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REST represses miR-124 and miR-203 to regulate distinct oncogenic properties of glioblastoma stem cells

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Abstract

Background. Glioblastoma (GBM) is one of the most common, aggressive, and invasive human brain tumors. There are few reliable mechanism-based therapeutic approaches for GBM patients. The transcriptional repressor RE1 silencing transcriptional factor (REST) regulates the oncogenic properties of a class of GBM stem-like cells (high-REST [HR]-GSCs) in humans. However, it has been unclear whether REST represses specific targets to regulate specific oncogenic functions or represses all targets with overlapping functions in GSCs.

Methods. We used genome-wide, biochemical, and mouse intracranial tumorigenic assays to identify and determine functions of microRNA (miR) targets of REST in 2 independent HR-GSC lines.

Results. Here we show that REST represses 2 major miR gene targets in HR-GSCs: miR-203, a new target, and miR-124, a known target. Gain of function of miR-124 or miR-203 in HR-GSCs increased survival in tumor-bearing mice. Importantly, the increased survival of tumor-bearing mice caused by knockdown of REST in HR-GSCs was reversed by double knockdown of REST and either miR-203 or miR-124, indicating that these 2 miRs are critical tumor suppressors that are repressed in REST-mediated tumorigenesis. We further show that while miR-124 and the REST–miR-124 pathways regulate self-renewal, apoptosis and invasion, miR-203 and the REST–miR-203 pathways regulate only invasion. We further identify and validate potential mRNA targets of miR-203 and miR-124 in REST-mediated HR-GSC tumor invasion.

Conclusions. These findings indicate that REST regulates its miR gene targets with overlapping functions and suggest how REST maintains oncogenic competence in GSCs. These mechanisms could potentially be utilized to block REST-mediated GBM tumorigenesis.

Key words

apoptosis | GSCs | invasion | miR-203 | miR-124 | REST | survival

Glioblastoma (GBM) is one of the most highly invasive human brain tumors; despite recent exciting advances in the field, patient outcomes for this common and aggressive brain tumor remain poor.[1–6] Genome-wide expression analysis has indicated that GBM is made up of different subclasses of tumor,[7–10] suggesting that GBM is not a single disease and that not all patients should be treated with the same drug. And yet, all patients with newly diagnosed GBM are treated with a similar therapeutic regimen irrespective of the molecular subtype of the tumor. Thus, there is an urgent need for therapeutic approaches that target the specific mechanisms in each GBM subtype.

"Stem-like" or "tumor-initiating" cells were found to influence GBM tumorigenesis by impacting specific signaling pathways, microenvironments, and drug-resistance mechanisms.[10,11] That discovery, along with the generation of invaluable new GBM mouse models[12] and the advent of preclinical and clinical approaches resulting in promises and challenge,[2,13–16]

has brought a new perspective to our understanding of GBM biology and therapy. The transcriptional repressor RE1 silencing transcriptional factor (REST) is a repressor of neuronal differentiation genes and a suppressor of neurogenesis in nonneural cells.[17,18] REST has been found to suppress numerous genes and can potentially regulate many biological processes in a context-dependent manner.[17–20] REST was recently found to regulate the tumorigenic properties of a subclass of GBM-derived stem cells (GSCs) called high-REST GSCs (HR-GSCs) and to produce distinct brain tumors.[21,22] However, how REST performs its oncogenic functions in GSCs is unknown, and this information is critical for developing targeted therapies for REST-regulated GBM.

Previously, we found that microRNA (miR) played a critical role in embryonic stem cells as well as in GBM.[23,24] Because REST can target many genes,[20] we first focused on its miR targets because miR genes are less abundant than coding genes. Here, we show that REST directly targets miR-124 and -203 genes to regulate overlapping oncogenic properties in GSCs. We further identify potential targets that are common to miR-124– and miR-203–mediated tumorigenesis.

Materials and Methods

Details of methods are provided in the Supplementary material.

GSC Culture and Biochemical Assays

All methods were followed from our previous publications.[22] Patient-derived GSCs were isolated with patient consent according to the protocol approved by the institutional review board. GSC neurospheres were cultured in Dulbecco's modified Eagle's medium–F12/B-27/glutamine/epidermal growth factor/fibroblast growth factor. Loss- and gain-of-function manipulations were performed by transduction of GSCs with lentiviruses containing appropriate molecules as described in the text and confirmed by real-time (RT) quantitative (q)PCR from total cellular RNA extracted using Trizol reagent (Invitrogen) and western blotting using REST antibody (Millipore). Luciferase assays were performed using pGL3 reporter plasmids containing RE1 or control sites as described in the text. Chromatin immunoprecipitation (ChIP) assays were performed from sheared cellular chromatin, immunoprecipitated using REST antibody; qPCR was performed, and the analysis was done by the fold enrichment method. Self-renewal assays were performed as described earlier (REF). In vitro proliferation and invasion assays were performed using a bromodeoxyuridine assay kit (Roche) and invasion assay kit (BD biosciences), respectively. GSCs were differentiated in 10% serum.

Genome-wide Expression Analysis of GSCs with Loss and Gain of Function

Total RNA and miR microarrays were used to identify the targets of REST with a GeneChiP human genome U-133 plus 2.0 array (Affymetrix). The GeneSpring GX11.5 software programs (Agilent) and R program were used to identify the miRs with a 2-fold increase upon REST knockdown when compared with control. ChIP-sequence (Seq) analysis was performed using HR-GSC2.shNT and HR-GSC2.shREST cells.

Identification and Validation of REST Binding Sites in the Promoter Regions of MiR-124 and MiR-203

REST binding sites on the promoter region of miR-124 and miR-203 were determined using Mat Inspector (Genomatix software suite v3.3) and confirmed by qPCR using site-specific primers as described.

Self-renewal and Annexin V Staining Assays

This was performed for 4 generations as described previously.[22] For annexin V, cells were labeled with annexin V Alexa Fluor 488 antibody and propidium iodide and analyzed by flow cytometry.

Mouse Orthotopic GBM Models

All the mouse experiments were performed according to protocols approved by the institutional animal care and use committee. Brain orthotopic tumor models were generated as described previously by implanting 50 000 cells and analyzing the mice for survival using a Kaplan–Meier plot, for mouse brain sections for tumor characteristics as described,[22] and tumors for transcript expression analysis by RT-qPCR.

Statistical Analysis

An unpaired 2-tailed Student's *t*-test was performed to evaluate the differences between the control and treatment groups using GraphPad Prism software. All quantified data represent at least 3 independent experiments.

