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***miR-29b* and *miR-125a* Regulate Podoplanin and Suppress Invasion in Glioblastoma**

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Abstract

Glioblastoma is the most frequent and malignant brain tumor, characterized by an elevated capacity for cellular proliferation and invasion. Recently, it was demonstrated that podoplanin membrane sialo-glycoprotein encoded by *PDPN* gene is over-expressed and related to cellular invasion in astrocytic tumors; however the mechanisms of regulation are still unknown. MicroRNAs are noncoding RNAs that regulate gene expression and several biological processes and diseases, including cancer. Nevertheless, their roles in invasion, proliferation, and apoptosis of glioblastoma are not completely understood. In this study, we focused on *miR-29b* and *miR-125a*, which were predicted to regulate *PDPN*, and demonstrated that these microRNAs directly target the 3' untranslated region of *PDPN* and inhibit invasion, apoptosis, and proliferation of glioblastomas. Furthermore, we report that *miR-29b* and *miR-125a* are downregulated in glioblastomas and also in CD133-positive cells. Taken together, these results suggest that *miR-29b* and *miR-125a* represent potential therapeutic targets in glioblastoma.

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INTRODUCTION

Glioblastoma is an aggressive and fatal tumor that represents the majority of primary brain tumors in adults (Ohgaki and Kleihues, 2009). The main causes for treatment failure include resistance to apoptosis and ability to invade adjacent normal tissues. Several proteins have been related to the invasion process in glioblastoma. We and others previously reported that *PDPN*, which encodes the mucin-type transmembrane sialoglycoprotein podoplanin, is upregulated in glioblastoma and is correlated with invasion and malignancy in astrocytic tumors (Scrideli et al., 2008). Additionally, podoplanin was suggested as a putative marker for neural stem cells and progenitor cells (Nakamura et al., 2006). Podoplanin is overexpressed in a variety of cancers, including squamous cell carcinomas (Kato et al., 2005), malignant mesotheliomas (Ordonez, 2006), colorectal adenocarcinomas (Kato et al., 2003), and central nervous system tumors (Mishima et al., 2006; Shibahara et al., 2006). However, the role of podoplanin in the pathogenesis of glioblastoma remains elusive.

Despite only modest improvement in patient outcomes, new insights in our understanding of glioblastoma molecular pathogenesis have opened the possibility of new therapeutic approaches. One of them is the connection between micro-RNAs and cancer. MicroRNAs are small non-coding RNAs that regulate gene expression at the posttranscriptional level by degrading or repressing translation of mRNA targets (Bartel, 2004). Mature microRNAs regulate gene expression through imperfect complementary base-pairing within the targeted transcript's 3'-untranslated region (UTR) (Ambros, 2004). Recent studies suggest that microRNA function is not limited to repressing individual key targets, but may also include fine-tuning target activity, and modulating multiple genes simultaneously (Flynt and Lai, 2008; Spizzo et al., 2009).

Although the biological functions of most microRNAs have not been completely revealed, several reports have demonstrated that aberrant expression levels of microRNAs are involved in glioblastoma initiation and progression. Notably, *miR-21* is highly overexpressed in glioblastoma and has important roles in cellular proliferation, invasion, and apoptosis (Chan et al., 2005; Ciafrè et al., 2005; Zhou et al., 2010). Alternately, important downregulated microRNAs have also been identified in glioblastoma, such as *miR-124*, *miR-128* (Godlewski et al., 2008) and *miR-137* (Silber et al., 2008). Nonetheless, little is yet known about how altered microRNA expression influences glioblastoma on a molecular basis. Thus, we investigated the potential roles of differentially expressed microRNAs in *PDPN* regulation and invasion, apoptosis, and proliferation in glioblastoma.

MATERIALS AND METHODS

PDPN microRNA Target Prediction

Potential microRNAs regulators of *PDPN* were identified by comparing information from the known target prediction databases: RNA22 (<http://cbcsrv.watson.ibm.com/rna22.html>) (Miranda et al., 2006), a pattern-based approach for the discovery of microRNA binding sites and their corresponding microRNA/mRNA complexes; miRanda (<http://www.microrna.org/microrna/home.do>) which target predictions incorporate current biological knowledge on target rules and on the use of an up-to-date compendium of

mammalian microRNAs, and TargetScan (<http://www.targetscan.org/>) which predicts biological targets of microRNAs by searching for the presence of conserved sites that match the seed region of each microRNA.

