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ORIGINAL PAPER

Downregulation of miR-383 promotes glioma cell invasion by targeting insulin-like growth factor 1 receptor

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Abstract Invasiveness is a major clinical feature of glioma, an aggressive brain tumor with poor prognosis. Although there is emerging evidence that some microRNAs are involved in the glioma cell invasion process, it remains necessary to find functional microRNAs and elucidate the underlying molecular mechanisms. Here, we reported that a microRNA, miR-383, was downregulated in gliomas and inversely correlated with glioma pathological grades. Downregulation of miR-383 enhanced, whereas upregulation of miR-383 inhibited, the glioma cell invasive ability. Furthermore, we found that downregulation of miR-383 activated the AKT signaling following upregulation of MMP2 expression by directly targeting insulin-like growth factor 1 receptor (IGF1R). Importantly, we demonstrated that IGF1R expression is critical for miR-383 downregulation-induced cell invasion. Taken together, these findings uncover a novel regulatory mechanism for constitutive IGF1R signaling activation in glioma cancer and may provide miR-383 as a useful diagnostic marker or therapeutic target.

Keywords miR-383 · Glioma · Invasion · IGF1R

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Instruction

Gliomas make up ~30 % of all brain and central nervous system tumors and 80 % of all malignant brain tumors [1]. Gliomas are rarely curable. The cumulative 2-year survival rate is <25 %, and the median survival time of the grade 4 glioma, glioblastoma multiforme, is less than 12 months [2–4]. Such poor prognosis of malignant gliomas is largely attributed to a high tendency to diffusely infiltrate and migrate into surrounding brain tissue [5, 6]. Several major acquired genetic mutations, including p53, PTEN and EGFR, have been found in glioma progression [7]. However, the microRNA-involved mechanisms are still unclear and remain to be elucidated. Thus, exploring functional microRNAs and delineate the mechanisms that regulate the glioma invasion might allow the identification of novel targets for prognosis and therapeutic intervention.

The type 1 insulin-like growth factor (IGF) receptor (IGF1R) signaling is constitutively activated in various types of human cancer, including lung, breast, prostate and glioma cancers [8–11]. Meanwhile, IGF1R has been demonstrated to play critical roles in multiple biological processes of tumor diseases, including malignant transformation, proliferation, anti-apoptosis, vascularization, invasion and distant metastasis [12, 13]. Previously, it has been reported that the IGF/IGF1R signaling could induce tumor invasion via activation of AKT kinase following upregulation of matrix metalloproteinase-2 (MMP-2) [14–16]. However, the regulatory mechanism of IGF1R expression and the IGF1R/AKT/MMP2 axis have not been investigated in gliomas.

MicroRNAs are a class of 21- to 24-nucleotides (nt) noncoding RNA molecules which are able to control gene expression at the posttranscriptional level by specifically interacting with their target mRNA molecules [17, 18].

Emerging evidence identifies microRNAs as key regulatory molecules in tumor progression, functioning as oncogene or tumor suppressor [18–20]. Herein, by analyzing the microRNA expression profiles, miR-383 was screened out to be downregulated in gliomas. Further, we confirmed its low expression in tissues and cell lines when compared with that in normal brain tissue. The potential tumor suppressor role of miR-383 was then investigated. As expected, upregulation of miR-383 inhibited, whereas inhibition of miR-383 promoted glioma cell invasion. Moreover, we found that miR-383 directly targeted the IGF1R. Downregulation of miR-383-induced IGF1R, activation of AKT signaling following upregulation of MMP2, leading to highly invasive ability. Taken together, our results suggest that downregulation of miR-383 promotes glioma cell invasion by targeting IGF1R.

Materials and methods

Cell culture

Glioma cell lines, U87MG and A172, were supplied by the "The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CBTCCCAS)" and routinely maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (HyClone, Logan, UT).