Results

REST Directly Targets MiR-124 and MiR-203 in GSCs

To determine potential immediate direct miR targets of REST in GSCs, we performed REST loss-of-function manipulations by treating 2 HR-GSC cell lines (HR-GSC1 and HR-GSC2)[22] with small-interfering (si)RNA (siREST) or a nontargeting control (siNT). Four cell lines were created: HR-GSC1/siNT, HR-GSC1/siREST, HR-GSC2/siNT, and HR-GSC2/siREST. REST knockdown was confirmed by RT-PCR (Fig. S1A) and western blotting (Fig. S1B). We then performed a genome-wide miR expression analysis to identify miRs with at least 2-fold higher expression in siREST-treated cells than in siNT control cells, as REST is a transcriptional repressor. We observed that siREST-treated cells had higher expression of 128 miRs in HR-GSC1 cells

and 43 miRs in HR-GSC2 cells than the siNT controls. However, only 7 miRs were common between HR-GSC1 and HR-GSC2 in this differential analysis (Fig. 1A), with miR-203 showing the greatest difference in expression in both cell lines (Table S1). MiR-124, a well-known target of REST,[25] was also identified in our screen. The capture of only a few miRs by this analysis suggested that most of the miR targets of REST are cell-line dependent and that REST function is highly context dependent, as we found earlier.[26]

To biochemically validate which of the 7 miRs are targeted by REST, we performed additional REST loss-of-function manipulations by transducing the 2 HR-GSC stable lines with short hairpin (sh)Rest (causing knockdown of REST expression) or nontargeting shNT control, as opposed to the transient knockdown using siREST as in the previous experiments. We had previously shown that shREST expression in these GSC lines decreased their oncogenic properties and prolonged survival in tumor-bearing mice.[22] We first confirmed the knockdown of REST protein by using western blot assays (Fig. 1B) and analyzed the RNA expression levels of the 7 miRs by using RT-qPCR (Fig. 1C). Only 2 of the 7 microRNAs—miR-203 and miR-124—were significantly upregulated upon REST knockdown in both HR-GSC lines. We further confirmed these targets by measuring mature miR transcripts using TaqMan assay (Fig. S1C).

We then performed REST gain-of-function manipulations in 2 low-REST (LR-) GSC lines by introducing either exogenous REST or green fluorescent protein control and confirming REST overexpression using western blotting (Fig. 1D). We then determined the expression levels of miR-203 and miR-124 in these cells. As shown in Fig. 1E, overexpression of REST consistently repressed the expression of both miRs in both LR-GSC lines. Thus, both loss- and gain-of-function results indicated that REST regulates the expression of both miR-124 and miR-203 in GSCs.

To determine whether the expression of miR-124 or miR-203 was suppressed by direct binding of REST on the gene chromatin, we determined the potential REST binding sites (RE1s) present on the miR-124 (Fig. 2A) or miR-203 (Fig. 2B) promoter elements 3000 bp upstream and downstream of the miR-124 or miR-203 transcription start site (TSS) using Mat Inspector (Genomatix software suite v3.3). We found 1 potential RE1 on miR-124 and 4 potential RE1s on miR-203 gene chromatin. We then performed ChIP analysis using either REST or immunoglobulin G (control) antibody and performed qPCR using primers corresponding to sites on

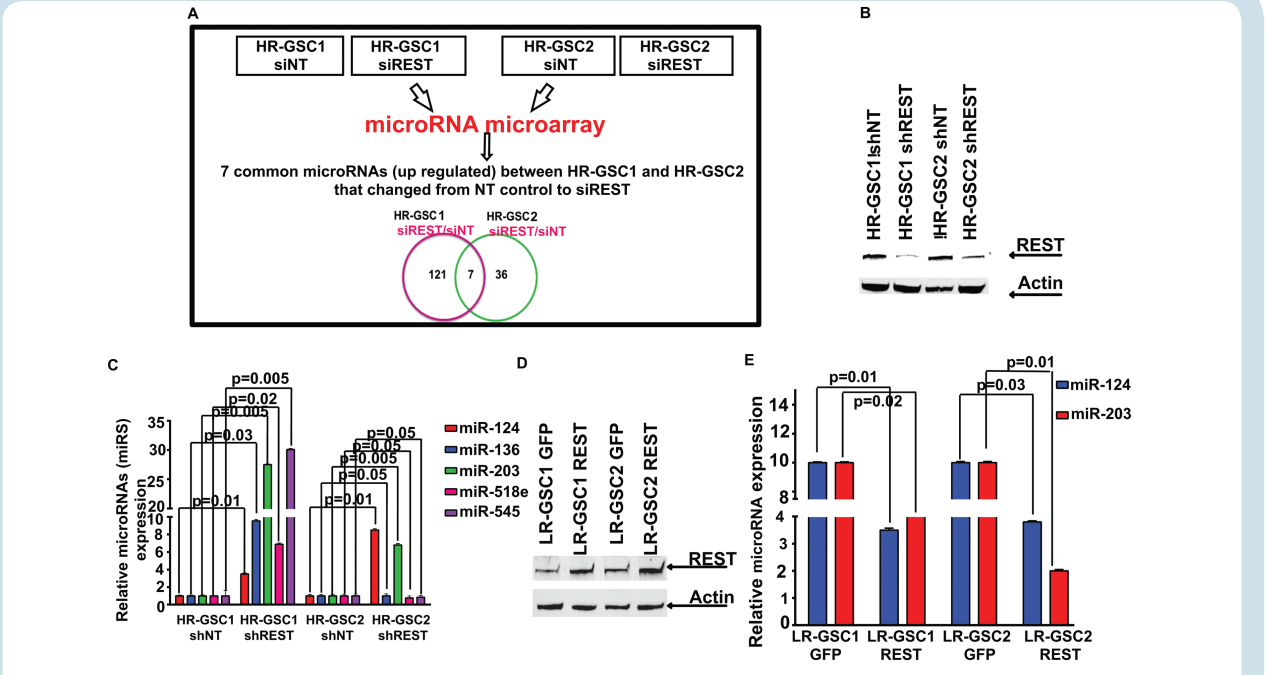

Fig. 1 Identification of miR-124 and miR-203 as REST targets in GSCs. (A, B) Genome-wide microRNA expression analysis. (A) Schematic representation of genome-wide microRNA expression analysis of the 2 HR-GSC cell lines representing REST loss-of-function using siREST manipulations (a total of 4 lines) resulted in only 7 microRNAs common between HR-GSC1 and HR-GSC2 in which REST expression differed between the nontargeting control (siNT) and siREST groups. (B–E) Functional validation of REST–miR-124 and REST–miR-203 pathways. (B, C) Loss-of-function manipulations: only 2 of the 7 microRNAs—miR-203 and miR-124—were significantly upregulated upon REST knockdown in both HR-GSC lines. (B) Stable cell lines were generated using lentiviruses carrying either the NT control or shREST and knockdown of REST protein was confirmed by western blotting assays. (C) MiR expression levels were determined using RT-qPCR. (D, E) Gain-of-function manipulations: expression levels of both miR-124 and miR-203 were decreased upon expression of exogenous REST. Stable lines of LR-GSC1 and LR-GSC2,[22] which express low levels of the REST protein, were infected with retroviruses carrying either green fluorescent protein or exogenous REST gene and the expression levels of REST determined by western blotting assays (D). Analysis of the resulting cells by RT-qPCR showed that overexpression of REST causes lowered miR-124 and miR-203 expression in both the LR-GSC lines (E).