Tumor and Normal Brain Samples

We analyzed tumors from two independent sets of patients. Samples from nine macrodissected glioblastomas [World Health Organization (WHO) grade IV] obtained from the Department of Pathology, The University of Texas M. D. Anderson Cancer Center, were analyzed by microRNA microarrays from patients ranged in age from 17 to 73 years (average age 54 years, median, and standard deviation [SD] 54 ± 16 years). The samples were compared with three normal adult brain samples purchased from Ambion (Austin, Texas). The microarray data were validated by quantitative real-time polymerase chain reaction (qRT-PCR) in another set of patient samples, microdissected WHO grade IV glioblastomas from 15 patients ranged in age from 29 to 73 years (average age 57 years, median, and SD 60 ± 13 years) obtained from the Department of Anatomy, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo. Diagnoses were based on 2007 WHO criteria (Louis et al., 2007). The study was approved by the Research Ethics Committee of the University Hospital of the Faculty of Medicine of University of Sao Paulo (process number 7645/99). As controls, we used three samples [FirstChoice[®] Human Brain Reference RNA (Applied Biosystems, Foster City, CA), Human Brain MVPTM Total RNA (Stratagene Agilent Technologies, Santa Clara, CA), and Human Brain Total RNA [BioChain Institute, Hayward, CA]].

RNA Extraction, Retrotranscription, and Analysis of Mature microRNAs by RT-qPCR

Total RNA was isolated from tissues and cell lines using Trizol reagent (Invitrogen) for both *PDPN* mRNA and microRNA analyses according to the manufacturer's protocol. For mature micro-RNA expression analysis, total RNA was retro-transcribed with microRNA-specific primers using TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA), and then qRT-PCR was performed using Taqman[®] micro-RNA assays according to the manufacturer's protocol. For *PDPN* expression study, mRNA was retrotranscribed by using high capacity cDNA reverse transcription kit (Applied Biosystems) and analyzed by qRT-PCR using Taqman[®] specific probe for *PDPN* (Applied Biosystems) according to the manufacturer's protocol. The comparative cycle time (Ct) method was used to calculate the relative abundance of *PDPN* and microRNAs compared with *RNAU6B* small nuclear RNA expression (Schmittgen and Livak, 2008).

Isolation of CD133-Positive Cells from U87 Cell Line

U87 glioblastoma cells (20×10^6) were resuspended in 1 mL of PBS solution and dissociated to a single cell suspension in 35- μ m BD Falcon round-bottom tubes (BD-Becton, Dickinson, Franklin Lakes, NJ). Cells were subjected to a centrifugation and resuspended in separation autoMACS[™] running buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). FcR blocking reagent (50 μ L) was added to each sample, after and before addition of specific antibodies. The CD133/2 (293C3) antibody (50 μ L) conjugated with R-phycoerythrin was used for CD133-positive cells and 20 μ L of mouse IgG2b was used as control. Cells were incubated for 30 min at 4°C, resuspended in separation buffer, and sorted

by FAC-Scalibur flow cytometer (San Jose, CA). CD133-positive cells were grown in Dulbecco's modified Eagle's medium (DMEM/F12) (Mediatech, Manassas, Virginia) supplemented with B-27 (Gibco Invitrogen), 20 ng/mL epidermal growth factor, and 20 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ) at 37°C in a humidified 5% CO₂ incubator.

Cell Lines

Established glioblastoma cell lines LN229, U87, and U251 were acquired from the American Type Culture Collection, whereas cell line LN319 was kindly provided by Dr. Frank Furnari (Ludwig Institute for Cancer Research, La Jolla, CA). Cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ incubator. Cell count and viability were determined by using a Vi-Cell XR cell viability analyzer (Beckman Coulter, Mississauga, ON, Canada).

