIF4E phosphorylation, thus supporting a role for MNK1 signaling in lymphoma cell survival (14). A more recent study using CGP57380 compound and an array of 263 prostate carcinoma-related genes identified new translationally regulated MNK targets involved in the response to hypoxia-inducible factor (HIF1α) and cell-cycle regulation (cyclin-dependent kinases and inhibitors) in prostate cancer cells (38). Our screen used a genetic approach together with a genome-wide microarray to identify MNK1-specific–regulated genes. The fact that this screen did not identify previously described MNK targets may be due to GBM cell specificity. In addition, by targeting MNK1 (but not MNK2 or eIF4E phosphorylation), we identified an MNK1-specific subset of mRNAs

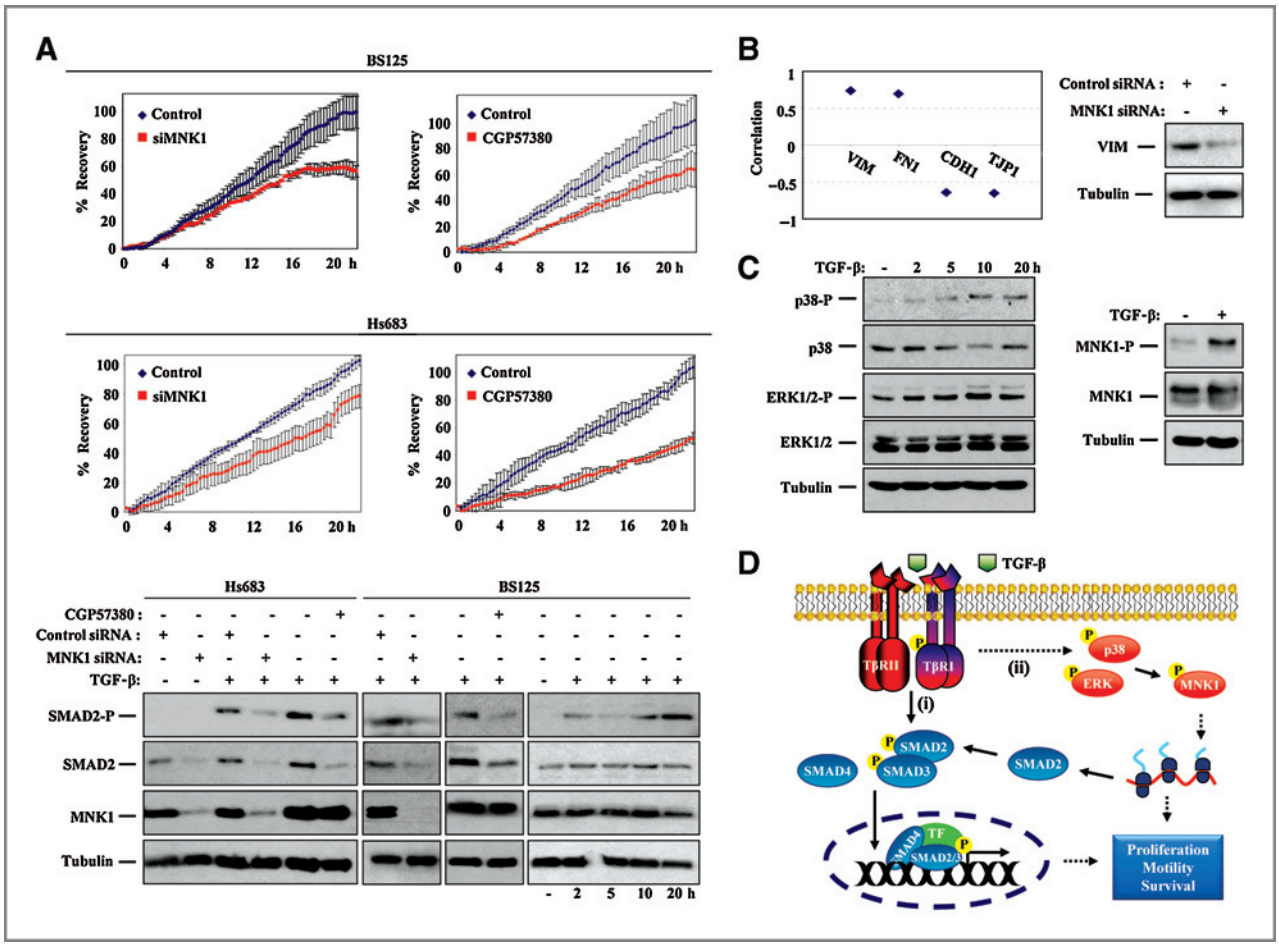

Figure 6. MNK1-regulated, SMAD2-dependent, TGF-β–induced glioma cell motility. A, BS125 and Hs683 cells were transfected with duplex siRNA oligonucleotides against the *MNK1* gene (si) or luciferase (control). A scratch was made 48 hours after transfection or 24 hours after treatment with 10 μmol/L CGP57380 or DMSO (control) and cellular motility was monitored in the presence of 5 nmol/L TGF-β. After 22 hours, the scratch "wound" recovery was set to 100% in control cells. All experiments were assayed in triplicate and results shown as means ± SD. Below, whole protein lysates isolated from transfected and/or treated cells (as described above) were subjected to Western blot analysis using p-SMAD2 (Ser245/250/255), SMAD2, MNK1, and tubulin antibodies. B, correlation coefficients of the comparison of MNK1 and EMT markers in 15 GBMs and 2 normal brain samples. VIM, vimentin; FN1, fibronectin; CDH1, E-cadherin; and TJP1, tight junction protein 1. Right, MNK1-dependent expression of vimentin analyzed by Western blotting in MNK1-depleted BS125 cells after duplex siRNA transfection and TGF-β treatment as described above. C, expression of p-p38 (Thr180/Tyr182), p38, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-MNK1 (Thr197/202), and MNK1 was analyzed by Western blotting in BS125 cells treated with 5 nmol/L TGF-β at indicated time points or for 24 hours. D, model for MNK1 and TGF-β pathway convergence. Overexpressed and activated MNK1 kinase increases SMAD2 translation thereby contributing to signaling of canonical (i) TGF-β pathways via SMAD2/3/4 complexes that interact with transcription factors (TF) and induce expression of genes involved in proliferation, motility, and survival of malignant GBM cells. Hyperactivated TGF-β receptors (TβRI and TβRII) can also activate noncanonical pathways (ii) leading to phosphorylation of ERKs and p38 kinases and activation of MNK1 that can further increase translation of specific mRNAs involved in cancer progression.

