

## MiR-328 promotes glioma cell invasion via SFRP1-dependent Wnt-signaling activation

Sabit Delic, Nadine Lottmann, Anja Stelzl, Franziska Liesenberg, Marietta Wolter, Silke Götze, Marc Zapatka, Yuzuru Shiio, Michael C. Sabel, Jörg Felsberg, Guido Reifenberger, and Markus J. Riemenschneider

Department of Neuropathology, Regensburg University Hospital, Regensburg, Germany (S.D., N.L., A.S., M.J.R.); Department of Neuropathology, Heinrich Heine University, Düsseldorf, Germany (F.L., M.W., J.F., G.R.); Department of Structural Biology, Max Planck Institute of Molecular Physiology, Dortmund, Germany (S.G.); Division of Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany (M.Z.); Greehey Children's Cancer Research Institute, The University of Texas Health Science Center, San Antonio, TX USA (Y.S.); Department of Neurosurgery, Heinrich Heine University, Düsseldorf, Germany (M.C.S.); Wilhelm Sander – NeuroOncology Unit, Regensburg University Hospital, Regensburg, Germany (M.J.R.); German Cancer Consortium (DKTK), partner site Essen/Düsseldorf, German Cancer Research Center (DKFZ), Heidelberg, Germany (G.R.)

**Corresponding author:** Markus J. Riemenschneider, MD, Department of Neuropathology, Regensburg University Hospital, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany (markus.riemenschneider@ukr.de).

**Background.** Diffusely infiltrative growth of human astrocytic gliomas is one of the major obstacles to successful tumor therapy. Thorough insights into the molecules and pathways signaling glioma cell invasion thus appear of major relevance for the development of targeted and individualized therapies. By miRNA expression profiling of microdissected human tumor biopsy specimens we identified miR-328 as one of the main miRNAs upregulated in invading glioma cells in vivo and further investigated its role in glioma pathogenesis.

**Methods.** We employed miRNA mimics and inhibitors to functionally characterize miR-328, 3' untranslated region luciferase assays, and T-cell factor/lymphoid enhancer factor reporter assays to pinpoint miR-328 targets and signaling pathways, and analyzed miR-328 expression in a large panel of gliomas.

**Results.** First, we corroborated the invasion-promoting role of miR-328 in A172 and TP365MG glioma cells. Secreted Frizzled-related protein 1 (SFRP1), an inhibitor of Wnt signaling, was then pinpointed as a direct miR-328 target. *SFRP1* expression is of prognostic relevance in gliomas with reduced expression, being associated with significantly lower overall patient survival in both the Repository of Molecular Brain Neoplasia Data (REMBRANDT) and The Cancer Genome Atlas. Of note, miR-328 regulated both *SFRP1* protein expression levels and Wnt signaling pathway activity. Finally, in human glioma tissues miR-328 appeared to account for the downregulation of *SFRP1* preferentially in lower-grade astrocytic gliomas and was inversely related to *SFRP1* promoter hypermethylation.

**Conclusion.** Taken together, we report on a novel molecular miR-328 – dependent mechanism that via *SFRP1* inhibition and Wnt activation contributes to the infiltrative glioma phenotype at already early stages of glioma progression, with unfavorable prognostic implications for the final outcome of the disease.

**Keywords:** astrocytoma, brain tumor, epigenetic, glioblastoma, miRNA.

Gliomas are the most common primary tumors of the central nervous system. Despite multimodal treatment options, the overall prognosis for most patients is poor, in particular in the case of glioblastoma.<sup>1</sup> Histologically, most gliomas are characterized by a diffuse infiltration of the surrounding nonneoplastic brain tissue, turning the local tumor into a brain-systemic disease.<sup>2</sup> Thus, the basic knowledge of molecules and signaling pathways involved in glioma cell invasion is of particular relevance to the development

of novel targeted and individualized therapies. One major signaling pathway that has been linked to glioma invasion is Wnt (wingless-type mouse mammary tumor virus integration site family). *Wnt5A*, for example, activates Wnt-downstream targets and thereby promotes glioma cell invasion.<sup>3</sup> On the other hand, secreted Frizzled-related protein 1 (SFRP1), an antagonist of Wnt signaling, has been shown to reduce invasiveness of glioma cells in vitro.<sup>4</sup> In gliomas and several other human cancers, silencing

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of negative regulators of Wnt signaling, such as *SFRP1*, by aberrant promoter methylation and loss of function mutations commonly serves to enhance tumor cell invasiveness.<sup>5–8</sup>

While glioma invasion-associated molecular changes at the DNA and RNA levels have been extensively studied,<sup>9</sup> less is known about the contribution of small non-protein-coding RNA molecules, so-called microRNAs (miRNAs), to the invasive phenotype of gliomas. MiRNAs are capable of regulating target gene expression by pairing with complementary sequences in the 3' untranslated region (UTR) of mRNAs and thereby inhibiting translation or leading to enhanced mRNA degradation.<sup>10</sup> One miRNA suspected of having cancer-related functions is miR-328. In breast cancer cells, breast cancer resistance protein/ATP-binding cassette sub-family G member 2, an efflux transporter responsible for cellular drug disposition, was identified as a miR-328 target.<sup>11</sup> In the human epithelial carcinoma cell line A431, miR-328 overexpression resulted in reduced cell adhesion, aggregation, and capillary formation by silencing of CD44.<sup>12</sup> Expression of miR-328 in myeloid progenitors is suppressed by the oncoprotein Bcr/Abl, while this miRNA in bone marrow cells influences the granulocytic maturation and interferes with RNA binding protein activity.<sup>13</sup> In lung adenocarcinomas, miRNA expression profiling revealed elevated miR-328 expression.<sup>14</sup> In non-small cell lung cancer, overexpression of miR-328 resulted in increased cell migration and was associated with brain metastasis.<sup>15</sup> The only study reporting on miR-328 in gliomas was a screening approach based on homogenized glioma tissues, which suggested a decrease in expression upon malignant progression from primary low-grade to recurrent high-grade gliomas.<sup>16</sup>

We performed miRNA expression profiling on a unique set of microdissected glioma tissues,<sup>17</sup> enabling the study of differential miRNA expression in the subpopulation of invading glioma cells. Of note, miR-328 was one of the major miRNAs upregulated in infiltrating tumor cells. We here report on the expression pattern as well as the functional implications of miRNA-328 in human gliomas.

## Materials and Methods

### Patient Samples

Microdissection of infiltrating glioma cells was described in detail elsewhere.<sup>17</sup> MiRNA was extracted either from the infiltration zone (INF) or from central areas (CENs) of tumor samples of 7 malignant glioma patients (5 males, 2 females; median age, 49 y; age range, 16–67 y; 5 glioblastomas, World Health Organization [WHO] grade IV, and 2 anaplastic astrocytomas, WHO grade III). In addition, miR-328 expression data from homogenized glioma tissues were derived from an independent series of 79 glioma patients, comprising 7 diffuse astrocytomas, WHO grade II (AII); 11 anaplastic astrocytomas, WHO grade III (AAIII); 8 secondary glioblastomas, grade IV (sGBIV); and 53 primary glioblastomas, WHO grade IV (pGBIV). Data on *SFRP1* promoter methylation and *SFRP1* mRNA expression were available from a previous study.<sup>5</sup> MiR-328 expression and *SFRP1* promoter methylation were correlated in an overlapping panel of 38 gliomas (5 AII, 9 AAIII, 5 sGBIV, and 19 pGBIV).

### MiRNA Expression Analyses

The Micro Fluidic Card System (Life Technologies) was used according to the manufacturer's protocol for miRNA expression profiling. MiRNA expression was calculated by StatMiner software (Integromics). Technical validation

of miR-328 expression was performed using TaqMan quantitative PCR (Life Technologies). After calculating the relative expression, miRNA expression in the microdissected glioma samples as well as in the independent series of 79 gliomas was normalized using the 4 miRNAs showing the most stable expression over all analyzed samples,<sup>18</sup> namely miR-30a-5p, miR-30b, miR-30c, and miR-30d. Seven nonneoplastic control RNA samples were obtained from commercial sources (#540005 from Stratagene; #636567 and #636561 from Clontech; #R1234051-50, #R1234035-50, #R1234062-50, and #R1234078-50 from Biochain).