the miR-124 and miR-203 promoter elements. For miR-124, we used the consensus RE1 site upstream of TSS (site#1 at -2648) and a random site (site#2 at -512). For miR-203, we used the 4 consensus RE1 sites upstream of TSS (site#1 at -223, site#2 at -512, site#3 at -762, and site#4 at -1223) and a random site (-2167). As shown, REST was found to bind only to site #1 gene chromatin of miR-124 and to site#4 of miR-203, indicating specific REST binding on these miR genes. No significant REST binding was observed on any of the control random sites.

To determine whether there are additional RE1s on the 2 miR gene chromatins, we performed unbiased, genome-wide REST ChIP-Seq profiles (Fig. S2A–E) using HR-GSC2/shNT and HR-GSC2/shREST cells. We examined for REST binding within 3000 bp upstream and downstream of the TSS of the miR genes. Similar to our bioinformatic and biochemical validation results, we found 1 potential RE1 present on miR-124 (Fig. 2A) and 2 potential RE1s present on miR-203 gene chromatins (Fig. 2B). As expected, we did not see REST binding on the other 5 miR gene chromatins under similar conditions.

To determine whether REST controlled expression of miR-124 and miR-203 via the single miR-124 and miR-203 gene chromatin, we subcloned the binding site found by ChIP assay in front of a luciferase reporter gene. For comparison, we also created reporter gene plasmids with no