Transfection of microRNAs and *PDPN*-siRNA

Pre-microRNAs *miR-29b*, *miR-125a*, *miR-101*, and negative control (scrambled oligos) (Ambion) and two siRNAs specific for *PDPN* and negative control (Ambion) were reverse-transfected into glioblastoma cell lines using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 nM. The microRNAs transfection efficiency was monitored by qRT-PCR at 24 hr. The efficiency of siRNAs transfection was verified by qRT-PCR and Western blot for *PDPN* at 24, 48, 72, and 96 hr.

Protein Extraction and Western Blot Analysis

Total protein was extracted by using NP40 lysis buffer (0.5% NP40, 250-mM NaCl, 50-mM HEPES, 5-mM ethylenediaminetetraacetic acid, 0.5-mM egtazic acid) supplemented with protease inhibitor cocktails (Sigma-Aldrich, St. Louis, Missouri). Lysates were subjected to centrifugation at 12,000 rpm for 10 min, and the supernatant was collected for experiments. Protein lysates (40 µg) were resolved on denaturing sodium dodecyl sulfate-polyacrylamide gels ranging from 4 to 20% and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with the following antibodies: primary antibodies antipodoplanin and antivinculin (Santa Cruz Biotechnology, Santa Cruz, CA), and secondary antibody labeled by horseradish peroxidase (Amersham GE Healthcare, Chalfont St Giles, United Kingdom). The secondary antibody was visualized by using the ECL chemiluminescent reagent kit (Amersham GE Healthcare).

Luciferase Assay and Vector Construction

For the luciferase assay, three regions predicted to harbor interactor sites (700–1400 base pairs (bp), 1400–2200 bp, and 2200–2400 bp) of *PDPN* 3'-UTR (NM_001006624.1) were amplified by PCR from human genomic DNA and inserted into the pGL3 control vector (wild-type) (Promega, Madison, Wisconsin). The primers used are shown in Supplementary Table 1. Three mutant vectors were generated with deletions by using the Quik-Change site-directed mutagenesis kit (Stratagene Agilent Technologies) using the primers shown in Supplementary Table 2. LN229 cells were plated in 96-well dishes at 10⁴ cells/well. Cells were cotransfected with *miR-29b*, *miR-125a*, or scrambled (100 nM) with pGL3 *PDPN* 3'-

UTR constructs (wild-type or mutant) and with pRLTK vector. Thirty-six hours after transfection, cells were incubated for 10 min with 20 μ L/well $1 \times$ Passive Lysis Buffer (Promega). Firefly and renilla luciferase activities were measured sequentially using dual-luciferase assays (Promega) by a Veritas microplate luminometer (Turner BioSystems, Sunnyvale, CA). The experiments were performed in quadruplicate in three independent sets. Values are shown as mean $\pm$ SD.

Invasion Assay

Glioblastoma cells were transfected with micro-RNAs or the two siRNAs specific for *PDPN* or scrambled oligos and cultured for 48 hr, and were then transferred to the top of Matrigel-coated invasion chambers (24-well insert, 8- μ m pore size; BD-Becton, Dickinson) according to the manufacturer's protocol. The invasive cells attached to the lower surface of the membrane insert were fixed in 100% methanol at room temperature for 2 min and stained with toluidine blue 1% (Sigma-Aldrich) before being counted under an inverted microscope. The experiments were performed in triplicate in three independent sets. Values are shown as mean $\pm$ SD.

Proliferation Assay

Glioblastoma cells were seeded in 96-well plates at 5,000 per well and transfected with microRNAs or control. At different time points (24, 48, 72, and 96 hr), the culture medium was removed and replaced with culture medium containing 60 μ L of XTT dye (3 mg/mL) (XTT II; Roche Molecular Biochemicals, Indianapolis, IN) in each well. The plates were incubated for 2 hr at 37°C and the formazan product was measured at 450 nm by using an iMark microplate reader (Bio-Rad Laboratories). The experiments were performed in triplicate in four independent sets. Values are shown as mean $\pm$ SD.

Apoptosis Assay

After transfection with microRNAs or control, glioblastoma cells (1×10^6) were incubated with 5 μ L of NucViewTM 488 caspase-3 substrate and 5 μ L of sulforhodamine 101-annexin V (Biotium, Hayward, CA) at room temperature in the dark for 30 min. Cells were also labeled with DAPI and examined by fluorescence microscopy. Levels of apoptosis were expressed as percentages of NucView-positive cells over the total cell counts. The values of four independent experiments are shown as mean $\pm$ SD.