### Cell Culture and MiRNA Transfection of Glioma Cells

The glioma cell lines A172 and T98G were obtained from American Type Culture Collection. The cell line TP365MG was kindly provided by Prof V. Peter Collins (Cambridge, UK). Cells were grown as monolayer cultures and maintained under standard conditions (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin/streptomycin). Cells were transfected with siPORT NeoFX transfection agent (Life Technologies) following the manufacturer's reverse transfection protocol. Anti-miRNAs (hsa-miR-328 Anti-miR miRNA Inhibitor and Anti-miR miRNA Inhibitor Negative Control #1) and pre-miRNAs (hsa-miR-328 Pre-miR miRNA Precursor and Pre-miR miRNA Precursor Molecules Negative Control #1) were all from Life Technologies. The RNeasy Plus kit (Qiagen) was used for RNA extraction, and miRNA expression was analyzed 48 h after transfection using TaqMan quantitative real-time PCR (Life Technologies). For functional assays, transfections were performed in 6-well plates with 300 000 cells/well. All experiments were performed with 100 nM anti- or pre-miRNAs. The *SFRP1* expression vectors used in this study (pcDNA3-huSARP2 and pcDNA3-SFRP1-C140Y) have been described elsewhere.<sup>19,20</sup> The commercially available pcDNA3 plasmid was used as an empty control vector (Life Technologies). The amount of transfected plasmids was adjusted to a final concentration of 200 ng/mL.

### Assays for Tumor Cell Invasion, Cell Proliferation, and Apoptosis

For investigating the impact of miR-328 silencing and overexpression on the invasive properties of A172 and TP365MG glioma cells, we used a 24-well modified Boyden chamber assay (BD Biosciences) as described.<sup>17</sup> Cell proliferation was assessed using a commercially available bromodeoxyuridine (BrdU) incorporation assay (Roche) with chemiluminescence measured on a plate reader. Differences in apoptotic properties were assessed by a commercially available fluorometric caspase-3/7 assay (Promega) according to the manufacturer's protocol. All experiments were performed in triplicates and confirmed in at least 1 second independent experiment.

### 3' UTR Luciferase Assay

Putative miR-328 target genes were identified by use of public prediction software (<http://pictar.mdc-berlin.de>, <http://targetscan.org>, <http://mirdb.org>, and <http://microRNA.org>). The 3' UTR sequences from 5 predicted target genes (*SFRP1*, *DNAJ3*, *NF2*, *PTEN*, and *ITGA5*) containing potential miR-328 binding sites were cloned into the psiCHECK-2 vector (Promega). Eight thousand T98G glioma cells per well were cotransfected in 96-well plates with 200 ng of the corresponding psiCHECK-2 plasmid and 100 nM of the indicated pre- or anti-miRNA using Lipofectamine 2000 (Life Technologies) as the transfection reagent. Forty-eight hours after transfection, the Dual-Glo Luciferase Assay System (Promega) was used to determine firefly and Renilla luciferase activity measured as chemiluminescence on a plate reader. The primers for cloning of the *SFRP1* 3' UTR fragment were *SFRP1*-forward: AATAAACCTCGAGTGCACATACGAGCCTTTG and *SFRP1*-reverse: CAGGAAGCGCCCTACCCTGGGAGAAGTTGA. Primers for cloning of the other putative target 3' UTRs are available on request. Specificity of miR-328-mediated regulation of the fusion mRNA containing the *SFRP1* 3'

UTR fragment was assessed by mutating the potential miR-328 binding site (seed sequence of miR-328) by overlap extension PCR (Fig. 3A).<sup>21</sup> Primer sequences for site-directed mutagenesis were mutSFRP1-forward: CTAAT TATCGCTAGGTGGGATTG and mutSFRP1-reverse: CAAATCCCACCTAGCGA TAATTAG.

### Western Blotting

Western blotting was performed according to standard protocols with a primary antibody directed against SFRP1 (1:1000 dilution; #4690, Cell Signaling Technology). Anti- $\alpha$ -tubulin was used as a loading control (#T9026, Sigma). Chemiluminescence was detected on the LAS 4000 mini imaging system (GE Healthcare), and western blotting results were reproduced in an independent experiment.

### TCF/LEF Reporter Assay

Activity of Wnt signaling was determined as luciferase transcription dependent on T-cell factor/lymphoid enhancer factor (TCF/LEF) with the Cignal Reporter Assay Kit (SA Biosciences). Lipofectamine 2000 (Life Technologies) was used to cotransfect 20 000 T98G glioma cells per well in 96-well plates with 100 ng of the TCF/LEF reporter plasmid and 100 nM of either anti- or pre-miR-328 or respective nontargeting controls. Resulting firefly and Renilla luciferase activities were determined with the DualGlo Luciferase Assay System (Promega). All conditions were measured in triplicates and repeated in an independent experiment. Functionality and transfection efficiency were controlled using the positive control plasmid contained within the Cignal Reporter Assay Kit and additionally by inducing TCF/LEF-dependent luciferase transcription with 30  $\mu$ M lithium chloride (LiCl) for 16 h (data not shown).

### Immunohistochemistry

Immunohistochemistry on formalin-fixed and paraffin-embedded tissue samples was performed using a standard protocol. In brief, 5- $\mu$ m sections were cut and slides were deparaffinized and, after microwave antigen retrieval, stained using the EnVision+ Dual Link System-HRP (DAB+) (#K4065, Dako) according to the manufacturer's protocol. Anti-SFRP1 primary antibody (#3534, Cell Signaling) was used 1:100 in phosphate buffered saline supplemented with 1% bovine serum albumin. Finally, the immunostained sections were counterstained with hematoxylin. Tumor center and INF of each individual patient were sampled on different tissue blocks. The histology of the tumor center and INF is clearly visible by the different cell density in both areas (Fig. 5). For maximum comparability, all blocks were cut and slides were stained at the same timepoint and according to an identical immunohistochemical protocol. Negative controls were performed with normal rabbit immunoglobulin G (#3900, Cell Signaling). For survival correlations, immunoreactivity was quantified using a previously reported composite numerical score based on the percentage of positive stained tumor cells multiplied by staining intensity, potentially ranging from 0 to 12.<sup>22</sup>

### Correlation of SFRP1 Expression With Overall Patient Survival

The Repository of Molecular Brain Neoplasia Data (REMBRANDT; <http://rembrandt.nci.nih.gov>) was used to determine the correlation between *SFRP1* expression and glioma patient survival. *SFRP1* mRNA expression levels were used to stratify glioma patients into 2 groups, one with a >2-fold higher *SFRP1* expression and the other with >2-fold reduced *SFRP1* transcript levels compared with the mean *SFRP1* expression in all glioma patients. The correlation between *SFRP1* mRNA/miR-328 expression and patient survival solely in the group of glioblastoma patients was assessed within the database of The Cancer Genome Atlas (TCGA; <http://tcga-data.nci.nih.gov>) using SPSS software (IBM).

### Statistical Analyses

For statistical analysis of WHO grade, we used dependent miR-328 and *SFRP1* expression one-way ANOVA and (where indicated) Tukey's multiple comparison as a posttest. For the statistical analysis of the correlation of *SFRP1* expression with overall patient survival, we used the log-rank test. Multivariate survival analyses by use of Cox proportional hazards models were performed to adjust for the effects of potential confounding factors. In all other cases, Student's *t*-test was used to assess statistical significance.  $P < .05$  was set as the cutoff for significance in all statistical analyses. Data points that were more extreme than quartile 1 (Q1) - 1.5 $\times$  interquartile range (IQR) or Q3 + 1.5 $\times$  IQR were designated as mild outliers. Extreme outliers were defined as data points more extreme than Q1 - 3 $\times$  IQR or Q3 + 3 $\times$  IQR.