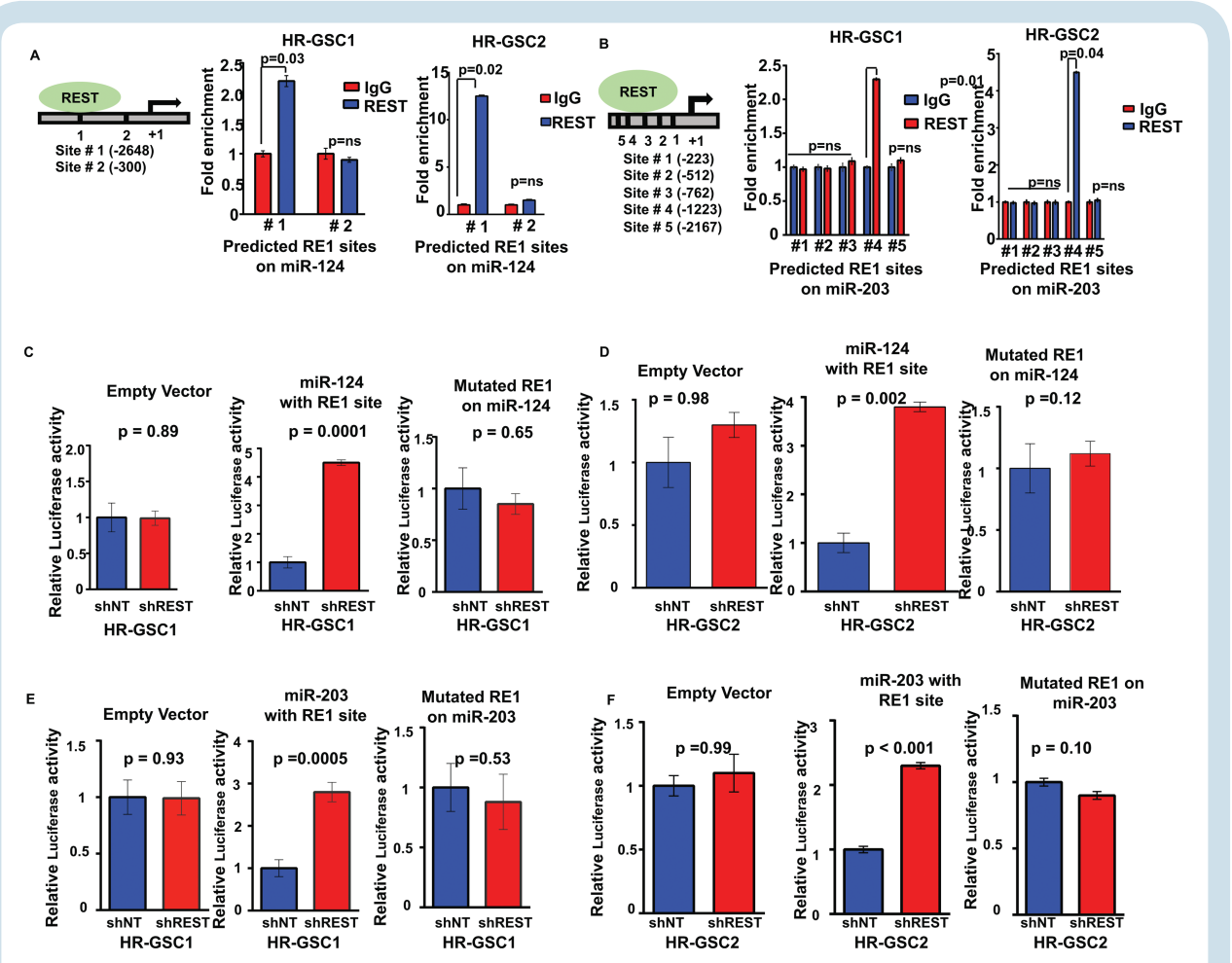

Fig. 2 REST directly targets miR-124 and miR-203 in GSCs through specific sites present in their gene chromatin. (A, B) REST binds to miR-124 (A) and miR-203 (B) gene chromatins in both HR-GSC1 and HR-GSC2. Chromatin immunoprecipitation analysis was performed using either REST or immunoglobulin G (control) antibody followed by RT-qPCR using primers corresponding to potential RE1 sites present on the miR-124 gene chromatin (site #1 at -2648 upstream of the transcriptional start site and site #2, a random site, at -512) and miR-203 gene chromatin (site #1 at -223, site #2 at -512, site #3 at -762, and site #4 at -1223, and a random site #5 at -2167). REST binds to specific RE1 sites on these miR genes. (C–F) REST represses both miR-124 and miR-203 gene expression through the RE1 site present in its gene chromatin. We performed reporter gene analysis using a plasmid containing a luciferase gene downstream of either the specific REST binding site present on the miR-124 (C, D) or miR-203 (E, F) gene chromatins, a mutated version of the sites, or no site, and transfected the resulting plasmids into HR-GSC1/shNT and HR-GSC1/shREST (C, E), and HR-GSC2/shNT and HR-GSC2/shREST (D, F) cells and measured luciferase activity. Results showed that while the luciferase activity remained primarily unaltered when the plasmid with no site was expressed in either shNT- or shREST-expressing cells, it increased in shREST- compared with shNT-expressing cells when the plasmid contained the REST binding site. This increase in luciferase activity in shREST cells was reversed when the plasmid contained the mutated REST binding site.

binding site or with a mutated version of the site. We then transfected these plasmids into HR-GSC1/shNT, HR-GSC1/shREST, HR-GSC2/shNT, and HR-GSC2/shREST cells and measured luciferase activity in the resulting cells. Results shown for miR-124 (HR-GSC1: Fig. 2C; HR-GSC2: Fig. 2D) and miR-203 (HR-GSC1: Fig. 2E; HR-GSC2: Fig. 2F) indicated that when no site was present or when the site was mutated, luciferase activity was similar in the shNT- and sh/Rest-expressing HR-GSC cells. In contrast, when the REST binding site was present, luciferase activity was higher in the shREST-expressing cells than in the shNT-expressing cells. Thus, taken together, these results indicated that REST directly targets miR-124 and miR-203 in GSCs.

Both MiR-124 and MiR-203 Regulate Survival of Mice Harboring GSC-Derived Brain Tumors

To determine whether miR-124 and/or miR-203 is relevant in the regulation of tumorigenesis, we studied whether manipulation of these miRs in GSCs affected the survival of mice bearing brain tumors derived from the altered GSCs. We performed miR gain-of-function experiments in HR-GSC1/shNT and HR-GSC2/shNT cells, which have high REST expression and low miR-124 and miR-203 expression. We transduced both HR-GSC cell lines with lentiviruses containing either the vector (V) control or pre-miR-124 or pre-miR-203 to obtain the following stable cell lines. For HR-GSC1, we generated HR-GSC1.shNT/V, HR-GSC1.shNT/pre-miR-124, and HR-GSC1.shNT/ pre-miR-203 and for HR-GSC2, we generated HR-GSC2.shNT/V, HR-GSC2.shNT/pre-miR-124, and HR-GSC2.shNT/ pre-miR-203. We then selected cells expressing the virus-encoded drug resistance and confirmed the overexpression of miR-124 and miR-203 in the stable cell lines by using RT-qPCR (Fig. S3A). We then transplanted these cells into the brains of nude mice as described before[22] and performed Kaplan–Meier survival analyses. As shown in Fig. 3A (HR-GSC1) and 3B (HR-GSC2), overexpression of either miR-124 or miR-203 in the HR-GSC lines increased the survival of tumor-bearing mice, indicating that both miR-124 and miR-203 have tumor-suppressor functions in GSCs.

Both REST–MiR-124 and REST–MiR-203 Pathways Regulate Survival of Mice Harboring GSC-Derived Tumors

We then determined whether the tumor-suppressor functions of miR-124 and miR-203 are mechanistically connected to REST. We used the 2 previously studied HR-GSC stable lines that were transduced with shREST: HR-GSC1/

