Moesin is a glioma progression marker that induces proliferation and Wnt/β-catenin pathway activation via interaction with CD44

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ABSTRACT

Moesin is an ERM family protein that connects the actin cytoskeleton to transmembrane receptors. With the identification of the ERM family protein NF2 as a tumor suppressor in glioblastoma, we investigated roles for other ERM proteins in this malignancy. Here we report that overexpression of moesin occurs generally in high-grade glioblastoma in a pattern correlated with the stem cell marker CD44. Unlike NF2, moesin acts as an oncogene by increasing cell proliferation and stem cell neurosphere formation, with its ectopic overexpression sufficient to shorten survival in an orthotopic mouse model of glioblastoma. Moesin was the major ERM member activated by phosphorylation in glioblastoma cells, where it interacted and co-localized with CD44 in membrane protrusions. Increasing the levels of moesin competitively displaced NF2 from CD44, increasing CD44 expression in a positive feedback loop driven by the Wnt/β-catenin signaling pathway. Therapeutic targeting of the moesin-CD44 interaction with the small molecule inhibitor 7-cyanoquinocarcinol (DX-52-1) or with a CD44-mimetic peptide specifically reduced the proliferation of glioblastoma cells overexpressing moesin where the Wnt/β-catenin pathway was activated. Our findings establish moesin and CD44 as progression markers and drugable targets in glioblastoma, relating their oncogenic effects to activation of the Wnt/β-catenin pathway.
INTRODUCTION

Gliomas, or tumors with features of glial differentiation, account for more than 50% of all brain tumors (1). Among these, glioblastoma (WHO grade IV) is the most aggressive and commonly occurring primary intracranial tumor in adults, with an average incidence of 4-5 per 100,000 persons per year. Glioblastoma either arises de novo or progresses over time from lower grade tumors. The median survival is 12 to 15 months, with survival rates of 20% and 5% at 2 and 5 years, respectively (2). The conventional treatment consists of surgical resection followed by radiotherapy and adjuvant chemotherapy with temozolomide, an oral alkylating agent (3). Some glioblastoma tumor cells express an enzyme called O-6-methylguanine-DNA methyltransferase (MGMT) that repairs the type of DNA damage induced by temozolomide, and tumors in which MGMT levels are elevated show a poor response to this chemotherapy (4). The relatively high incidence of glioblastoma, its resistance to treatment and the dismal patient prognosis rendered this tumor a forefront for the drug discovery efforts.

Ezrin, radixin, moesin (ERM) and neurofibromatosis type 2 (NF2) proteins form a distinct subfamily of cytoskeletal members, belonging to the larger family of Band 4.1 proteins (5). The ERM-NF2 proteins share a conserved structure, consisting in an amino (N)-terminal FERM domain, an extended $\alpha$-helical intermediate region and an actin-binding carboxyl (C)-terminal region, the latter being modified in NF2. The amino acid identity between ezrin and radixin, moesin, or NF2 is 86%, 86% and 62%, respectively, in the FERM domain, and 63%, 60% and 25%, respectively, in the rest of the molecule. It is apparent that the homology within the FERM domain is higher than in the other regions, and indeed the ERM-NF2 proteins share common ligands that bind to two defined pockets in the FERM domain (6). One pocket accommodates the juxtamembrane polybasic motif of some transmembrane receptors, such as CD44 and NEP/CD10, or a different consensus motif in ICAM2 and CD43 (7-10). A non-overlapping neighbouring pocket binds to the Na/H exchanger 3
regulatory factors (NHERF) 1 and 2 adaptor proteins (6). The FERM domain pockets are also the binding site for the C-terminal self-association region of the ERM proteins (11), and this head-to-tail interaction can be either intramolecular or intermolecular, resulting in “closed” monomers or in dimers, respectively. In the monomeric “closed” conformation, the binding sites for FERM ligands, as well as for actin, are masked, and the inactive ERM proteins are most likely localized in the cytoplasm (5, 12, 13). At least two events have been reported to induce a molecular opening, phosphorylation of a conserved C-terminal Thr residue (Thr567/564/558 for ezrin/radixin/moesin, respectively) (14), and binding of a phosphatidyl inositol-4,5-bisphosphate (PIP2) molecule in yet another area of the FERM domain (15, 16). In open conformation, the ERM proteins are able to function by connecting plasma membrane proteins to the actin cytoskeleton (7).