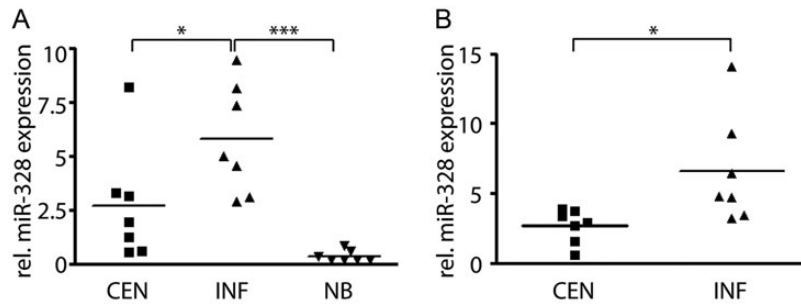
## Results

### MiR-328 Is Upregulated in Infiltrating Glioma Cells In vivo

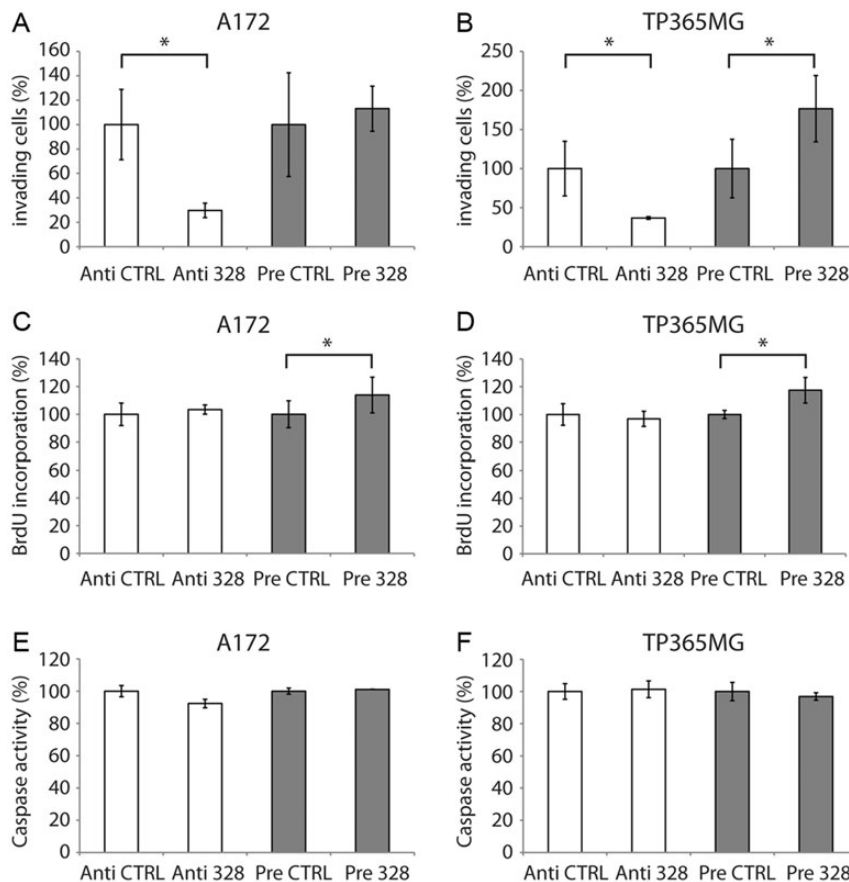
Glioma cells were microdissected from the INFs and CENs of 7 human malignant gliomas and used for miRNA extraction followed by expression profiling of 365 distinct miRNA species using the Micro Fluidic Card System. The raw data are available at the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE37737. In this screen, miR-328 was identified as differentially regulated with consistent and significant upregulation in the infiltrating glioma cell population compared with the corresponding tumor center (CEN vs INF, paired *t*-test,  $P = .044$ ; Fig. 1A). Additionally, 7 commercially available RNAs from nonneoplastic brain (NB) tissues were analyzed for miR-328 expression. MiR-328 in the infiltrating glioma cell population was also significantly upregulated compared with the nonneoplastic control tissue samples, arguing against the possibility of elevated miR-328 expression caused by an increased element of nonneoplastic tissue in the samples from the tumor periphery (INF vs NB nonpaired *t*-test,  $P = .0007$ ; Fig. 1A). The upregulation of miR-328 in invasive glioma cells was validated by TaqMan PCR (paired *t*-test,  $P = .032$ ; Fig. 1B). For a summary of significantly differentially regulated miRNAs between tumor INFs and tumor CENs, see Supplementary Table S1 (all fold change >2 and  $P < .05$ ). The 5 miRNAs for validation, including miR-328, were selected according to literature hints pointing toward a potential involvement into invasion-related cell processes, cancer gene/pathway regulation, or glioma pathogenesis.<sup>15,23-26</sup>

### MiR-328 Promotes Glioma Cell Invasion and Proliferation In vitro

Next, we performed cell-based assays using the glioma cell lines A172 and TP365MG to determine miR-328 functions in vitro. We reduced miR-328 expression in glioma cells by transfection of miRNA inhibitors (anti-328) or upregulated miR-328 expression by transfecting miRNA precursors (pre-328). Control transfections were performed with the corresponding nontargeting miRNA inhibitor or precursor. In both cell lines, A172 and TP365MG, transfection with the miR-328 inhibitor (anti-328) led to >80% reduction in miR-328 expression compared with the corresponding control transfected cells. Transfection with miR-328 precursor resulted in a >100-fold increase of miR-328 expression in comparison with control transfected cells (Supplementary Fig. S1).



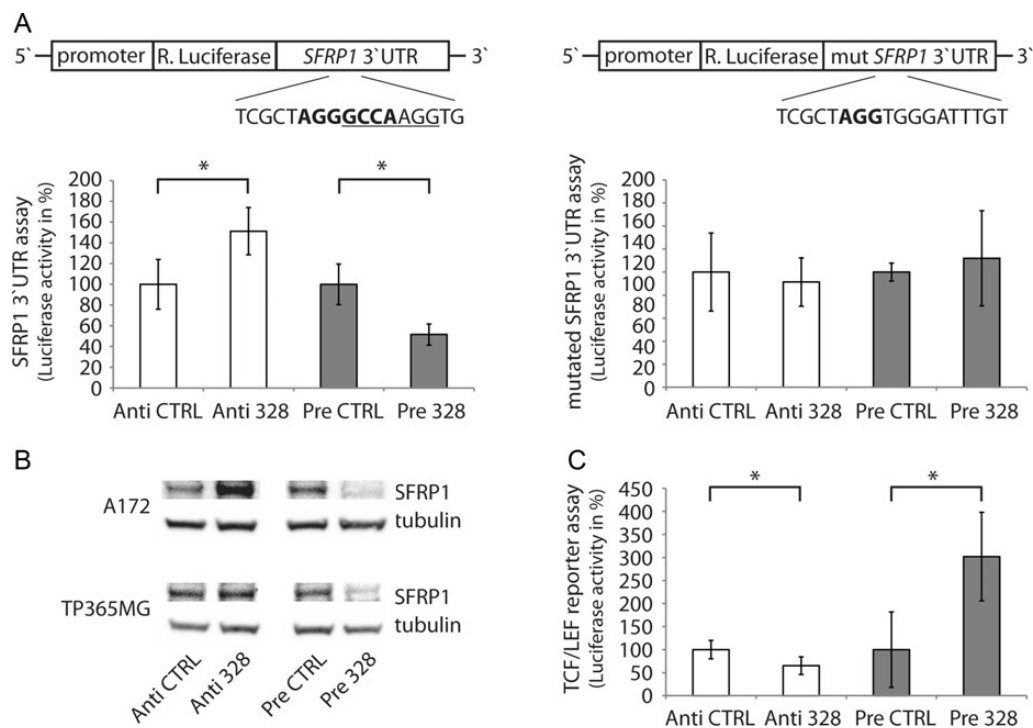
**Fig. 1.** MiR-328 expression is upregulated in infiltrating glioma cells in vivo. (A) MiR-328 expression in microdissected tumor areas from the infiltration zone (INF) and more central cell-rich tumor regions (CEN) of 7 malignant gliomas was analyzed by quantitative PCR assays based on Micro Fluidic Cards and additionally compared with miR-328 expression in 7 commercially available nonneoplastic brain (NB) samples. (B) Results were validated for microdissected INFs and CENs by singleplex microRNA assays (miR-328 expression normalized to RNU48) (*t*-test with pairwise comparison for INF vs CEN, unpaired *t*-test for INF vs NB. \*  $P < .05$ , \*\*\*  $P < .001$ ).