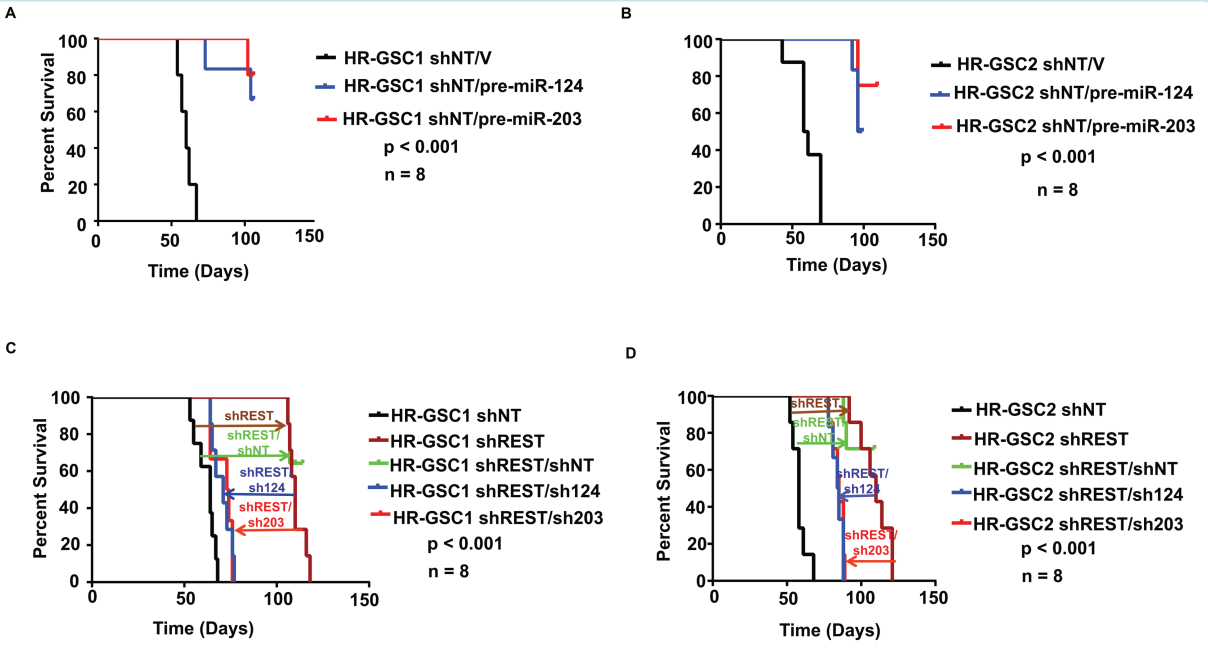

Fig. 3 MiR-124, miR-203, REST-miR-124, and REST-miR-203 pathways regulate survival of mice harboring brain tumors derived from both HR-GSC1 and HR-GSC2 cells. Kaplan–Meier survival plots of mice harboring HR-GSC1.shNT/V, HR-GSC1.shNT/pre-miR-124, and HR-GSC1.shNT/pre-miR-203 (A) and HR-GSC2.shNT/V, HR-GSC2.shNT/pre-miR-124, and HR-GSC2.shNT/pre-miR-203 (B) cells show that overexpression of either miR-124 or miR-203 in either HR-GSC1 or HR-GSC2 cells increased the survival of tumor-bearing mice, indicating that both miR-124 and miR-203 have tumor-suppressor functions in GSCs. Kaplan–Meier survival plots of mice harboring HR-GSC1 lines (HR-GSC1.shNT, HR-GSC1.shREST, HR-GSC1.shREST/shNT, HR-GSC1.shREST/shmiR-124, HR-GSC1.shREST/shmiR-203) (C) and HR-GSC2 lines (HR-GSC2.shNT, HR-GSC2.shREST, HR-GSC2.shREST/shNT, HR-GSC2.shREST/shmiR-124, HR-GSC2.shREST/shmiR-203) (D). Knockdown of REST by shREST in HR-GSC1 and HR-GSC2 cells caused increased survival compared with their shNT controls, as expected.[22] Additional expression of shNT in these shREST-expressing cells did not significantly alter survival. In contrast, the double-knockdown shREST/shmiR-124 or shREST/shmiR-203 cells reversed the increased survival caused by single shREST in both the HR-GSC1 and HR-GSC2 cell lines, indicating that both the REST-miR-124 and REST-miR-203 pathways regulate the tumorigenesis of GSCs.

shREST and HR-GSC2/shREST (expressing low REST, high miR-124, and high miR-203); control cells were HR-GSC1/shNT and HR-GSC2/shNT (Figs. 1C, 1D). As described before, REST knockdown with shREST in these cells increases survival duration in tumor-bearing mice.[22] To determine whether additional knockdown of miR-124 or miR-203 can attenuate these effects of shREST in GSCs, we performed shREST/shmiR-124 and shREST/shmiR-203 double knockdown in these cells. We transduced each of the HR-GSC.shREST lines with lentiviruses containing shNT control, shmiR-124, or shmiR-203; selected cells expressing the virus-encoded drug resistance; and confirmed the knockdown of miR-124 and miR-203 by RT-qPCR (Fig. S3B). We then transplanted these cells into the brains of nude mice, as described in the preceding paragraph, and performed survival analyses. As shown in Fig. 3C, mouse survival was longer in the shREST-expressing HR-GSC1 cells than in the shNT controls, as expected (purple arrow). Also, as expected, expression of additional shNT in the shREST-expressing cells (shREST/shNT: as a control for the expression of shmiRs) did not alter survival significantly (green arrow). However, the double knockdown of either shREST/shmiR-124 (blue arrow) or shREST/shmiR-203 (red arrow) attenuated the increase in survival caused by shREST in these cells. Similarly, in HR-GSC2 cells (Fig. 3D), the double knockdown of either shREST/shmiR-124 or shREST/shmiR-203 decreased the survival duration compared with the control shREST/shNT cells. These results indicated that the REST-mediated tumorigenesis of GSCs operates via both the REST–miR-124 and REST–miR-203 pathways.

REST–MiR-124 Pathway, but Not REST–MiR-203 Pathway, Regulates Self-Renewal in GSCs

We previously found that REST regulates self-renewal of HR-GSCs[22] and, when treated with shREST causing knockdown of REST, both HR-GSC1 and HR-GSC2 showed lowered self-renewal compared with shNT control-treated cells. To examine the roles of miR-124 and miR-203 in REST-mediated tumorigenesis, we determined whether their manipulation in GSCs affected self-renewal in vitro. We first performed gain-of-function experiments. We took both HR-GSC1/shNT and HR-GSC2/shNT cells expressing either the vector control, pre-miR-124, or pre-miR-203 and performed neurosphere assays for 4 generations. As shown, pre-miR-124, but not pre-miR-203, lowered self-renewal of both HR-GSC1 (Fig. 4A) and HR-GSC2 (Fig. 4B) lines.

We then examined whether the REST–miR-124 or REST–miR-203 pathways impacted self-renewal of HR-GSCs. As shown, knockdown of REST using shREST lowered self-renewal, as was seen before, in both HR-GSC1 and HR-GSC2.[22] Further, double knockdown of shREST/shmiR-124, but not shREST/shmiR-203, rescued the lowered self-renewal caused by shREST alone in both HR-GSC1 (Fig. 4C) and HR-GSC2 (Fig. 4D). Thus, the REST–miR-124 pathway