that may be different to MNK2- or phospho-EIF4E–regulated target gene groups.

Genetic or pharmacologic targeting of MNK1 reduced total and phosphorylated SMAD2 levels as well as TGF-β–associated cell motility of glioma cells indicating that MNK1 regulates not only one of the main transducers of TGF-β signals but also an TGF-β/SMAD2–dependent phenotype. The correlation between EMT markers and MNK1 expression in GBM patients, and the reduced vimentin expression in MNK1-depleted GBM cells, further support MNK1 regulation of TGF-β–induced gene expression. In addition, the correlation found between MNK1 and SMAD2 protein expression in human GBMs further supports our model in GBM patients. SMAD2 is one of the major signal transducers during TGF-β pathway activation. Previous studies showed that high TGF-β/SMAD activity in human glioma patients correlated with poor prognosis and was dependent on platelet-derived growth factor (PDGFB) expression (39) and a more recent report demonstrates that TGF-β can induce the self-renewal capacity of glioma-initiating cells in a SMAD-dependent manner (40). In our study, MNK1 expression correlated with PDGFB in primary GBM patients (correlation coefficient = 0.56, data not shown) and targeting MNKs reduced GBM-derived spheres growth

suggesting that MNK1 signaling supports TGF-β activity in malignant gliomas.

In invasive cancers, TGF-β signaling has a tumor-promoting effect supporting cell motility, invasion, angiogenesis, immunosuppression, and EMT (41). Interestingly, noncanonical TGF-β signaling pathways can activate ERKs and p38 kinases (42) that phosphorylate and activate MNKs. In agreement with previous study, TGF-β induced ERK and p38 activity as well as MNK1 phosphorylation in GBM cells. Therefore, our data together with previous observations propose a model whereby activation of MNK1 and TGF-β pathways and their mutual regulation support GBM progression (Fig. 6D).

A balance between MNK activity and mTOR pathways was reported in prostate and lung cancer cells, where downregulation of one pathway was correlated with the activation of another, resulting in a defined level of translation that supported cancer cell survival (38, 43). In agreement with a recent study, simultaneous blocking of the MNK and mTOR pathways significantly blocked GBM cell proliferation, colony formation, and tumor sphere growth. We observed a marked additive effect on translation inhibition and G_1 cell-cycle arrest compared with the single treatment, thus also indicating a therapeutic potential of targeting MNK and mTOR pathways against certain tumor entities including malignant gliomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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