**Fig. 2.** MiR-328 promotes glioma invasion and proliferation in vitro. (A and B) MiR-328 downregulation leads to significantly decreased invasion of the glioma cell lines A172 and TP365MG in modified Boyden chamber assay experiments. In TP365MG, miR-328 upregulation resulted in significantly increased invasion. (C and D) Upregulation of miR-328 expression significantly increased cell proliferation of A172 and TP365MG glioma cells. (E and F) Apoptosis remained unaffected by miR-328 up- or downregulation in A172 and TP365MG cells (anti-control [CTRL]: transfected with nontargeting miRNA inhibitor; anti-328: transfected with miR-328 inhibitor; pre-CTRL: transfected with nontargeting miRNA precursor; pre-328: transfected with miR-328 precursor; *t*-test. \*  $P < .05$ ).

To test whether miR-328 expression influences the invasive behavior of glioma cells, we performed modified Boyden chamber assays with 10% fetal calf serum as chemoattractant. MiR-328

downregulation significantly reduced invasiveness of both glioma cell lines (Fig. 2A and B) to  $29.7\% \pm 5.9\%$  (*t*-test,  $P = .010$ ) invasive A172 cells and  $36.7\% \pm 1.7\%$  ( $P = .018$ ) invasive TP365MG cells



**Fig. 3.** *SFRP1* and Wnt signaling is a target of miR-328 in glioma cell lines. (A) A *SFRP1* 3' UTR luciferase reporter gene assay containing a wild-type *SFRP1* 3' UTR fragment or a mutated *SFRP1* 3' UTR fragment was used to analyze the effect of miR-328 up- and downregulation in T98G glioma cells. The *SFRP1* 3' UTR sequence corresponding to the miR-328 seed sequence is indicated in bold letters, and the 7 base pairs missing in the mutated *SFRP1* 3' UTR are underlined. With the luciferase reporter gene assay containing the wild-type *SFRP1* 3' UTR, downregulation of miR-328 expression resulted in higher luciferase activity, while miR-328 upregulation decreased luciferase activity. There is no significant effect of miR-328 on luciferase activity if the potential miR-328 binding site is mutated. (B) The expression of endogenous *SFRP1* protein was analyzed by western blotting after transfection with either miR-328 inhibitor (anti-328) or miR-328 precursor (pre-328) in A172 and TP365MG glioma cells (shown is a representative western blot). Note that transfection of anti-328 resulted in markedly higher *SFRP1* expression, while transfection with pre-328 nearly completely abolishes *SFRP1* expression in both cell lines. (C) TCF/LEF reporter assay was applied to assess Wnt signaling activity in T98G cells in relation to miR-328 expression. Transfection with anti-328 significantly decreased Wnt signaling pathway activation, while transfection with pre-328 increased Wnt pathway activation (anti-control [CTRL]: transfection with nontargeting miRNA inhibitor; anti-328: transfection with miR-328 inhibitor; pre-CTRL: transfection with nontargeting miRNA precursor; pre-328: transfection with miR-328 precursor; *t*-test. \*  $P < .05$ ).

compared with the corresponding control transfected cells (100%). Upregulation of miR-328 significantly increased invasiveness of TP365MG glioma cells to  $176.6\% \pm 42.4\%$  ( $P = .028$ ) invasive cells compared with the corresponding control transfected TP365MG cells (100%). In A172 cells, upregulation of miR-328 expression resulted in increased invasiveness ( $113.1\% \pm 18.4\%$ ) compared with control transfected cells (100%) but did not reach statistical significance.

In both cell lines (A172 and TP365MG), miR-328 upregulation caused a moderate but significant increase in cell proliferation as assessed by BrdU incorporation (Fig. 2C and D). After miR-328 upregulation, BrdU incorporation in A172 cells increased to  $113.9\% \pm 12.9\%$  ( $P = .041$ ) and in TP365MG cells to  $117.4\% \pm 9.2\%$  ( $P = .002$ ) compared with corresponding control transfected cells (100%). MiR-328 downregulation, in contrast, did not influence cell proliferation. Also, no relevant or uniform differences in apoptotic activity, as measured by caspase-3/7 activity, were observed when comparing A172 and TP365MG glioma cells after miR-328 up- or downregulation with corresponding control transfected cells (Fig. 2E and F).

### *SFRP1* Is a Target of MiR-328 in Glioma Cells

To identify target mRNAs directly regulated by miR-328, we consulted freely available miRNA target prediction software (<http://pictar.mdc-berlin.de>, <http://targetscan.org>, <http://mirdb.org>, and <http://microRNA.org>). In the selection of candidate mRNAs, we focused on predicted targets that could potentially mediate the proinvasive (and proliferative) function of miR-328 in glioma cells. We selected the 5 genes *SFRP1*, *DNAJ3*, *NF2*, *PTEN*, and *ITGA5* and cloned the respective 3' UTRs or part thereof into luciferase-reporter plasmids. The luciferase assay was performed in T98G glioma cells and revealed the *SFRP1* 3' UTR as a potential target of miR-328 (core target sequence: nucleotides 1465–1471 in NM\_003012.4; OFig. 3A). Transfection with miR-328 inhibitor led to a significant increase in luciferase activity ( $151.27\% \pm 22.87\%$ ,  $P = .028$ ), while transfection with miR-328 precursor significantly reduced luciferase activity ( $51.57\% \pm 10.19\%$ ,  $P = .016$ ). Luciferase assays after cloning of a second proposed miR-328 target site within the *SFRP1* 3' UTR (core target sequence: nucleotides 3760–3766 in NM\_003012.4) did not reveal

regulation through miR-328 (data not shown). Also, no significant differences in luciferase activity on miR-328 were seen for the cloned 3' UTRs of the other 4 genes (data not shown). Specificity of the miR-328-mediated regulation of luciferase activity was tested by deleting most of the potential miR-328 binding site within the cloned *SFRP1* 3' UTR fragment (Fig. 3A). In contrast to the results with the luciferase-reporter plasmid containing the wild-type *SFRP1* 3' UTR fragment, transfection with miR-328 inhibitor as well as with miR-328 precursor did not significantly change luciferase activity when the binding site was mutated (miR-328 inhibitor: 102.55% ± 11.47%,  $P = .891$ ; miR-328 precursor: 122.54% ± 26.11%,  $P = .520$ ).

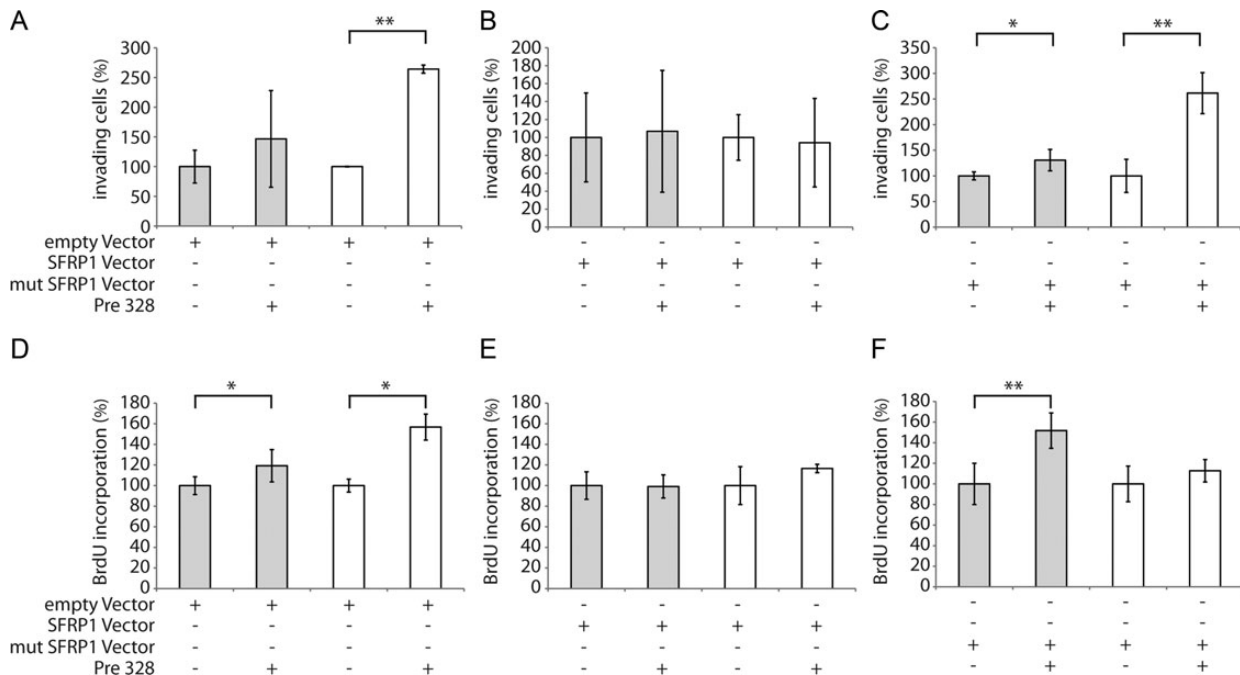
MiR-328-mediated regulation of endogenous *SFRP1* expression in glioblastoma cells was further verified by *SFRP1* protein expression analysis using western blotting. In both glioma cell lines, A172 and TP365MG, miR-328 downregulation caused increased *SFRP1* expression (115.27% in A172; 106.45% in TP365MG), while miR-328 upregulation diminished expression of endogenous *SFRP1* protein to 16.04% in A172 and 57.13% in TP365MG cells (Fig. 3B).