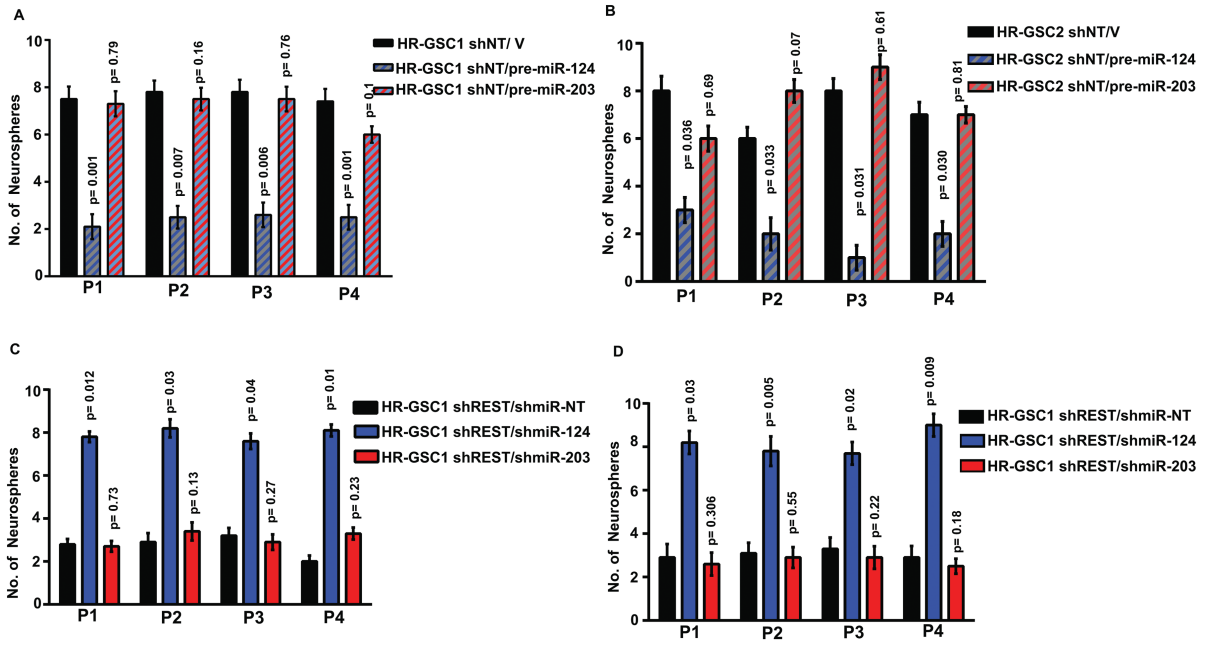

Fig. 4 While MiR-124 and the REST–miR-124 pathway regulate self-renewal of HR-GSCs, miR-203 and the REST–miR-203 pathway do not. MiR-124 and miR-203 gain-of-function in HR-GSC1 cells (A: HR-GSC1.shNT/ pre-miR-124; HR-GSC1.shNT/ pre-miR-203) and HR-GSC2 cells (B: HR-GSC2.shNT/ pre-miR-124; HR-GSC2.shNT/ pre-miR-203) were subjected to neurosphere assays for 4 generations (P1–P4). While the exogenous miR-124 decreased self-renewal as compared with the control cells (HR-GSC1.shNT/Vector and HR-GSC2.shNT/Vector), the exogenous miR-203 did not. Knockdown of REST with shREST in either HR-GSC1 (C: HR-GSC1 shREST/shmiR-NT) or HR-GSC2 (D: HR-GSC2 shREST/shmiR-NT) caused lowered self-renewal, as was observed before (REF). Additional knockdown of miR-124 with shmiR-124 in either HR-GSC1 (C: HR-GSC1 shREST/shmiR-124) or HR-GSC2 (D: HR-GSC2 shREST/shmiR-NT) significantly rescued the lowered self-renewal cause by shREST alone in these cells. In contrast, shmiR-203 had negligible effect in double knockdown cells.

impacted self-renewal, whereas the REST–miR-203 pathway did not. Similar outcomes were also observed for the REST, REST–miR-124 pathway, and REST–miR-203 pathways when we assayed these cells for cell proliferation (Fig. S4A–D). Interestingly, both shREST and pre-miR-124–treated cells maintained self-renewal properties, albeit at a significantly lower efficiency, even after 4 generations. In support of this finding, expression of marker genes for neuronal (microtubule-associated protein 2), glial (glial fibrillary acidic protein), and oligodendrocyte (myelin basic protein) differentiation was not significantly altered in these cells when they were cultured under differentiation conditions (Fig. S5A, B). These results further suggest that although REST is a critical factor in the maintenance of self-renewal of HR-GSCs, there are additional regulators of this property.

REST–MiR-124 Pathway, but Not REST–MiR-203 Pathway, Regulates Apoptosis in GSCs

To determine the roles of miR-124 and miR-203 in REST-mediated tumorigenicity, we determined whether its manipulation in GSCs affected apoptosis in vitro. We first subjected the REST, miR-124, and miR-203 manipulated cells as described in the preceding section to terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assays to determine the cells' apoptotic status in vitro (Fig. S6A–D). As shown, the miR-124 and REST–miR-124 pathway, but not the miR-203 or REST–miR-203 pathway, regulated apoptosis in both the HR-GSC1 and HR-GSC2 cells.

To determine whether the properties of the REST–miR-124 and REST–miR-203 pathways seen in in vitro assays were also present in mouse brain tumors, we took the HR-GSCs with various manipulations described in the preceding sections, transplanted them into the brains of nude mice, waited 40 days, euthanized all the mice, and performed immunofluorescence analysis of the mouse brain tumor sections using anti–nuclear mitotic apparatus protein (NuMA) antibody that selectively stains human cells and for apoptosis using TUNEL assays. Quantification data showed that the overexpression of miR-124, but not miR-203, in the 2 HR-GSC tumors increased apoptosis (Fig. 5A: HR-GSC1; Fig. 5B: HR-GSC2) and the knockdown of miR-124, but not miR-203, in the 2 HR-GSC/shREST tumors significantly lowered apoptosis (Fig. 5C: HR-GSC1; Fig. 5D: HR-GSC2). We then performed complementary assays for apoptosis using annexin V