The physiological roles of ERM proteins are related to the structuring of the cell cortex, being involved in cell differentiation and morphogenesis (7). In cancer, except for NF2, which has been incontestably established as a tumor suppressor (17, 18), the role of the other ERM proteins is not entirely clear. We show here that moesin is distinctively upregulated in glioblastoma and acts as an oncoprotein in this malignancy. The dissection of moesin’s pro-oncogenic mechanism of action identified CD44, a hyaluronan transmembrane receptor and stem cell marker, as a transducer of signals upstream the Wnt/β-catenin pathway. Importantly, we show that this oncogenic pathway can be drugged by specifically targeting the interaction between moesin and CD44.
MATERIALS AND METHODS

**Human Tissue Specimens.** Frozen glioma specimens classified as based on World Health Organization (WHO) criteria were previously described (19). The paraffin-embedded human normal brain, grade II (oligodendroglioma, low grade astrocytoma, mixed oligoastrocytoma), grade III (anaplastic astrocytoma, anaplastic oligodendroglioma), and grade IV (glioblastoma) glioma tissue microarray (TMA) was also described (20, 21).

**Immunohistochemistry and immunofluorescence.** Immunohistochemistry and confocal immunofluorescence were performed as described (20, 21) (see also Supplemental Material).

**Plasmids and small hairpin (sh)RNAs.** Human moesin cDNA was obtained from Dr. Vijaya Ramesh (Addgene Inc., Cambridge, MA) and the constructs used are described in Supplemental Material.

**Cell lines, glioblastoma stem cells (GSC), retroviral infections, proliferation and neurosphere formation assays.** The glioblastoma cell lines LN229, LN18, LN308, LN373, LN428, U87-MG, D54, A172, MO59J and U251-MG were previously described (20). The normal human astrocyte (NHA) cell line was obtained from Dr. TJ Liu, MD Anderson Cancer Center. Patient-derived GSC2, GSC11 and GSC23 were kindly provided by Drs. Howard Colman and Fred Lang, MD Anderson Cancer Center, and propagated as neurospheres, as described (22, 23). All the cell lines were tested and authenticated (20). Transfections and retroviral infections were described (24). The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay was performed as described (20). The GSC neurosphere formation assays are detailed in Supplemental Material.

**Moesin-CD44 inhibitors.** An inhibitory peptide targeting the moesin interaction site on CD44 (CD44pep) was designed, SRRRCGQKKKLVINSGNGAV, and subsequently custom synthesized with amidated C-terminus (Biosynthesis, Lewisville, TX). The quinocarmycin analog DX-52-1 previously shown to bind ERM proteins and inhibit the interaction of radixin with CD44 (25, 26)
was obtained from the National Cancer Institute, Bethesda, MD. Briefly, \(2 \times 10^3\) cells in 100 \(\mu\)l DMEM containing 5\% FBS were plated in triplicate in 96-well plates, allowed to attach overnight, and subsequently treated with DMSO vehicle (0) and serial concentrations of DX-52-1 or CD44pep. For GSC neurosphere assays, the inhibitors were added at plating (day 0).

**Orthotopic tumorigenicity assay.** The orthotopic tumorigenicity assay was performed in SCID mice by inoculating \(10^6\) LN229 cells as described (19).

**Protein analysis and antibodies.** Cell lysis, immunoprecipitation, immunoblotting and cellular fractionation were previously described (21). Antibodies are listed in Supplemental Material.

**Luciferase assay.** TOP/FOP \(\beta\)-catenin luciferase reporter assays were performed as described (27).

**RNA extraction and quantitative real time PCR microarray analysis.** The transcription target microarray protocol is provided in Supplemental Material.

**Statistical analysis.** The statistic analysis is detailed in Supplemental Material.
RESULTS

Moesin is progressively increased in gliomas and behaves as an oncoprotein.

In order to analyze the expression of ERM-NF2 proteins in tumors of increasing malignant phenotype, we initially examined 17 glioma samples by Western blot (Fig.1A). Distinctively, moesin showed a marked expression increase in glioblastoma compared to lower grade gliomas (Fig.1A). NF2 had an opposite trend, confirming our previous observations (21), whereas ezrin and radixin did not show significant differences (Fig.1B). Exploration of the same samples for putative ERM ligands revealed increased CD44 expression and decreased NHERF1 expression in glioblastoma (Fig.1B). Importantly, the CD44 and moesin expression patterns exhibited strong direct correlation (Fig.1B, lower graph). The expression of the epidermal growth factor receptor (EGFR) and EGFRv3 constitutive active splice variant in a subset of the glioblastoma samples (Fig.1B, red arrow) confirmed the high grade of the tumors, but did not correlate with moesin expression. Subsequent immunohistochemistry analysis of a glioma TMA containing normal brain and grades II-IV gliomas, confirmed the gradual increase of moesin staining in gliomas of increasing severity (Fig.1C). Additional expression analysis of a panel of 10 glioblastoma cell lines in comparison to a normal human astrocyte (NHA) cell line showed increased moesin levels in all glioblastoma lines and higher CD44 levels in the majority (Fig.1D). These combined results indicate moesin and CD44 as glioma progression markers, with highly correlated expression levels.