The specificity of miR-328-mediated *SFRP1* knockdown was validated by use of the randomly selected non-*SFRP1*-targeting miR-375. MiR-375 regulation had no significant effect on *SFRP1*, neither in the *SFRP1* 3' UTR luciferase assay nor on the expression of endogenous *SFRP1* in western blot analyses (Supplementary Fig. S2).

To demonstrate that the level of miR-328 expression directly influences the Wnt signaling pathway, we performed a TCF/LEF reporter assay that is an established test for Wnt signaling pathway

activity. In line with our results regarding *SFRP1* expression, the TCF/LEF reporter assay revealed significantly decreased Wnt signaling activity after miR-328 downregulation (65.23% ± 19.18%,  $P = .048$ ) and significantly increased Wnt signaling activity after miR-328 upregulation (301.93% ± 96.25%,  $P = .042$ ) compared with the corresponding control cells (100%; Fig. 3C).

To further corroborate the role of *SFRP1* in mediating the effects of miR-328 in invasion and proliferation, A172 and TP365MG glioma cells were transfected with an *SFRP1* expression vector, leading to overexpression of the coding sequence of the *SFRP1* gene. The 3' UTR where the miR-328 target site is located is missing in the *SFRP1* expression vector. Cotransfecting the 2 glioma cell lines A172 and TP365MG with the empty vector together with miR-328 precursor resulted in significantly increased invasion of TP365MG cells (A172: 146.70% ± 81.34%,  $P = .169$ ; TP365MG: 264.46% ± 6.82%,  $P = .009$ ) and significantly increased proliferation for both cell lines (A172: 119.29% ± 15.67%,  $P = .035$ ; TP365MG: 156.81% ± 12.61%,  $P = .031$ ) compared with cells transfected with only the empty vector (100%; Fig. 4A and D). Of note, after cotransfecting the *SFRP1* expression vector together with the miR-328 precursor, there were no significant changes in invasion (A172: 106.88% ± 67.93%,  $P = .438$ ; TP365MG: 94.13% ± 49.33%,  $P = .433$ ) or proliferation (A172: 99.17% ± 11.27%,  $P = .466$ ; TP365MG: 116.70% ± 4.03%,  $P = .352$ ) compared with the cells transfected with the *SFRP1* expression vector alone (100%; Fig. 4B and E). Thus, cotransfection of *SFRP1* rescues the invasive and proliferative phenotype induced by miR-328 overexpression.



**Fig. 4.** MiR-328 modifies invasion and proliferation of glioma cells by regulating *SFRP1*. (A–C) Effects on glioma cell invasion: cotransfection of a miR-328 precursor (pre-328) and an empty control vector increases invasion of TP365MG (white bars) and slightly also of A172 glioma cells (gray bars) (A). When cotransfecting an *SFRP1* expression vector instead of the empty control vector, the stimulating effect of miR-328 overexpression on tumor cell invasion is decreased or completely abolished (B). When cotransfecting C140Y mutated *SFRP1*, the stimulating effect of miR-328 overexpression on tumor cell invasion remains unaffected (C). Same applies to proliferation using BrdU incorporation assays (D–F): miR-328 effect in presence of the empty vector (D), rescue of the miRNA-328 effect through presence of the *SFRP1* expression vector (E), and no rescue through coexpression of mutated *SFRP1* (F). \*  $P < .05$ ; \*\*  $P < .01$ .

This rescue of the invasive and proliferative phenotype was not achieved by cotransfecting miR-328 precursor with a mutated (C140Y) SFRP1 protein (Fig. 4C and F). In the 2 glioma cell lines A172 and TP365MG, cotransfection resulted in a significantly increased invasion (A172:  $130.72\% \pm 20.80\%$ ,  $P = .048$ ; TP365MG:  $261.43\% \pm 39.92\%$ ,  $P = .003$ ) and an increased proliferation of both cell lines (A172:  $151.76\% \pm 17.19\%$ ,  $P = .001$ ; TP365MG:  $112.74\% \pm 10.92\%$ ,  $P = .083$ ) compared with cells transfected with the expression vector for C140Y mutated SFRP1 alone (100%; Fig. 4C and F).

In the nonneoplastic epithelial cell line FHs74Int, it had been shown that *SFRP1* mRNA expression increased after LiCl-mediated Wnt signaling activation, suggesting a negative feedback loop.<sup>27</sup> To test whether also in glioma cells Wnt signaling activation leads to induction of *SFRP1* or affects miR-328 expression, we treated A172 and TP365MG cells with LiCl (10 mM for 8 h). *SFRP1* expression remained unaffected in both cell lines on the mRNA as well as on the protein level, and miR-328 expression was not altered after LiCl treatment (Supplementary Fig. S3).

To investigate whether the upregulation of miR-328 in the tumor rim also translates into a downregulation of its target in this location, we performed *SFRP1* immunohistochemistry on 3 of the 7 glioma patients who had been subjected to microdissection for miRNA expression profiling and for which sufficient and representative formalin-fixed paraffin-embedded material from both regions (tumor CEN and tumor INF) was available. Indeed, the spatial distribution of *SFRP1* protein expression reflected mainly the expected relationship, with lower *SFRP1* expression levels in the tumor periphery in 2 patients (Fig. 5C–F). A third patient had equally low or absent *SFRP1* protein expression in both the tumor center and periphery, potentially caused by additional *SFRP1* inactivating mechanisms, such as promoter hypermethylation.

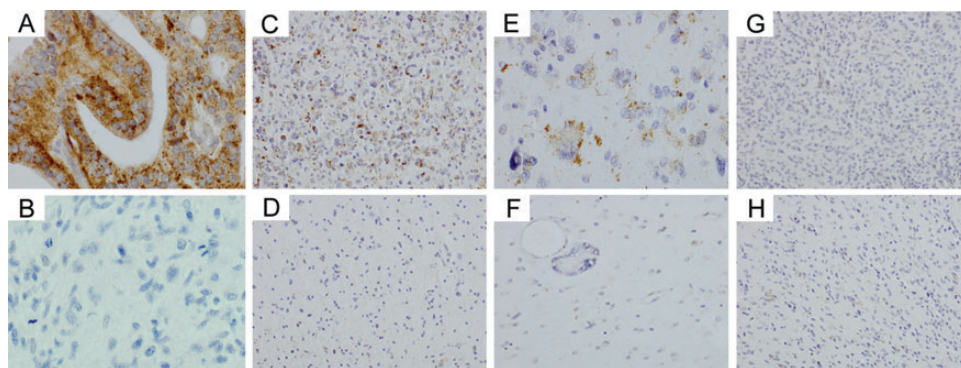
### MiRNA-328 Expression Is Preferentially Elevated in Low-grade Astrocytic Gliomas and Inversely Related to SFRP1 Promoter Hypermethylation

As previously shown, *SFRP1* expression in gliomas can also be epigenetically silenced by promoter hypermethylation, in particular