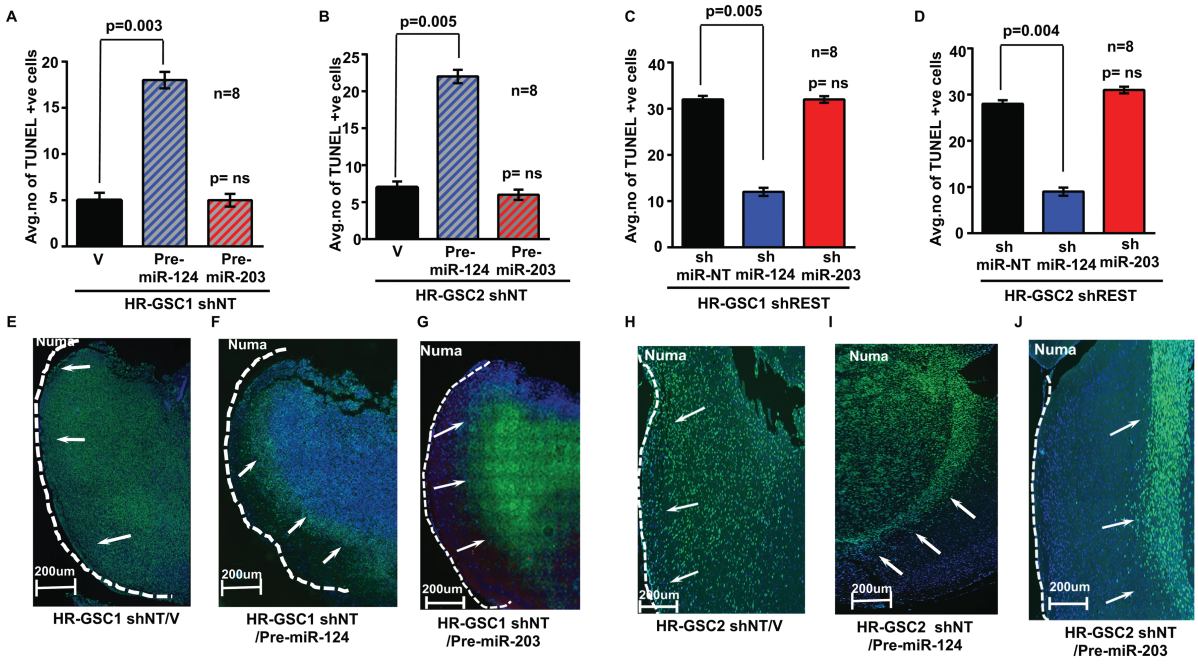

Fig. 5 While miR-124 and the REST–miR-124 pathway regulate both apoptosis and invasion, miR-203 and the REST–miR-203 pathway regulate invasion in GSC-derived tumors in mouse brains. Quantification of double immunofluorescence staining of brain sections with anti-NuMA antibody that selectively stains human cells and TUNEL (A–D) showed increased apoptosis with the overexpression of miR-124 in HR-GSC1 (A) and HR-GSC2 (B) tumors and decreased apoptosis with knockdown of miR-124 in HR-GSC1/shREST (C) and HR-GSC2/shREST (D) tumors. In contrast, neither overexpression of miR-203 in either of the HR-GSC tumors (A and B, respectively) nor double knockdown of miR-203 in either the HR-GSC1/shREST (C) or HR-GSC2/shREST (D) tumors significantly altered tumor cell apoptosis. (E–J) Both MiR-124 and miR-203 regulate invasion in GSC-derived tumors in mouse brains. We stained the mouse brain tumor sections with anti-NuMA antibody and performed immunofluorescence analysis. Both HR-GSC1 and HR-GSC2 cells produced highly invasive tumors with cell invasion from the core of the tumor to the pial surface (E: HR-GSC1.shNT/Vector [V], H: HR-GSC2.shNT/Vector [V]), similar to what was seen previously.[22] Overexpression of either miR-124 (F, I) or miR-203 (G, J) in both HR-GSC1 (F, G) and HR-GSC2 (I, J) tumors resulted in the blockade of invasion and the formation of circumscribed tumors (F: HR-GSC1.shNT/Pre-miR-124, G: HR-GSC1.shNT/Pre-miR-203, I: HR-GSC2.shNT/Pre-miR-124; J: HR-GSC2.shNT/Pre-miR-203).

staining (Fig. S7A–D). As shown, both REST and miR-124 impacted apoptosis, whereas miR-203 did not.

Both REST–MiR-124 and REST–MiR-203 Pathways Regulate Invasion in GSCs

To determine the role of miR-124 and miR-203 in invasion in vitro, we took the REST, miR-124, and miR-203 manipulated cell lines as described above and measured the impact of these manipulations on cellular invasion using invasion chamber assays. The addition of both exogenous pre-miR-124 (Fig. S8A) and pre-miR-203 (Fig. S8B) to either HR-GSC1 or HR-GSC2 cells decreased invasion compared with the control vector, indicating that both miR-124 and miR-203 negatively regulate this process. To determine whether the REST–miR-124 pathway or the REST–miR-203 pathway regulates cellular invasion, we took both the HR-GSC1 and HR-GSC2 cell types with shREST/shmiR-124 (Fig. S8C) or shREST/shmiR-203 (Fig. S8D) double knockdown. The results indicated that the decrease in cellular invasion caused by shREST could be significantly reversed by the addition of either shmiR-124 or shmiR-203. Thus, both the REST–miR-124 pathway and the REST–miR-203 pathway control cellular invasion in HR-GSCs in vitro.

We then examined the impact of these manipulations on invasion of HR-GSC-derived mouse brain tumors. To differentiate the human GSC cells from the mouse brain cells, we again stained the tumor sections with anti-NuMA antibody and performed immunofluorescence analysis of sections cut through the middle of the tumor. As shown, we found that invasion of HR-GSC1 (Fig. 5E–G) and HR-GSC2 (Fig. 5H–J) tumors was suppressed by overexpression of either miR-124 (Fig. 5F, I) or miR-203 (Fig. 5G, J) compared with the V control. Previously, we had reported that REST causes a high degree of migration, causing cell invasion from the core of the tumor to the pial surface,[22] and knockdown of REST caused tumors with lower invasiveness. We now reproduced those results and when we expressed shREST in either HR-GSC1 or HR-GSC2, the tumors showed significantly lowered invasion (Fig. S9). Further, double knockdown of REST and either miR-124 or miR-203 significantly reversed the effect, resulting in highly invasive HR-GSC1 (Fig. S9A–C) and HR-GSC2 tumors (Fig. S9D–F). Thus, these results indicated that both the REST–miR-124 pathway and the REST–miR-203 pathway regulate invasion in HR-GSC tumors.

Identification of Potential Downstream Targets of the REST–MiR Axis in GBM Tumorigenesis

To determine the potential targets, we analyzed our transcriptome dataset of REST knockdown in HR-GSCs.[22] Because REST is a transcriptional repressor, we considered only those genes that are downregulated upon REST knockdown with a fold change of 2 or more. We then downloaded the predicted targets of miR-124 and miR-203 using miRwalk 2.0 and compared the genes that are common targets of miR-124 and miR-203 with the genes that are downregulated upon REST knockdown. The comparison yielded 18 common genes (Table S2). We performed ingenuity pathway analysis on these genes. Because GBM invasion is a common characteristic of both miR-124 and miR-203, we focused on 8 genes that were suggested by immunoprecipitation assay to regulate cellular movement.

For biochemical validation of the role of these miR targets, we made transcripts from mouse brain tumors generated from various gain- and loss-of-function cells and analyzed them by RT-qPCR. *REST* transcripts in the tumors were not significantly different from the injected cells (Fig. S10A, B). When pre-miR-124 and pre-miR-203 were individually overexpressed in HR-GSC1/shNT and HR-GSC2/shNT cells, the tumors overexpressed miR-124 and miR-203 compared with vector control (Fig. 6A), validating maintenance of molecular properties of the injected cells in the tumor. We then analyzed the expression of the 8 miR targets in these cells. Only 4 of the targets (*KITL, SEMA6D, NRP2, and THBS1*) were significantly downregulated by both pre-miR-124 and pre-miR-203 compared with the vector control (Fig. 6B: HR-GSC1.shNT; Fig. 6C: HR-GSC2.shNT). The other 4 potential miR-targets showed no significant change in expression (Fig. S11A: HR-GSC1.shNT, Fig. S11B: HR-GSC2.shNT). We next performed the corollary loss-of-function experiments. We examined the tumors expressing shmiR-NT control, shmiR-124, or shmiR-203 in HR-GSC1/shREST and HR-GSC2/shREST and confirmed the miR knockdown (Fig. 6D) in these tumors by RT-qPCR. We also analyzed the expression of the same 8 miR targets in these tumors. Corroborating the gain-of-function results, *KITL, SEMA6D, NRP2, and THBS1* were significantly upregulated compared with the shmiR-NT control (Fig. 6E: HR-GSC1.shREST; Fig. 6F: HR-GSC2.shREST). The other 4 potential targets showed no significant change (Fig. S11C: HR-GSC1.shREST; Fig. S11D: HR-GSC2.shREST). Thus, these results support *KITLG, SEMA6D, NRP2*, and *THBS1* as common downstream targets of miR-124 and miR-203 in HR-GSC–mediated tumor invasion.