To define the role of moesin as oncoprotein in glioma, we examined the effect of moesin overexpression on tumor cell growth. Stable overexpression of Myc-tagged moesin in NHA and LN229 cells that express lower levels of endogenous moesin than other glioblastoma cells increased cell proliferation relatively to vector control cells (Fig.2A). To compare moesin’s growth-promoting effects to those of another ERM protein, we established stable Myc-tagged ezrin overexpressing cells (Fig.2A). The proliferation of these cells was intermediate between that of moesin-expressing
cells and control cells, suggesting a stronger oncogenic effect of moesin. Alternatively, stable depletion of moesin by two different shRNAs in LN229 and NHA cells did not significantly alter cell proliferation (Fig.2B), suggesting possible compensation by endogenous ezrin.

As established cell lines are less representative of human gliomas than primary tumor cells propagated as neurospheres that retain stem-cell characteristics such as self-renewal (28, 29), we examined the effect of moesin on two patient-derived primary GSC neurosphere lines (Fig.2C). Moesin overexpression significantly increased neurosphere size in both GSC2 and GSC23 and also the neurosphere self-renewal ability in GSC2. As a global measure of GSC growth, the differences in the total number of cells were quantified in an MTT assay (Fig.S1). As for cell lines, partial knockdown of moesin in GSC11, which express higher levels of endogenous moesin than other GSCs, did not significantly alter their proliferation (Fig.S2). Collectively, these results showed that moesin has marked growth-promoting consequences on GSCs.

To confirm the proliferative effect of moesin overexpression in vivo, we used our previously described brain tumor model (19). In this orthotopic model, the moesin-everexpressing LN229 cells were highly proliferative and induced rapidly developing tumors that significantly shortened the animal survival (Fig.2D). These results relate moesin overexpression to a fast-proliferating glioma phenotype, characteristic for glioblastoma.

Moesin is constitutively phosphorylated in glioblastoma cells.

To investigate the mechanism underlying the specific effect of moesin on glioblastoma growth, we first analyzed the activation status and subcellular localization of ERM proteins. As mentioned, ERM proteins reside in inactive closed conformation in the cytosolic compartment, and following activation through phosphorylation and PIP₂ binding, interact with their ligands at the plasma membrane in an active open conformation (Fig.3A) (5, 7). To examine ERM
phosphorylation and localization, we developed shRNAs for all ERM proteins (Fig. S3A). The phosphorylation of the Thr567/564/558 site was examined in cytosolic and membrane fractions from LN229 and U251 glioblastoma cells depleted of individual ERM proteins (Fig.3B and S3B). The phospho-ERM antibody detected a band in the membrane fractions of LN229 cells that disappeared following moesin knockdown (Fig.3B), indicating that moesin is the ERM protein phosphorylated in these cells. Similarly, in U251, a stronger faster band disappeared following moesin knockdown, and a lighter upper band disappeared following ezrin knockdown (Fig.S3B). This suggested that both moesin and ezrin are phosphorylated in these cells, moesin being the main phosphorylated species. Radixin was not phosphorylated in either cell line. We confirmed these results by cellular fractionation (not shown) and by immunofluorescence with phospho-ERM antibody of ERM-depleted cells with a second set of shRNAs (Fig.S4), and observed that phosphorylated moesin is localized in microspike-like plasma membrane protrusions (Figs.S4-S5).

We also investigated the ERM protein phosphorylation in cells with Myc-moesin overexpression, and noted that it increased, and corresponded to the membrane fraction of overexpressed moesin (Fig.3C). Overall, these results indicate that moesin is the ERM protein preferentially phosphorylated in glioblastoma cells.

To appreciate the importance of ERM phosphorylation in glioma, the glioma TMA was examined by immunofluorescence with phospho-ERM antibody (Fig.3D). Strong positivity was observed in close to 50% of glioblastoma samples, in contrast with mild positivity in 10-15% of lower grade gliomas. Both atypical glial and microvascular endothelial cells were P-ERM positive in glioblastoma (Fig.3D-inset). These results revealed high levels of ERM phosphorylation, most likely of moesin, specifically present in glioblastoma samples, indicating an activated ERM state in tumors.
Moesin enhances the expression of CD44 and competes with NF2 for CD44 binding.

In phosphorylated active conformation, ERM proteins interact with their ligands (Fig.3A). Since moesin levels paralleled CD44 levels in glioma samples (Fig.1A), we examined the effect of moesin overexpression on CD44 expression. In LN229 cells, endogenous and overexpressed moesin co-localized with CD44 at plasma membrane microspike protrusions (Fig.4A). At higher magnification, the co-localization was concentrated at the base of the microspikes and in discrete areas along these structures (Fig.S6). Overexpressed moesin also enhanced CD44 expression in these cells (Fig.4A). To quantify the latter effect, a time course mitogenic stimulation of LN229 cells with and without moesin overexpression showed marked increase of CD44 expression levels in moesin-overexpressing cells (Fig.4B). As observed, in the absence of serum, CD44 expression was undetectable in LN229 cells (Fig.4B, upper panel). Similarly, in GSCs growing in serum-free conditions, CD44 expression was undetectable, in contrast to that of four other neural stem cell markers investigated (Figs.4C and S7). Moesin overexpression considerably raised CD44 expression in both LN229 cells and GSCs (Fig.4B-C) and also Sox2 expression in GSCs (Fig.4C), consistent with a shift towards a more undifferentiated and malignant phenotype.