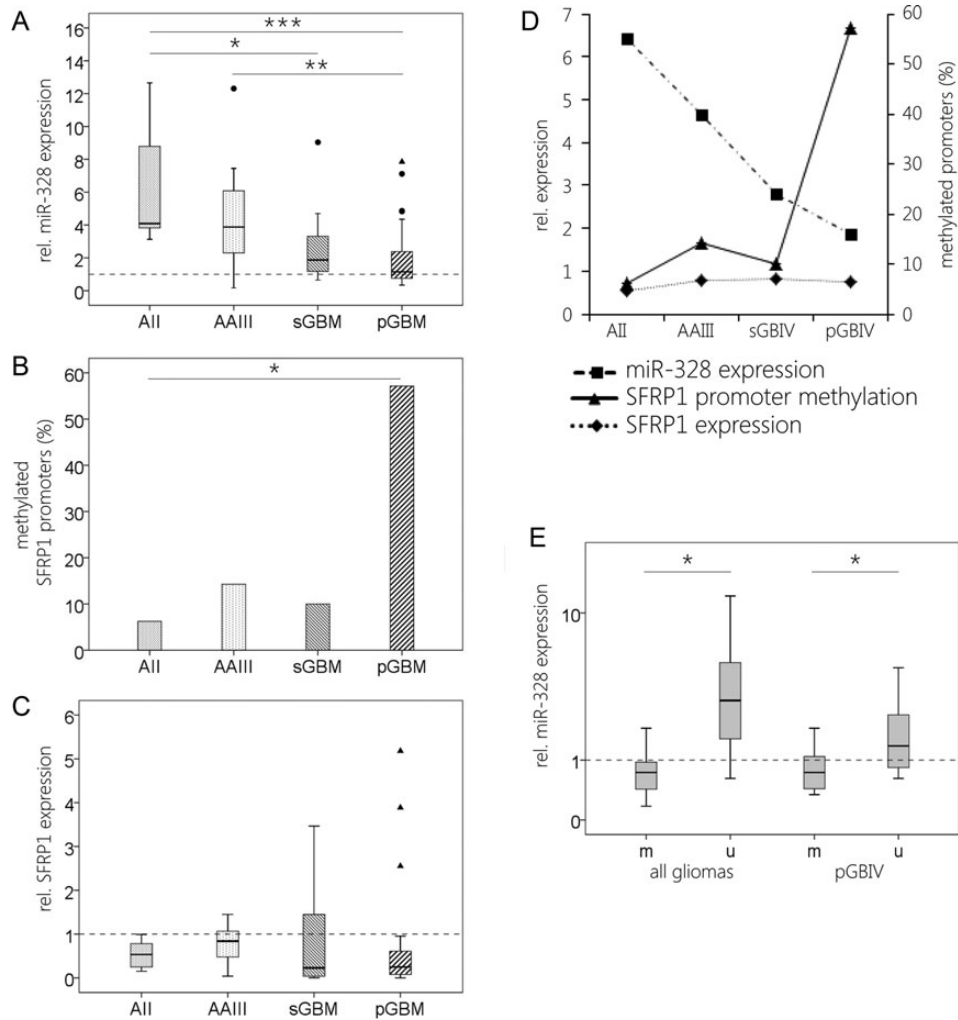
in primary glioblastomas.<sup>5</sup> We therefore investigated the expression of miR-328 in 79 human astrocytic gliomas of different WHO grades and compared these findings with a previously published dataset on *SFRP1* mRNA expression ( $n = 59$  gliomas) and promoter hypermethylation ( $n = 70$  gliomas).<sup>5</sup> In comparison with NB, miR-328 showed the highest median expression levels in AII (median value = 4.09, IQR = 8.76) but to a lower extent was also elevated in AAI (median = 3.88, IQR = 4.03) and sGBIV (median = 1.87, IQR = 2.14). In primary glioblastomas, miR-328 expression did not relevantly differ from the expression levels observed in nonneoplastic control tissues (pGBIV: median = 1.15, IQR = 1.74). After one-way ANOVA ( $P < .0001$ ), Tukey's multiple comparison posttest revealed the following significant  $P$ -values: AII versus NB,  $P = .002$ ; AAI versus NB,  $P = .004$ ; AII versus sGBIV,  $P < .05$ ; AII versus pGBIV,  $P < .001$ ; AAI versus pGBIV,  $P < .01$  (Fig. 6A).

Contrary to the observed miRNA-328 expression patterns, *SFRP1* methylation was most frequent in primary glioblastomas (57.14%) and observed at only low frequencies in diffuse astrocytomas of WHO grade II (6.25%), anaplastic astrocytomas (14.29%), and secondary glioblastomas (10.0%) (AII vs pGBIV,  $P = .001$ , Fisher's exact test; Fig. 6B).<sup>5</sup> *SFRP1* mRNA expression was uniformly decreased in all investigated glioma entities and there were no significant expression differences among the grades (AII median = 0.53, IQR = 0.53; AAI median = 0.84, IQR = 0.65; sGBIV median = 0.23, IQR = 1.41; pGBIV median = 0.25, IQR = 0.65; Fig. 6C).

To further substantiate the hypothesis of *SFRP1* promoter hypermethylation and miR-328-mediated *SFRP1* inhibition as 2 complementary and mutually exclusive mechanisms explaining the generally decreased median *SFRP1* expression in gliomas (Fig. 6D), we directly analyzed for an inverse correlation between *SFRP1* promoter hypermethylation and miR-328 expression in an overlapping subset of glioma patients ( $n = 39$ ) for which both miR-328 expression as well as *SFRP1* promoter hypermethylation data were available (Fig. 6E). Strikingly, tumors lacking *SFRP1* promoter hypermethylation showed significantly higher miR-328 expression levels than tumors bearing a methylated *SFRP1* promoter, both when comparing all gliomas (14 methylated and 25 unmethylated cases; Student's  $t$ -test,  $P = .001$ ) as well as solely primary



**Fig. 5.** *SFRP1* expression in the tumor center vs the tumor periphery. (A) Prostate adenocarcinoma as positive control of *SFRP1* immunoreactivity. (B) Negative control of a malignant glioma tissue with normal rabbit immunoglobulin G as the primary antibody. (C–H) Examples of matched pairs of tumor center and periphery from 3 malignant glioma patients. Note that *SFRP1* staining in the tumor periphery/infiltration zone (D, F, and H) is generally low. In sample pairs C–F, *SFRP1* is decreased in the tumor rim (D, F) relative to the tumor center (C, E), most likely due to miR-328 upregulation. In sample pair G and H, *SFRP1* is low also in the tumor center (G), which may be explained by alternative mechanisms of *SFRP1* downregulation, such as aberrant promoter methylation.



**Fig. 6.** Synopsis of miR-328 expression with *SFRP1* expression and promoter methylation in gliomas. (A) MiR-328 expression is highest in WHO grade II astrocytomas and decreases with malignant progression to secondary glioblastoma. Primary glioblastomas exhibit median miR-328 expression at about the level observed in nonneoplastic brain tissue. (B) *SFRP1* promoter hypermethylation exhibits an inverse pattern to miR-328 expression, with the by far highest methylation frequency in primary glioblastomas. Data shown in B are from Götze et al.<sup>5</sup> (C) *SFRP1* mRNA expression levels do not significantly differ among WHO grades. However, median *SFRP1* expression levels for each individual WHO grade are decreased relative to nonneoplastic brain tissue. (D) Synopsis of the molecular changes illustrated in A–C indicating that miR-328 overexpression and *SFRP1* promoter hypermethylation are mutually exclusive, depending on tumor type and grade, and may alternatively explain the uniformly low *SFRP1* transcript levels in gliomas. (E) Concomitant analysis of miR-328 expression and *SFRP1* hypermethylation in 38 gliomas (left) and 19 pGBIVs (right) revealed significantly higher miR-328 expression in tumors with unmethylated *SFRP1* promoter (u.) compared with tumors with *SFRP1* promoter methylation (m.) (AII: diffuse astrocytoma, WHO grade II; AAIII: anaplastic astrocytoma, WHO grade III; sGBIV: secondary glioblastoma, WHO grade IV; pGBIV: primary glioblastoma, WHO grade IV; \*  $P < .05$ ; \*\*  $P < .01$ ; \*\*\*  $P < .001$ ; ● marks mild outlier, ▲ marks extreme outliers; dotted line in A, C, and E marks expression levels in nonneoplastic brain tissue).

glioblastomas (11 methylated and 8 unmethylated cases; Student's *t*-test,  $P = .03$ ).