Discussion

Identifying the mechanisms that REST employs to regulate the oncogenic properties of GSCs is critical for the development of more effective therapeutic approaches to block GSC-mediated tumorigenesis. Here, we show that REST represses 2 major miR genes to regulate overlapping oncogenic properties in GSCs: miR-124 and miR-203. Whereas the REST–miR-124 pathway regulates cell proliferation, apoptosis, and invasion, the REST–miR-203 pathway regulates invasion of GSC-mediated tumorigenesis. These mechanisms could potentially be utilized to block REST-mediated GBM tumorigenesis—for example, by expression of mimics of miR-124 or miR-203 or both. Such miR mimics are being tested in open-label phase I clinical trials (clinicaltrials.gov).

MiR-124, a known REST target,[25] was suggested to be a downstream target of REST in GSCs[21] and in suppressing pro-survival stress responses in glioblastoma.[27] Our findings describe the novel REST-miR-203 pathway, which regulates invasion in HR-GSC tumors, with REST suppressing miR-203 gene expression and miR-203 functioning as a tumor suppressor. The recent observation that miR-203 expression was significantly lower in a large number of high-grade GBM tumor tissues than in low-grade glioma tissues or normal brain tissues[28,29] supports miR-203's role

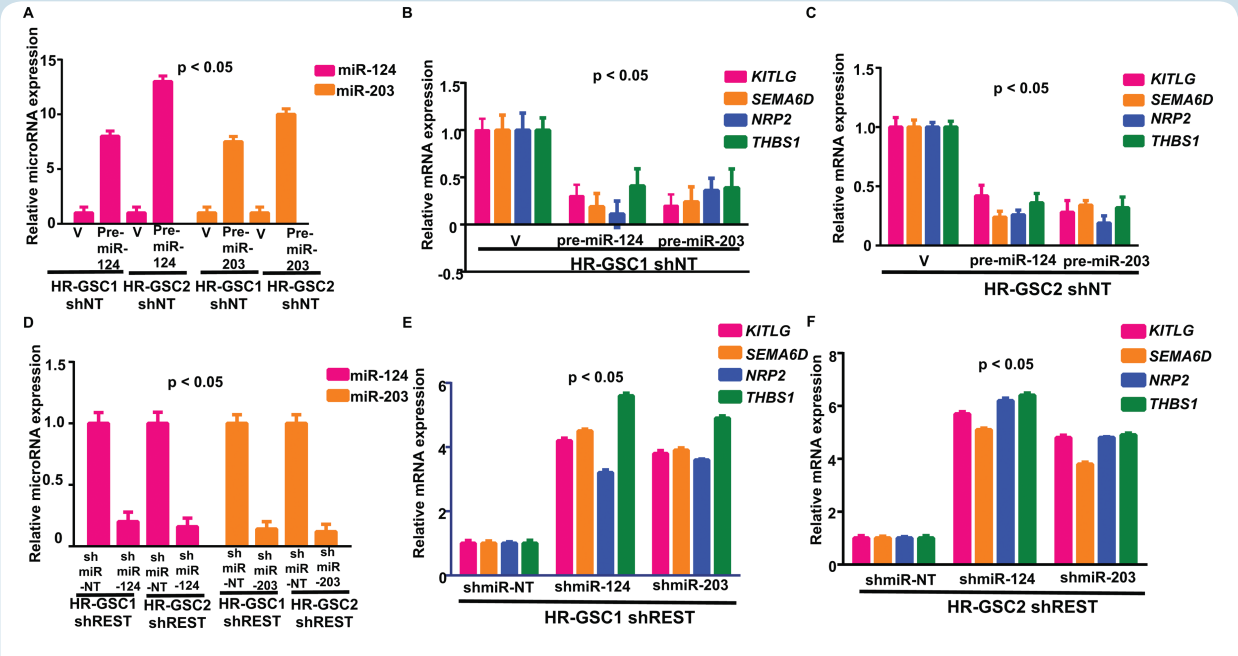

Fig. 6 **Identification of downstream targets of REST–microRNA axis in GBM tumorigenesis.** (A–C) Transcripts from mouse brain tumors generated from HR-GSC1/shNT (A, B) and HR-GSC2/shNT (A, C) cells expressing pre-miR-124, pre-miR-203, or the control vector were analyzed by RT-qPCR for the expression of miR-124 and miR-203 (A), *KITLG, SEMA6D, NRP2, and THBS1* (B, C). (D–F) Transcripts from mouse brain tumors generated from HR-GSC1/shREST (D, E) and HR-GSC2/shREST (D, F) cells expressing either shmiR-124, shmiR-203, or shmiR-NT control were analyzed by RT-qPCR for the expression of miR-124 and miR-203 (D), *KITLG, SEMA6D, NRP2, and THBS1* (D, F).

as a tumor suppressor in GBM. In addition, miR-203 is known to act as a tumor suppressor in other cancers.[30–32]

Interestingly, in two previous publications, miR-203 positively regulated invasion as well as cell proliferation in some glioma cell lines.[28,29] However, our studies indicate that the REST–miR-203 pathway specifically regulates invasion but not cell proliferation or apoptosis in HR-GSCs in vitro and in mouse orthotopic tumor models. It is unclear whether this difference in activity is due to (1) the use of GSCs derived from primary GBM tumors in the current study rather than the glioma cell lines used in the other studies, (2) cell culture conditions that can affect various signaling pathways,[26] or (3) a special property of the HR class of GSCs, in which REST has yet-unknown functions in the cell such that the invasion property is exacerbated in these GSCs.

Because our studies involved tumor-derived GSCs, they could not determine whether REST targets both miR-124 and miR-203 in all cells in the tumor or whether it targets one over the other in distinct tumor cells depending on the microenvironment. One conjecture would be that when the tumor requires both cell proliferation and invasion, REST would selectively repress miR-124. In contrast, when the need of the tumor is essentially invasion, rather than cell proliferation, REST would selectively repress miR-203. Single-cell analysis of the tumor would shed light on such mechanisms.

Supplementary material

Supplementary material is available at *Neuro-Oncology* online.

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Conflict of interest statement. No conflicts of interest.

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