An important aspect of the interaction between CD44 and ERM-NF2 proteins is that whereas ezrin have been reported to enhance CD44 growth signaling (30), NF2 has been shown to repress it (31). As in approximately 1/3 of glioblastoma tumors, NF2 expression is maintained at normal high levels (21), we examined the possibility of a competition between moesin, ezrin and NF2 for CD44 occupancy (Fig.4D). Immunoprecipitation of CD44 from lysates of vector, Myc-moesin and Myc-ezrin cells, as well as from vector and moesin knockdown cells, showed that moesin overexpression displaced NF2 and ezrin from CD44, whereas moesin depletion had an opposite effect (Fig.4D, graphs). Ezrin overexpression displaced moesin and, to a lesser extent NF2, from
CD44. These results suggested that moesin overexpression has a double impact on CD44 signaling, first, by enhancing its expression, and second, by decreasing its occupancy for NF2.

**The moesin-CD44 interaction constitutes a potential therapeutic target in glioblastoma.**

To examine whether the oncogenic effect of moesin is mediated through the interaction with CD44, we treated cells overexpressing moesin with a CD44-derived peptide, CD44pep, known to strongly interact in vitro with the FERM domain of ERM proteins (9, 32). At a concentration of 0.5 μM, CD44pep had no effect on the proliferation of control or Myc-moesin-overexpressing cells (Fig.5A, left graph). Remarkably, at 10 μM, CD44pep specifically suppressed the proliferation of Myc-moesin-overexpressing cells to levels slightly under those of control cells (Fig.5A, right graph). This result indicated that the effects of moesin on proliferation are mediated through the interaction with CD44, and that this interaction can be specifically targeted for inhibition.

The DX-52-1 drug has been developed for melanoma treatment in the 1990s (33-35). Fenteany’s group has shown that DX-52-1 binds tightly to radixin and less to ezrin and moesin (25), and is able to disrupt the interaction between radixin and CD44 (26). Based on these observations, we treated vector-control and moesin-overexpressing cells with serial concentration of DX-52-1 (Fig. 5B). A sharp and specific decline of moesin-overexpressing cell proliferation to control levels was apparent at 100 nM. Higher concentrations stopped proliferation in both control and moesin-overexpressing cells.

To facilitate a more rapid translation into the clinic, these results were confirmed in GSCs (Fig.5C-E). Both inhibitors significantly reduced the cell growth of moesin-overexpressing neurospheres in 3-day-proliferation assays (Fig.5C-E, left graphs). Interestingly, the growth of vector-control GSCs, which do not express CD44, was unchanged, supporting inhibition specificity for the moesin-CD44 interaction. In 5-day-neurosphere-formation assays, the size of moesin-
overexpressing neurospheres was reduced by both inhibitors to levels similar to vector neurospheres (Fig.5C-E, right graphs). The neurosphere count was not significantly changed (not shown), suggesting that moesin might increase self-renewal independently of CD44. Mechanistically, treatment with CD44pep decreased the levels of moesin, CD44 and Sox2 (Fig.5D), implicating the stability of the moesin-CD44 complex as key regulator of GSC growth. Collectively, these experiments clearly showed that the oncogenic effects of moesin overexpression in glioblastoma, most likely through the interaction with CD44, could be targeted with therapeutic drugs.

Large-scale analysis identifies the Wnt/β-catenin pathway as specific signaling downstream of the moesin-CD44 interaction.

Signaling through CD44 has been previously investigated and downstream ERK and PI3K-Akt pathway activation or Hippo cascade inhibition were described (36-40). Moesin overexpression in glioblastoma and NHA cells activated the PI3K-Akt, ERK and p38 MAPK pathways (Fig.6A). To gain a better insight on the signaling triggered by moesin in glioblastoma, we interrogated a transcription-target-microarray containing direct targets of transcription factors downstream of signaling pathways involved in tumor growth (Fig.6B and Table S1). This profiling revealed that: (1) growth and metastasis-promoting factors were upregulated by moesin, whereas the inhibitors of growth were generally not modified, and (2) Wnt/β-catenin, a major growth pathway not previously implicated downstream of CD44 signaling, was activated by moesin. The profiled PI3K-Akt, ERK, JNK, NF-κB or Hippo pathways were engaged at various extents, as determined by FOXO, AP1, NF-κB and YAP/TAZ target variations, respectively (Fig.6B).