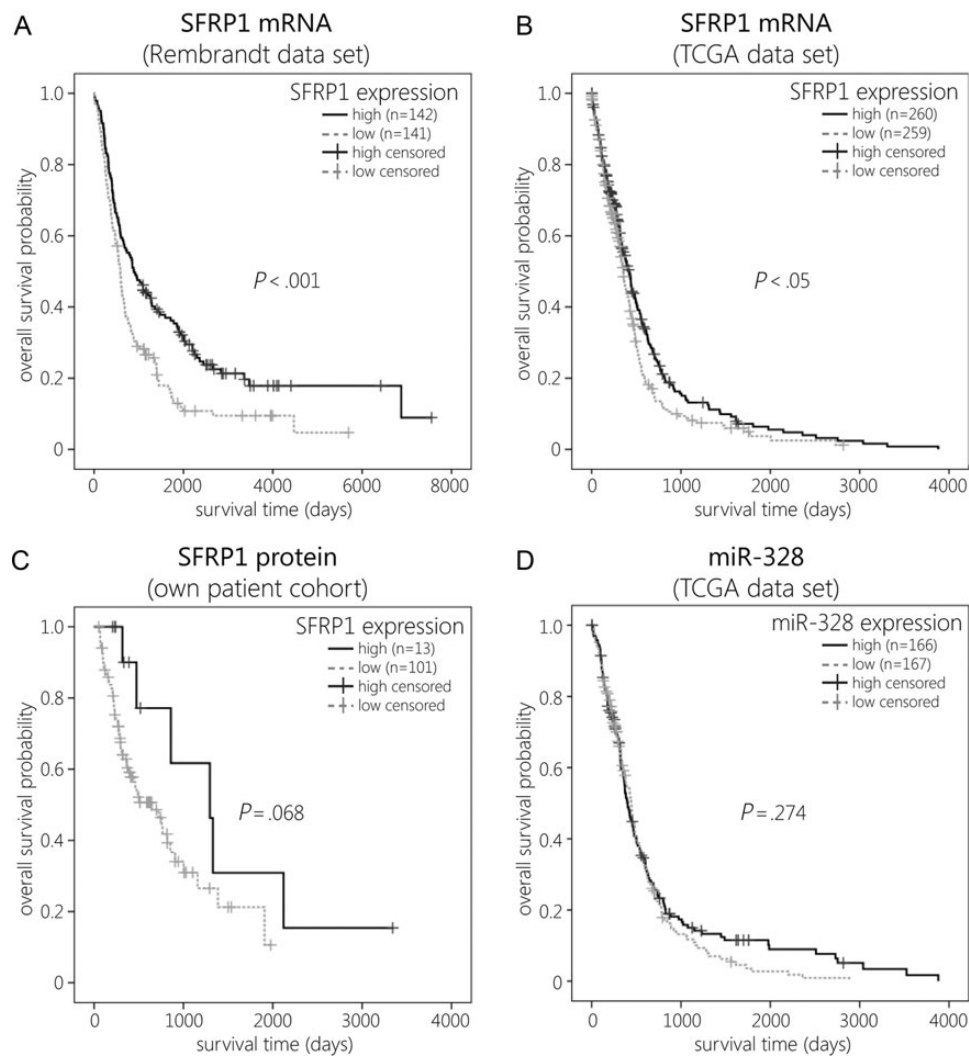
### Low *SFRP1* Expression Is a Negative Prognostic Factor in Gliomas

To evaluate the prognostic impact of epigenetically silenced *SFRP1* in gliomas, we analyzed the association between *SFRP1* mRNA expression and patient overall survival in the REMBRANDT and TCGA databases. REMBRANDT contains data from 283 glioma samples (66 grade II, 58 grade III, 148 grade IV). Kaplan–Meier curves of

patients stratified for “high” and “low” *SFRP1* mRNA expression revealed a significantly shorter overall survival of glioma patients with low *SFRP1* expression compared with patients with high *SFRP1* transcript levels (log-rank test,  $P < .001$ ; Fig. 7A). Within TCGA, a second independent data set containing only glioblastoma samples ( $n = 519$ ), patient stratification according to high and low *SFRP1* expression similarly showed a significantly shorter overall survival probability for patients with low compared with high *SFRP1* mRNA expression (log-rank test,  $P < .05$ ; Fig. 7B).

In a multivariate analysis for *SFRP1* expression and age (TCGA) or *SFRP1* expression, age, and WHO grade (REMBRANDT), *SFRP1*





**Fig. 7.** Kaplan–Meier survival analysis in glioma patients stratified for *SFRP1* and miR-328 expression. (A) The REMBRANDT data set, containing astrocytomas, oligodendrogliomas, and mixed oligoastrocytic tumors of different WHO grades, reveals statistically significant overall survival differences among patients stratified for *SFRP1* mRNA expression. (B) Analysis within TCGA shows statistically significant overall survival differences depending on *SFRP1* transcript levels also in the group of WHO grade IV glioblastoma patients. (C) In the own cohort of 114 glioblastoma patients, there is a (nonsignificant) trend toward longer overall survival in the small subset of patients with high *SFRP1* protein expression. (D) MiR-328 expression does not significantly correlate with overall survival in the group of WHO grade IV glioblastoma patients (log-rank tests).

expression was prognostic of better outcome in TCGA, but not in REMBRANDT (Table 1).

We then assessed *SFRP1* protein expression by immunohistochemistry in a clinically annotated single-institution cohort of 114 primary glioblastoma patients (110 glioblastomas, 4 gliosarcomas), all treated according to the current standard of care, that is, open tumor resection followed by radiotherapy as well as concomitant and adjuvant chemotherapy with temozolomide. In line with the finding of an overall low *SFRP1* mRNA expression in glioblastomas (Fig. 6C), the majority of glioblastomas ( $n = 101$ ) exhibited low *SFRP1* protein expression levels (score  $\leq 6$ , range 0–12). Of note, however, when comparing these patients with the few patients ( $n = 13$ ) whose tumors had high *SFRP1* protein expression scores ( $>6$ ), we observed a trend toward a better overall survival in patients with high *SFRP1* protein expression levels (Fig. 7C).

For 333 patients from the TCGA cohort, miR-328 expression values were available. MiR-328 expression was not significantly correlated with overall survival probability after patient stratification into high and low miR-328 expression groups (Fig. 7D).

## Discussion

Diffusely infiltrative growth is one of the major obstacles to efficient and successful anti-glioma therapies. Glioma cells harbor an intrinsic ability to invade the NB tissue surrounding the focal tumor mass.<sup>28</sup> This diffusely infiltrative growth pattern is shared by gliomas of WHO grades II to IV, suggesting that the underlying molecular aberrations manifest early during tumor development.<sup>29,30</sup> In order to identify novel molecules and regulatory mechanisms

**Table 1.** Multivariate analysis of SFRP1 mRNA expression and patient outcome using Cox proportional hazards models

Variable	HR	SE	P	95% CI
TCGA				
SFRP1 expression (high vs low)	0.782	0.101	.015	0.642–0.953
Age (<65 y vs >65 y)	2.225	0.111	<.001	1.789–2.769
REMBRANDT				
SFRP1 expression (high vs low)	0.823	0.140	.164	0.626–1.083
Age (<65 y vs >65 y)	0.286	0.169	<.001	0.205–0.398
WHO grade (II+III vs IV)	2.419	0.152	<.001	1.795–3.258

Abbreviations: HR, hazard ratio; CI, confidence interval for hazard ratio. Adjustment for patient age in TCGA dataset (glioblastomas only) and for patient age and WHO grade in the REMBRANDT dataset.