Statistical Analysis

The results were analyzed by Student's two-tailed *t* test ($P < 0.05$ was considered significant). All statistical analyses and graphing were performed by the SPSS 12.0 Windows version software (SPSS, Chicago, IL).

RESULTS

***miR-29b*, *miR-125a*, and *miR-149* are Predicted to Target *PDPN* and are Downregulated in Glioblastomas and in CD133-Positive Cells**

We analyzed by in silico target prediction programs (see Methods) putative interactor microRNAs with 3'-UTR of the *PDPN* mRNA (Fig. 1A). On the basis of target prediction by at least two different programs, we selected three microRNAs, *miR-29b*, *miR-125a*, and *miR-149*, as the most likely potential regulators of *PDPN* that is known to be overexpressed in glioblastomas (Scrideli et al., 2008). These selected miRNAs were also significantly downregulated in our microarray screening (Supplementary Table 3 and data not shown). QRT-PCR analysis confirmed the micro-array results for *miR-29b* ($P = 0.03$) and *miR-125a* ($P = 0.001$) (Figs. 1B and 1C), whereas it showed that *miR-149* was downregulated in this set of glioblastomas, but without statistically significant P values (Fig. 1D). Because CD133-positive cells isolated from glioblastomas express significantly higher levels of neural stem cell genes among which also *PDPN* stands out and promote tumor initiation in in vivo models, we analyzed by qRT-PCR whether the downregulated glioblastoma microRNAs *miR-29b*, *miR-125a*, and *miR-149*, were also under-expressed in CD133-positive cells. CD133-positive and CD133-negative cells were isolated from the U87 glioblastoma cell line. Consistently, all three microRNAs, *miR-29b* ($P = 0.02$), *miR-125a* ($P = 0.0005$), and *miR-149* ($P = 0.014$), were also downregulated in CD133-positive cells compared with their negative counterparts (Figs. 1B–1D, respectively). On the basis of these results, we focused on *miR-29b* and *miR-125a* for functional studies.

PDPN* is a Direct Target of *miR-29b* and *miR-125a

To correlate the expression of *miR-29b* and *miR-125a* with that of *PDPN*, we analyzed by qRT-PCR their expression in LN319 cells. The results showed that LN319 overexpressed *PDPN*, as demonstrated by others (Kato et al., 2005; Scrideli et al., 2008), and also express very low levels of *miR-29b*; interestingly, this cell line expresses low levels of *miR-125a* and high levels of *PDPN* (Fig. 2A). To confirm the regulation of *PDPN* by *miR-29b* and *miR-125a*, we analyzed the expression of the podoplanin protein by Western blot 48 hr after transfection of these microRNAs in the LN319 cell line. Podoplanin expression decreased significantly after *miR-29b* and *miR-125a* restoration, but not for *miR-101* that is also predicted to target *PDPN* (Fig. 2B). Regulation of *PDPN* by *miR-29b* and *miR-125a* was confirmed by luciferase assay. The results showed a significant reduction of luciferase activity in cells transfected with *miR-29b* ($P = 0.002$) or *miR-125a* ($P = 0.003$), compared with scrambled control (Fig. 2C). Mutation of *miR-29b* and *miR-125a* interaction sites rescued the luciferase activity, thus confirming the direct interaction with *PDPN* 3'-UTR by these microRNAs. These results suggest that *miR-29b* and *miR-125a* directly regulate podoplanin expression in glioblastoma cells.

Restoration of *miR-29b* and *miR-125a* and Downregulation of *PDPN* Impaired Invasion in Glioblastoma Cells

Since *PDPN* was involved in invasion (Scrideli et al., 2008), we next analyzed the effect of the restoration of microRNAs levels on invasion in glioblastoma cells. We performed the Matrigel assay and found significant reductions of invasion, by 40% ($P = 0.0003$) and 23%

($P = 0.005$) compared with controls, in LN319 cells transfected with *miR-29b* or *miR-125a*, respectively (Fig. 3A). Furthermore, reductions of invasion by 23.5% ($P = 0.03$) and 56% ($P = 0.0008$) were observed also in U251 cells after transfection with *miR-29b* or *miR-125a*, respectively (Fig. 3B). The efficiency of microRNA transfection was confirmed by qRT-PCR (data not shown). The same significant reduction of invasion in LN319 cells was found after *PDPN* downregulation by specific siRNA treatment for 96 hr ($P = 0.03$) (Fig. 4). Therefore the effects on invasion by restoration of *miR-29b* and *miR-125a* are similar with the effects by down-regulation of the proved direct *PDPN* target.