We confirmed the Wnt/β-catenin activation by showing that c-Jun, the highest activated transcription target from the large-scale analysis, was specifically upregulated in moesin-
overexpressing cells but not in ezrin-overexpressing cells (Fig.6C). We next used the luciferase-reporter system with β-catenin-responsive (TOP) or mutated (FOP) elements and observed that β-catenin transcriptional activity was significantly elevated in moesin-overexpressing cells (Fig.6D). Importantly, this activation was abolished when moesin-overexpressing cells were treated with CD44pep (Fig.6D), indicating that the moesin-CD44 interaction specifically triggers the activation of β-catenin transcriptional activity. We further examined the phosphorylation and intracellular localization of β-catenin. β-catenin phosphorylation on residue Ser552 by Akt has been shown to induce β-catenin nuclear translocation and transcriptional activation (41, 42). This residue was phosphorylated by moesin more than by ezrin overexpression in LN229 cells (Fig.6E). The fractionation and immunofluorescence analysis of moesin-overexpressing cells showed that a fraction of β-catenin translocated from the membrane to the nuclear compartment in these cells (Fig.6F). Overall, this comprehensive analysis showed that moesin, through binding to CD44, induces β-catenin nuclear translocation and increased transcriptional activity, which can be inhibited by specifically targeting the moesin-CD44 interaction.
DISCUSSION

Glioblastoma is one of the relatively frequent cancers with very poor prognosis due to its invasiveness, tumor heterogeneity and resistance to chemotherapy. Efforts are focused on characterizing the molecular signature of these tumors in order to diagnose them and define new targets amenable to therapy. Our previous studies have defined a network of interacting tumor suppressors specifically disrupted in glioblastoma that include NF2 and NHERF1 (19-21). Continuing this analysis with a comprehensive examination of the other three ERM-NF2 members, we found that the expression levels of moesin, but not of ezrin or radixin, are significantly upregulated in glioblastoma. Moesin could thus be used as reliable marker of grade IV glioblastoma, and we pursue an extensive marker validation analysis for diagnostic purposes.

The ERM proteins have been implicated in the motility of cells and different aspects of metastasis (7, 43). We show here that moesin overexpression increases glioma cell growth, and this effect is translated in vivo by a significantly decreased animal survival due to massive tumor cell proliferation. Interestingly, moesin depletion did not consistently decrease proliferation, suggesting that moesin is an oncogenic protein when overexpressed, similarly to EGFR growth factor receptors, for example. A compensatory effect of ezrin in moesin-depleted cells and GSCs could also explain the lack of phenotype change (see also below). Analyzing the EGFR expression that is upregulated in roughly 40% of glioblastoma samples (44), we did not find a correlation with moesin levels that were consistently elevated in the majority of cases. In contrast, CD44, a transmembrane receptor that interacts directly with ERM-NF2 proteins, showed similar expression trend as moesin. This led us to hypothesize that the proliferative signals of moesin are dependent of its interaction with CD44. The findings of specific phosphorylation of moesin in glioblastoma on the conserved Thr558 residue
that dictates a permissive conformational state for interactions between the FERM domain and CD44 supported this model (Fig. 7).

CD44 is a hyaluronic acid receptor that has an intracellular tail with non-overlapping binding sites for ERM-NF2 and ankyrin (38). Because all ERM-NF2 members bind to the same site but have opposite effects on CD44 signal transduction, it has been hypothesized that they may compete for CD44 binding depending on the cell proliferative status which would determine their activation by phosphorylation (38). We brought evidence that moesin, ezrin and NF2 compete for CD44 binding in glioblastoma, suggesting that not only the cell proliferative status is important for selective CD44 occupancy by ERM-NF2 members but also the expression levels of the various ERM-NF2 proteins. Accordingly, NF2 acts as a natural inhibitor of CD44 signaling, with effects proportional to its expression levels. In glioblastoma, its levels are undetectable or decreased in two-thirds of cases (21, 45), favoring thus an enhanced signaling through complexes between CD44 and other ERM members. Interestingly, moesin is overexpressed in almost all glioblastoma cases, suggesting that its effect is not limited only to displacing the traces of NF2 from CD44. Indeed, it appears that moesin is required for sustaining a CD44 positive feedback loop, in which the signaling through CD44 augments CD44’s own expression. ERK activation has been previously shown to enhance CD44 transcription (46), and we have shown that serum stimulation of LN229 cells increases CD44 levels. However, moesin overexpression dramatically upregulates CD44, with consecutive activation of CD44-dependent downstream pathways, including ERK, indicating that moesin is a limiting factor for signaling through CD44. The question of why moesin, and not ezrin or radixin, is the preferred ERM protein upregulated in glioblastoma could open several avenues for exploration. The cell-type specific regulation of transcription in glioblastoma cells could be an explanation for increased moesin expression (see below). However, the interesting observation that moesin is the major ERM protein phosphorylated and thus activated in glioblastoma opens the possibility of a broader
signaling network in which the kinase responsible for ERM phosphorylation has more specificity for moesin than for ezrin. Taken together, these observations suggest a model in which moesin is preferentially transcribed and phosphorylated in glioblastoma, with major activating consequences on CD44 signaling (Fig.7).