that drive the invasive phenotype of gliomas, we focused on the investigation of microdissected tumor cell subpopulations from distinct spatial locations within malignant gliomas, namely the infiltrative rim versus more central, solid tumor areas.<sup>17</sup> By applying miRNA expression profiling, we identified miR-328 as significantly upregulated in infiltrative glioma cells (Fig. 1). We then validated the invasion-promoting role of miR-328 in vitro using miRNA inhibitors and precursors in glioblastoma cells (Fig. 2). Our results in gliomas are supported by 2 other published studies in lung cancers. In lung adenocarcinomas, miR-328 was elevated in miRNA expression profiling analyses<sup>14</sup>; and in non-small cell lung cancers, a promigratory and metastasis-promoting function of miR-328 was proposed.<sup>15</sup>

MiRNAs mainly mediate their cellular functions via posttranscriptional regulation of gene expression. In this respect, 2 genes, namely *BCR/ABCG2* in breast cancer cells<sup>11</sup> and *CD44* mRNA in human epithelial carcinoma cells,<sup>12</sup> have been experimentally validated as direct targets of miR-328. We thus initially focused on the investigation of these 2 potential targets in our glioma cell lines, but neither of them was influenced by miR-328 up- or downregulation in glioma cells as assessed by immunoblotting (data not shown), suggesting that the posttranscriptional effects exerted by miR-328 differ among cell types and tumor entities. In an approach using a combination of in silico miRNA target prediction and target confirmation by 3' UTR luciferase assays and western blotting, we identified *SFRP1* as a novel target of miR-328 in human glioblastoma cells (Fig. 3).

SFRP1 contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins and acts as a soluble inhibitory modulator of Wnt signaling.<sup>31</sup> In line with this function, we demonstrated by using TCF/LEF reporter assays that the upregulation of miR-328 enhances and the downregulation of miR-328 decreases Wnt signaling pathway activity in human glioblastoma cells (Fig. 3C). The importance of Wnt signaling in human gliomas has been indicated by several preceding studies. In high-grade gliomas, elevated expression of the activating ligands *Wnt2* and *Wnt5A* as well as the activating Wnt receptors *Frizzled 2* (*Fzd2*) and *Fzd9* have been reported.<sup>32,33</sup> Functionally, Wnt signaling activation by *Wnt2*, *Wnt5A*, and *Fzd4* promotes glioma cell proliferation and neurosphere formation in vitro and tumor growth after subcutaneous injection of U251 glioma cells.<sup>3,32,34</sup> These results are in line with our finding that Wnt signaling activation by miR-328 overexpression not only triggers invasion but also increases glioma cell proliferation in vitro (Fig. 2C, D).

In addition, dysregulation of inhibitory Wnt pathway components has been described. As such, expression of *Wnt inhibitory factor 1* (*WIF1*) is downregulated in high-grade gliomas,<sup>35</sup> resulting in enhanced glioma cell proliferation.<sup>36</sup> The Wnt-inhibiting SFRP1 that was identified as a miR-328 target in the present study had been previously shown to inhibit glioma cell motility in U87MG cells in vitro.<sup>4</sup> This is in line with our finding of a proinvasive function of miR-328-mediated *SFRP1* inhibition. Similarly, in mammary epithelial cells (7GN), small interfering RNA-mediated silencing of *SFRP1* expression increased cell migration and invasion as assessed by transwell assays.<sup>37,38</sup> Furthermore, *SFRP1* overexpression reduced the invasive behavior of cervical cancer cells (HeLa) and the migration of breast adenocarcinoma cells (MDA-MB-231).<sup>39,40</sup> Nevertheless, in contrast to the mentioned motility-inhibiting function of SFRP1, SFRP1 treatment increased migration of endothelial cells in a radial migration assay and invasion of renal cell carcinoma cells in a transwell invasion assay,<sup>41,42</sup> suggesting that the SFRP1 effects on tumor cell migration are diverse and that complex networks modulate Wnt signaling in different types of cancers.

Interestingly, in the nonneoplastic epithelial cell line FHs74Int, it had been shown that *SFRP1* expression was increased after Wnt signaling activation, suggesting a negative feedback loop.<sup>27</sup> In our glioblastoma cell lines (A172 and TP365MG), LiCl-mediated Wnt signaling activation had no significant effect on SFRP1 mRNA or protein expression. Also, miR-328 expression was not affected by LiCl treatment. Thus, in glioma cell lines, there is no evidence of Wnt activation affecting miR-328 and SFRP1 expression (Supplementary Fig. S3).

Inhibitory Wnt pathway components in human cancers are frequently inactivated by epigenetic events. As such, *WIF1* in high-grade gliomas is downregulated by promoter hypermethylation,<sup>35</sup> and the *SFRP1* promoter is commonly hypermethylated in human cancers,<sup>6,43</sup> including gliomas, among which *SFRP1* promoter hypermethylation is most commonly detected in primary glioblastomas.<sup>5</sup> As we found that silencing of the Wnt signaling inhibitor SFRP1 by miR-328 represents an additional regulatory epigenetic mechanism of Wnt signaling in gliomas, we were interested in how our miR-328 expression data would correlate with *SFRP1* promoter hypermethylation and expression data when comparing gliomas of different subtypes and grades (Fig. 6). Of note, the highest upregulation of miR-328 expression was found in diffuse WHO grade II astrocytomas, with expression levels decreasing in the progression over anaplastic astrocytomas to secondary glioblastoma, but still being elevated in all these tumors relative to NB tissue. Primary glioblastomas, in contrast, showed miR-328 expression at about the level of the NB tissue controls. This miR-328 expression pattern was inversely related to the reported distribution of methylated *SFRP1* promoters, the percentage of which was by far highest in primary glioblastomas (Fig. 6D). In confirmation of this observation, in a panel of 38 gliomas for which we had overlapping data on miR-328 expression and *SFRP1* promoter hypermethylation, the lack of *SFRP1* promoter hypermethylation was associated with significantly higher miR-328 expression levels, suggesting that both epigenetic inactivation mechanisms are inversely correlated and mutually exclusive (Fig. 6E). This is probably also the reason why miR-328 expression alone is not prognostic for patient overall survival (Fig. 7D). With miR-328 being only 1 of 2 mechanisms of epigenetic *SFRP1* regulation in gliomas, a direct correlation of miR-328 expression with patient survival would thus not necessarily be expected.

MiR-328 in lower-grade astrocytic gliomas appears to downregulate *SFRP1* expression in a similar fashion as *SFRP1* hypermethylation in primary glioblastomas, and when evaluating *SFRP1* this bears prognostic implications. In bivariate analyses, low *SFRP1* mRNA expression in both the REMBRANDT and TCGA databases is associated with significantly worse patient overall survival. Also on the protein level (in our own smaller clinically annotated patient cohort), high *SFRP1* expression showed at least a trend toward better overall survival that appears likely to reach significance in larger clinically annotated patient populations. For the mRNA data, multivariate analyses adjusting for patient age confirmed the prognostic relevance of *SFRP1* in WHO grade IV glioblastomas in TCGA (Table 1). However, within REMBRANDT, *SFRP1* was not prognostic after adjusting for patient age and WHO grade.

Interestingly in this context, previous data suggested that miR-328 expression levels decrease with increasing malignancy in gliomas.<sup>16</sup> However, in this study miR-328 expression was compared between primary low-grade and recurrent high-grade gliomas of individual patients and not normalized to NB tissue controls. Our present experiments extend the interpretation of this observation in the way that not the downregulation of miR-328 in higher-grade gliomas, but the upregulation of miR-328 expression in lower-grade gliomas (at least with respect to *SFRP1*) appears to contribute to Wnt activation and tumor cell infiltration at early stages of glioma progression. Similar to a number of other molecular alterations, miR-328 upregulation seems to be involved preferentially in the pathway to secondary glioblastomas that are molecularly distinct from primary glioblastomas and develop from lower-grade precursor lesion.<sup>44–46</sup>

Taken together, our data are a comprehensive molecular and functional analysis of the role of miR-328 in human gliomas and describe a novel molecular miR-328-dependent mechanism that via *SFRP1* inhibition and Wnt signaling activation promotes glioma cell invasion. Targeted therapies addressing Wnt signaling in gliomas have to consider different levels of epigenetic regulation and may be useful to apply at already low-grade stages of the disease to effectively prevent glioma spread and improve the final outcome of the disease.

## Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (<http://neuro-oncology.oxfordjournals.org/>).

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