***miR-29b* Induces Apoptosis and Inhibits Proliferation in Glioblastoma Cells**

In a previous study, *miR-29b* was reported to upregulate TP53 levels and induces apoptosis in a TP53-dependent manner (Park et al., 2009). Thus, we sought to determine the role of *miR-29b* in apoptosis in glioblastoma cells by investigating its effects in the mutant *TP53* glioblastoma cell line U251 (CGT to CAT/arginine to histidine) and the wild-type *TP53* cell line U87 (Van Meir et al., 1994). *miR-29b* induced apoptosis in U87 wild-type *TP53* cells ($P = 0.01$) (Fig. 5A). However, no significant differences were observed in U251 mutant *TP53* cells transfected with *miR-29b* compared with control (Fig. 5B). Moreover, we analyzed whether *TP53* status influences the effects of *miR-29b* in glioblastoma cells proliferation and found no significant differences in the two mutant *TP53* cell lines U251 and LN319, transfected with *miR-29b*, compared with controls (Figs. 5C and 5D); whereas, *miR-29b* was able to reduce proliferation of wild-type *TP53* cells ($P = 0.0002$) (Fig. 5E). LN319 cells contain a mutation of *TP53* that results in a change of arginine for histidine (CGC to CAC/ arginine to histidine) (Van Meir et al., 1994). These results may suggest that *miR-29b* induces apoptosis as well as reduces proliferation in a TP53-dependent manner in glioblastoma.

DISCUSSION

In this study, we demonstrate that over-expressed *PDPN* is regulated by microRNAs *miR-29b* and *miR-125a*, which are deregulated in glioblastomas and CD133-positive cells. We show, furthermore, that the altered signaling pathways of invasion, apoptosis, and proliferation that provide a selective advantage to tumors are affected by these microRNAs.

On the basis of our microarray screening and qRT-PCR results, we demonstrated that *miR-29b* and *miR-125a* are significantly downregulated in glioblastomas. *miR-29b* was also found downregulated in several other types of tumor, including invasive breast cancer (Iorio et al., 2005), lung cancer (Yanaihara et al., 2006), chronic lymphocytic leukemia (Calin et al., 2005), prostate cancer (Porkka et al., 2007), colon cancer (Cummins et al., 2006). To date, this is the first report of *miR-125a* downregulation in glioblastoma. Others studies showed that *miR-125a* is also downregulated in breast cancer (Iorio et al., 2005), neuroblastoma (Laneve et al., 2007), and medulloblastoma (Ferretti et al., 2009).

Recent reports demonstrated that some micro-RNAs related to glioblastoma regulate such important molecular mechanisms in neural stem cells as differentiation and self-renewal (for a review, see Nicoloso et al., 2009). Conversely, several studies revealed that genes related to self-renewal are also implicated in important pathways in carcinogenesis (Godlewski et al.,

2009). Our findings show that *miR-29b*, *miR-125a*, and *miR-149* are under-expressed in CD133-positive cells compared with their counterpart CD133-negative cells. Interestingly, other reports have indicated that these microRNAs are expressed during brain development and are related to differentiation of precursor cells in many types of tumors. Krichevsky et al., (2006) and others demonstrated that *miR-125* is expressed during mouse brain development and is strongly induced during neural differentiation of embryonic stem cells (Rybak et al., 2008). Moreover, *miR-125* induced development of P19 murine embryonal carcinoma cells to neurons (Wu and Belasco, 2005). The reconstitution of *miR-29* expression promoted differentiation of rhabdomyosarcoma RH30 cells in vivo (Wang et al., 2008). Taken together, these findings suggest that *miR-29b* and *miR-125a* have roles in differentiation of precursor cells in many tissues, including the brain. CD133-positive cells present a significant tumorigenic potential in vivo, capacity for angiogenesis and multilineage differentiation, and high resistance to radiation and chemotherapy (Bao et al., 2006). These results and the fact that glioblastomas are poorly differentiated tumors suggest a potential role for these microRNAs in regulation of signaling pathways related to maintenance of stem cell properties such as differentiation and self-renewal. Nonetheless, additional studies are necessary to elucidate these results.