CD44 does not have a catalytic activity and the mechanisms by which it signals relate to its interactions with receptor tyrosine kinases (RTKs), such as Met or the EGFR family (38). Although several pathways have been described downstream CD44 co-receptor function (38), we profiled a transcription factor target RT-PCR microarray that included outputs of known pathways but also other pathways involved in proliferation but not previously described for CD44 signaling. Surprisingly, the Wnt/β-catenin pathway was prominently and specifically activated downstream of the moesin-CD44 interaction. A possible mechanism of activation of this pathway by CD44 could transit through the activation of the PI3K by CD44-associated RTKs, with subsequent phosphorylation of β-catenin by Akt on residue Ser552, which has been shown to direct β-catenin to the nucleus (41, 42). In moesin overexpressing cells, all these modifications were present, strongly supporting this mechanism (Fig.7). Other moesin-independent but converging influences on the activation of Wnt-β-catenin pathway could come from the loss of NHERF1, which has been shown to destabilize β-catenin from the membrane and facilitate its nuclear translocation (27).

An extensive literature on glioma transcriptomes from public microarray databases is available (47-51). To examine whether moesin upregulation in glioblastoma is due to increased gene transcription, we reviewed these studies and found that moesin is on the short list of genes with mRNA levels consistently elevated in glioblastoma versus anaplastic astrocytoma (48, 50). Moreover, moesin transcript is part of the signature that differentiates glioblastoma-rich from oligodendroglia-rich groups (49). mRNA glioblastoma subgroups have been also described, including the proneural subtype with better prognosis and the mesenchymal subtype with increased
aggressiveness (47, 51). Although moesin transcript was not part of the mesenchymal subtype, it is present on the list of 884 genes in TCGA Worst Prognosis Signature from the glioblastoma TCGA dataset (47), suggesting that it is highly enriched in the poor-prognosis subsets of all glioblastoma subtypes. Similarly, CD44 expression, even if not present in the latter analysis, has been associated with poorer prognosis in a subset of glioblastoma (52, 53). While deemed a stem cell marker, CD44 is not consistently expressed in GSC neurospheres (22, 53). We hint that serum deprivation might underlie this. Importantly, moesin boosts CD44 expression in serum-deprived GSCs and also increases the expression of the neural stem cell marker Sox2, supporting a functional shift towards an aggressive stem-like phenotype in glioblastoma.

With the advent of personalized cancer therapy, the discovery of markers for disease staging, progression and response to therapy is front line. Although glioblastoma is a rapidly fatal disease, some patients benefit from temozolomide, whereas those with MGMT overexpression are poor responders (4). Since we found moesin as a glioma progression marker in conjunction with CD44, we also tested if it could constitute a therapeutic target. Although CD44 has been previously investigated as therapeutic target in various malignancies (54), the options considered for its inhibition were either eliminating its expression by miRNAs or interfering with the binding of ligands to its extracellular domain by a variety of blocking antibodies and peptides (55, 56). In this study, we show for the first time a new modality of efficient CD44 inhibition by disrupting its interaction with moesin (Fig.7). In principle, NF2 is the natural inhibitor of this interaction, and we have previously shown that NF2 overexpression suppresses the proliferation of glioblastoma cells (21). Replacing NF2 with a specific CD44 peptide induced a potent inhibition of cell proliferation, comparable to that achieved by NF2 overexpression (data not shown). Importantly, the DX-52-1 quinocarmycin analog that was shown to disrupt the binding between radixin and CD44 (26), had a similar effect on cell growth in moesin-overexpressing cells, including GSCs, providing a proof of
principle that a drug can accomplish the same effect as the specific peptide. DX-52-1 has been previously used in a clinical trial that was interrupted because of high toxicity (33). However, our data prompt to the design and/or high-throughput detection of new and less toxic compounds targeting the CD44-moesin interaction. These drugs could not only be beneficial in glioblastoma, but also in tumors with ezrin overexpression, such as osteosarcoma or rhabdomyosarcoma, or in tumors with NF2-inactivating mutations, such as meningiomas, schwannomas and mesotheliomas.

In conclusion, we described here moesin as a marker for glioma progression that induces oncogenic growth through a mechanism involving the interaction with CD44 and subsequent activation of a host of downstream signaling cascades, including the Wnt/β-catenin pathway. We described for the first time a new therapy target consisting of the moesin-CD44 interface, whose disruption represses both the proliferation and β-catenin-dependent signaling. This study adds moesin and CD44 on the map of relevant molecular changes in glioblastoma and provides a drugable target for glioblastoma therapy.
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FIGURE LEGENDS

**Fig1.** Moesin is a progression marker in glioma. **A-B.** Immunoblot with indicated antibodies of tissue extracts (8-10μg protein) from 10 low-grade glioma and 7 glioblastoma cases. The mean patient survival is indicated. LN229 with moesin knockdown (shMoe) or without (shV), and D54 glioblastoma cell lines were included as controls. The red arrow indicates EGFRv3. Actin-normalized ERM-NF2 and CD44 levels are shown in the upper graph as individual brain tumor specimen values distributed around the mean. The lower table indicates high correlation between moesin, CD44 and NF2 levels across the 17 samples. **C.** Immunohistochemistry (x200) with moesin antibody of glioma TMA. The diagram shows the number of the specimens and the intensity of moesin staining (means±SD) per glioma category. **C.** Western blot of cell extracts (20μg protein) from NHA and 10 human glioblastoma cell lines showing moesin and CD44 expression. The graph shows actin-normalized moesin and CD44 levels.

**Fig2.** Moesin increases cell proliferation and tumorigenicity. **A-B.** NHA and LN229 cells with stable expression of Myc-Moesin (MycMoe) and Myc-Ezrin (MycEz) (A) or stable moesin knockdown by two shRNAs (sh4 and sh7) (B) were subjected to MTT proliferation assay for the indicated time periods. Data are means±SD (n = 3). **C.** Establishment of Myc-Moesin-expressing GSC neurosphere lines (images at 100x). Neurosphere size and numbers were recorded. Data are means±SEM from 20 microscope-10x-fields. **D.** Control and Myc-Moesin-overexpressing LN229 cells were injected intra-cranially in 6-week-old SCID female mice. Mice were sacrificed at the indicated periods. Arrows highlight the brain tumors stained by hematoxilin and eosin (H&E) (images x20). Ki-67 staining of tumors from dying mice is shown (images x200). The Kaplan-Meier curve and the median survival of mice (n = 5) are shown.

**Fig3.** Moesin is constitutively phosphorylated in glioblastoma. **A.** Simplified view of the activation of ERM proteins, featuring the domains and the two events that lead to the “open” active
conformation. **B.** Purification of cytoplasmic (C) and membrane (M) fractions from control (shV), ezrin (sh8Ez), radixin (sh8Rad) or moesin (sh3Moe) knockdown LN229 cells shows the phospho-Thr567/564/558-ERM (P-ERM) species corresponding to moesin in the membrane compartment. Arrows indicate ERM proteins. Ns, non-specific band. ERK and N-cadherin were used as cytoplasmic- and membrane-fraction markers, respectively. **C.** Immunofluorescence (x400) with P-ERM and moesin antibodies of control and Myc-Moesin overexpressing LN229 cells shows signal overlap in membrane protrusions. **D.** Confocal immunofluorescence (x400) of the glioma TMA with P-ERM antibody and ToPro-3 nuclear staining, with quantification of P-ERM-positive specimens per glioma type. The inset shows P-ERM staining of endothelial cell membrane (arrowhead) and erythrocytes (green) inside the lumen.

**Fig4.** Moesin enhances CD44 expression and competes with NF2 for CD44 binding. **A.** Immunofluorescence (x400) of control and Myc-Moesin-overexpressing LN229 cells with moesin and CD44 antibodies. **B.** Time-course stimulation of serum-deprived vector and Myc-Moesin-overexpressing LN229 cells with 10% FBS for the indicated periods. Duplicate samples are shown. Random indicates unsynchronized growing cells in 10% FBS. Actin-normalized CD44 levels are plotted as means±SD (n=2). **C.** CD44 and Sox2 expression are upregulated in Myc-Moesin-overexpressing GSCs. Actin-normalized Sox2 and Olig2 levels are plotted as means±SD (n=2). **D.** Co-immunoprecipitation of moesin, ezrin and NF2 with CD44 and control IgG antibodies from protein lysates of vector, Myc-Moesin, Myc-Ezrin, shRNA vector (shV) and moesin knockdown (sh7Moe) LN229 cells shows competition between moesin, ezrin and NF2 for CD44 binding. The levels of co-immunoprecipitated moesin, ezrin and NF2 were normalized to immunoprecipitated CD44 levels and expressed as percentage from the levels of the vector-control co-immunoprecipitated proteins that were set to 100%.
**Fig5.** The moesin-CD44 interaction is a potential therapeutic target. **A-B.** Proliferation of vector and Myc-Moesin-expressing LN229 cells treated with two concentrations of CD44pep (A), or with serial concentrations of DX-52-1 (B). Note specific growth suppression of Myc-Moesin-expressing cells at 10 μM CD44pep and 100 nM DX-52-1. **C-E.** Treatment with CD44pep (C-D) and DX-52-1 (E) of control and Myc-Moesin-overexpressing GSC2 neurospheres. The global neurosphere growth is quantified as in Fig.S1. Images and neurosphere size quantification are presented as in Fig.2C.