Our results indicate that *miR-29b* and *miR-125a* specifically interact with the 3'-UTR of *PDPN* and regulate its expression levels. Important targets were validated for *miR-125a* and its homologue *miR-125b*, such as *lin-28* (Wu and Belasco, 2005), *ERBB2* and *ERBB3* (Scott et al., 2007), and a truncated form of *trkC* receptor (Laneve et al., 2007; Ferretti et al., 2009). Published reports have demonstrated that *TCL1* (Pekarsky et al., 2006), *MCL1* (Mott et al., 2007), *DNMT3A* and *DNMT3B* (Fabbri et al., 2007), p85 α , the regulatory subunit of phosphoinositol-3 kinase (Park et al., 2009), and *YY1* (Wang et al., 2008) are validated targets of *miR-29b*. Interestingly, Park et al., (2009) also demonstrated that *miR-29b* regulates *Cdc42*, a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions, including cell morphology and migration. Likewise, podoplanin is related to migration and invasion by modulating the activity of RhoA, a member of the Rho-subfamily like *Cdc42* (Martin-Villar et al., 2006; Wicki et al., 2006), confirming that microRNAs regulate multiples genes that participate in related biological processes.

In this report, we show that replacement of downregulated microRNAs *miR-29b* and *miR-125a* promoted significant reduction of invasion processes in glioblastoma cells. Previous studies demonstrated that *miR-29b* is related to invasion and metastasis in nasopharyngeal carcinoma via regulation of extracellular matrix proteins (Sengupta et al., 2008). Also, *miR-125a* and *miR-125b* reduced migration and invasion in SKBR3 neuroblastoma cells (Scott et al., 2007). The same study showed that *miR-125a* and *miR-125b* overexpression decreased motility in ERBB2-positive cells in breast cancer. Importantly, it was demonstrated that *miR-125a* is regulated by an epidermal growth factor signaling pathway and promoted inhibition of migration and invasion in lung cancer cells (Wang et al., 2008).

Park et al., (2009) showed that *miR-29* (a, b, and c) activate *TP53* and induce apoptosis in a *TP53*-dependent manner. In this study, they demonstrate that *miR-29b* induced apoptosis

only in wild-type *TP53* cell lines such as HeLa and MCF-7, to the detriment of MDA-MB-231 and SW480 cells, which express mutant *TP53*. Our results demonstrate that *miR-29b* induced apoptosis and also reduced proliferation in glioblastoma cells. In the mutant *TP53* glioblastoma cell lines, no effect on proliferation was observed and the differences in apoptosis were not significant compared with the control. *miR-29b* also reduced proliferation of rhabdomyosarcoma RH30 cells in vivo (Wang et al., 2008). *miR-29b* involvement in apoptosis was shown by a previous report that demonstrated that this microRNA regulates *MCL1*, a member of the BCL-2 family, and sensitized cholangiocarcinoma KMCH cells to apoptosis mediated by treatment with TRAIL (Mott et al., 2007). These results suggest that *miR-29b* has an important role in apoptosis. This is particularly important in glioblastoma because *TP53* mutations are significantly more frequent in secondary glioblastoma than in primary (Ohgaki and Kleihues, 2009). Thus, these findings might be taken into consideration in rational development of new therapeutic approaches in glioblastoma.