**Fig6.** Moesin activates Wnt/β-catenin pathway. **A.** Immunoblot of protein extracts with indicated antibodies shows pathway analysis in Myc-Moesin overexpressing cells. **B.** Quantitative SYBER-green-based real-time-PCR array showing changes in transcript expression of indicated signaling pathways target genes in Myc-Moesin-expressing LN1229 cells relatively to vector-control. Gene expression level was normalized to GAPDH expression set to 1. **C.** Immunoblot confirming increased c-Jun expression in Myc-Moesin-expressing cells. **D.** β-catenin TOP/FOP luciferase reporter assay with or without CD44pep treatment of vector and moesin-overexpressing LN229 cells. The luciferase activity was normalized to renilla activity. Data are means±SD (n = 3). **E.** Immunoblot of whole protein extracts shows increased β-catenin phosphorylation on Ser552 in Myc-Moesin-overexpressing cells. **F.** Increased β-catenin nuclear localization in Myc-Moesin cells shown by nuclear (N)/cytoplasm (C)/membrane (M) fractionation (left) and immunofluorescence (right) of LN229 cells. PARP and N-cadherin were used as nuclear and membrane-fraction markers, respectively. The graph shows nuclear PARP-normalized β-catenin levels.

**Fig7.** Model of moesin-CD44 signaling and inhibitory targeting in glioblastoma. It is assumed here that CD44-RTK complexes trigger CD44 signaling. LGG, lower grade gliomas (II-
III). Note a positive feedback loop in which CD44 is the upstream activator and the downstream transcriptional target of both ERK and Wnt/β-catenin pathways.
Figure 1

A

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<tr>
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B

- CD44
- Radixin
- Moesin
- NF2
- Ezrin
- NHERF1
- EGFR
- Actin

![Western blot images of proteins](image)

C

- Normal Brain
- Grade II glioma
- Grade III glioma
- GBM

![Histological images](image)

D

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<th>GBM cell lines</th>
<th>Expression Level (%)</th>
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![Expression level bar graph](image)
Figure 2

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C

GSC2

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GSC23

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D

Day 31st post-injection

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Day 48th post-injection

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Survival (%)

MycMoe 33 days

Vector 48 days

P=0.005
Figure 3

A

ERM inactive

C-term

α-helical

ERM active

PIP2 binding

Phosphorylation

Thr567/564/558

CD44 NHERF1

B

LN229

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</table>

C

Moesin

pERM

Merged

Vector

MycMoe

D

Normal Brain

Grade II (Astrocytoma)

Grade III (Anaplastic Astrocytoma)

Grade IV (Glioblastoma)

% P-ERM+ specimens

P<0.01

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Figure 4

A

Moesin | CD44 | Merge

Vector

MycMoe

15 μm

15 μm

15 μm

B

FBS stimulation (min)

Random 0 15 30 60 120 180

CD44

Actin

CD44

Actin

C

GSC2 GSC23

CD44

Moesin

Sox2

Olig2

Nestin

Actin

D

IP

IgG CD44 IgG CD44

Moesin

Ezrin

NF2

CD44

Actin

Input

Vector MycMoe MycEz shV shV sh7Moe sh7Moe

%ERM bound to CD44

Moesin Ezrin NF2

Vector

MycMoe

MycEz

shV

sh7Moe

Ezrin

NF2
Figure 5

A

Vector
Vector+CD44pep
MycMoe
MycMoe+CD44pep

LN229 proliferation (OD units)

0.5 μM

1 2 3 4 5 Days

10 μM

1 2 3 4 5 Days

B

Vector+DX-52-1
MycMoe+DX-52-1

0 nM

25 nM

50 nM

100 nM

200 nM

400 nM

0 0.2 0.4 0.6 0.8 1 Day 1 2 3 4 5

C

CD44pep (10 μM)

GSC2, MycMoe, Day 5

Neurosphere size (μm)

CD44pep

- - MycMoe

+ + Vect

P<0.0001

D

GSC2, MycMoe

Neurosphere size (μm)

P<0.0001

E

DX-52-1 (100 nM)

GSC2, MycMoe, Day 5

Neurosphere size (μm)

P<0.0001

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Figure 6

A

LN229  
Vector  MycMoe  MycEz  Vector  MycMoe  MycEz
P-Akt  
S473  
Akt  
P-Erk1/2  
Erk1/2  
P-p38  
p38

B

Pathways  
- WNT  
- AP1/NF-κB  
- FOXO/PI3K  
- Hippo  
- House-keeping  
- Others

C

LN229  
Vector  MycMoe  MycEz  Vector  MycMoe  MycEz

c-Jun  
Actin

D

Luciferase activity (units)

Vector  MycMoe

E

P-β-catenin  
(Ser552)  
β-catenin

F

Vector  MycMoe

β-catenin  
Parp  
N-Cad

Merged

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Figure 7
Moesin is a glioma progression marker that induces proliferation and Wnt/β-catenin pathway activation via interaction with CD44

Xiaoping Zhu, Fabiana Morales, Nitin Agarwal, et al.

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