In conclusion, microRNAs *miR-29b* and *miR-125a* are downregulated in glioblastoma and in CD133-positive cells and are related to important biological processes such as apoptosis, proliferation, and invasion. We showed here that *miR-29b* and *miR-125a* regulate *PDPN*, a putative marker of neural stem cells related to invasion and malignancy in glioblastoma. Our findings suggested that *miR-29b* and *miR-125a* are the potential regulators of invasion in these tumors, and also that *miR-29b* affected apoptosis and cell proliferation in glioblastoma cell lines. These results may be important for the development of future micro-RNA-based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MicroRNAs *miR-29b*, *miR-125a*, and *miR-149* are predicted to target the overexpressed *PDPN* and are downregulated in glioblastomas and in U87 CD133-positive cells. *miR-101* binding sites were also predicted for *PDPN* 3'-UTR. The predictions were performed as described in methods. (A) The *miR-29b* (900–906 bp; 1968–1974 bp), *miR-101* (868–889 bp; 2164–2185 bp), *miR-125a* (766–789 bp; 1169–1192 bp), and *miR-149* (1354–1376 bp; 1477–1499 bp) predicted target sites in *PDPN* 3'-UTR are shown as triangles. Levels of each microRNAs were measured by qRT-PCR in glioblastomas and adult normal brain samples and CD133-positive and -negative cells. The comparative Ct method was used to calculate the fold changes of microRNA *miR-29b* (B), *miR-125a* (C), and *miR-149* (D) compared with *RNAU6B* small nuclear RNA expression. Results are expressed as mean + SD. The asterisk (*) represents a significant difference ($P < 0.05$) by Student's *t* test.

Figure 2.

miR-29b and *miR-125a* downregulate *PDPN* levels and target the *PDPN* 3'-UTR. (A) Inverse correlation of *miR-29b* and *miR-125a* levels with *PDPN* expression in cell line LN319. LN319 expresses high *PDPN* and low *miR-125a* and *miR-29b* levels. (B) LN319 cells were transfected with *miR-29b*, *miR-125a*, *miR-101* or scrambled and cell lysates were collected and run on a sodium do-decyl sulfate polyacrylamide gel for Western blotting. Podoplanin expression decreases after *miR-29b* and *miR-125a* transfection. (C) LN229 cells were transfected with luciferase constructs which contain *miR-29b* and *miR-125a*-predicted targets. *miR-29b* and *miR-125a* downregulate target *PDPN* 3'-UTR. Values represent averages and bars represent SD of three independent experiments. Results are expressed as mean + SD. The asterisk (*) represents a significant difference ($P < 0.05$) by Student's *t* test.

Figure 3.

Invasion assay using transwell chambers coated with Matrigel was performed 48 hr after transfection of LN319 (A) and U251 (B) cells with *miR-29b*, *miR-125a*, or scrambled. LN319 cells presented a significant decrease in invasion rate after transfection with *miR-29b* or *miR-125a*, compared with scrambled. A decrease in invasion rate was also observed for U251 cells transfected with *miR-29b* or *miR-125a*, compared with scrambled. In (A) and (B), values represent averages and bars represent SD of three independent experiments. Results are expressed as mean + SD. The asterisk (*) represents a significant difference ($P < 0.05$) by Student's *t* test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4.

Silencing of podoplanin with specific siRNA impaired invasion in LN319 cells. (A) Analysis of podoplanin silencing by specific siRNA by qRT-PCR. After 48 hr, it was observed a reduction of 82% of *PDPN* expression. (B) Confirmation of podoplanin silencing by specific siRNA 72 and 96 hr after transfection by Western blotting. Vinculin was used as normalizer. The same results were obtained with two specific siRNAs (only one is shown here). (C) Invasion assay using transwell chambers coated with Matrigel was performed 96 hr after transfection of LN319 with specific siRNA. LN319 cells presented a significant decrease in invasion rate compared with control (D). Values represent averages and bars represent SD of three independent experiments. Results are expressed as mean + SD. The asterisk (*) represents a significant difference ($P < 0.05$) by Student's *t* test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5. *miR-29b* induces apoptosis and represses proliferation in glioblastoma cells. Apoptosis assay was performed 48 hr after transfection of U87 (A) and U251 (B) cells with *miR-29b* or scrambled. Cellular proliferation was assessed 24, 48, 72, and 96 hr after transfection of LN319 (C), U251 (D), and U87 (E) cells with *miR-29b* or scrambled control. Values represent averages and bars represent SD of three independent experiments. Results are expressed as mean + SD. The asterisk (*) represents a significant difference ($P < 0.05$) by Student's *t* test.