Plasmid, siRNA and transfection

The region of human IGF1R 3′UTR, from 5,073 to 5,616, generated by PCR amplification from DNA of the U87MG cells, was cloned into pGL3 vector (Promega, Madison, WI). The primers selected were as the following: IGF1R-3′UTR-luc-up, 5′-GCCCCGCGGAGATGCTGAAGATACAGAC C-3′; IGF1R-3′UTR-luc-dn, 5′-GCCCTGCAGAACTGAC TACCCGTAATCTA-3′. IGF1R-3′UTR-mutant-luc-up, 5′-ACCTAACATCCTACTCTGGAAACTGTACTCGGAGT TAAGGCGAATTGTT-3′; IGF1R-mutant-3′UTR-luc-dn, 5′-AACAATTCGCCTTAACTCCGAGTACAGTTT CCA GAGTAGGATGTTAGGT-3′. IGF1R siRNA: CAAAUUA UGUGUUUCCGA AUU. The miR-383 mimic and inhibitor were purchased from RiboBio (Guangzhou, Guangdong, China). Transfection of the plasmids, siRNAs, microRNA mimic and inhibitor were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA extraction and real-time quantitative PCR

Total miRNA from cultured cells and fresh surgical gastric tissues was extracted using the microRNA Fast Extraction Kit (BioTeke Corporation, Beijing, China) according to the manufacturer's instructions. Reverse transcription and quantification of miR-383 expression was assessed with the RiboBio miRNA kit. The expression of miRNA was defined based on the threshold cycle (Ct), and relative expression levels were calculated as 2-[(Ct of miR−383)−(Ct of U6)] after normalization with reference to expression of U6 small nuclear RNA.

Total RNA from cells was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction; 1 μg of RNA from each sample was used for cDNA synthesis primed with random hexamers. The primers used for gene expression are MMP2, forward, 5′-TTGCTGG AGACAAATTCTGG-3′, and reverse, 5′-AAGAA GTAGC TGTGACCGCC-3′. Expression data were normalized to the geometric mean of housekeeping gene GAPDH (forward, 5′-GAAGGTGA AGGTCGGAGTCA-3′, and reverse, 5′-TTG AGGTCAATGAAGGGGTC-3′) to control the variability in expression levels and calculated as 2-[(Ct of gene)−(Ct of GAPDH)], where Ct represents the threshold cycle for each transcript.

Transwell matrix penetration assay

Cells (1×10^4) to be tested were plated on the top side of polycarbonate Transwell filter (with Matrigel) in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA) and incubated at 37 °C for 20 h. Invaded cells on the lower membrane surface were fixed in 1 % paraformaldehyde, stained with crystal violet and counted (Ten random 100 × fields per well). Relative folds of invaded cell were normalized to control. Three independent experiments were performed, and the data are presented as mean ± standard deviation (SD).

Western blotting

Cells were harvested in cell lysis buffer (Cell Signaling Technology; Cat#: 9803) and heated for 5 min at 100 °C. Equal quantities of denatured protein samples were resolved on 10 % SDS–polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Roche). After blocking with 5 % nonfat dry milk in Tris-buffered saline/0.05 % Tween 20 (TBST), the membrane was incubated with a specific primary antibody, followed by the horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using ECL reagents (Pierce). The anti-IGF1R, anti-MMP2, anti-p-AKT and anti-AKT antibodies were purchased from Abcam (Cambridge, MA).

Luciferase assays

Cells (2×10^4) were seeded in triplicates in 48-well plates and cultured for 24 h. One hundred nanogram of

pGL3-IGF1R-3′UTR, or pGL3-IGF1R-3′UTR-mutant luciferase plasmid, plus 5 ng of pRL-TK renilla plasmid (Promega, Madison, WI) was transfected into indicated cells using the Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's recommendation. Luciferase and renilla signals were measured 36 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol.

Statistical analysis

The two-tailed Student's *t* test was used to evaluate the significance of the differences between two groups of data in all pertinent experiments. A p values <0.05 was considered significant.

Results

miR-383 is downregulated in human glioma tissues and cell lines

Accumulating evidence indicated that microRNAs play important roles in cancer progression, including human glioma. Here, we first analyzed a published microarray-based, high-throughput microRNA expression dataset (GSE25631) to screen out dysregulated microRNAs. Microarray analysis revealed that one microRNA, miR-383, was significantly downregulated in human glioma tissues ($n = 82$) when compared with that in normal brain tissues ($n = 5$) ($p < 0.001$) (Fig. 1a). Furthermore, real-time PCR analysis showed that miR-383 levels were low in all 3 glioma tissues of grade II and became markedly lower in glioma tissues of grade IV (Fig. 1b), suggesting that downregulation of miR-383 might play important roles in glioma progression. Consistently, we found that miR-383 was downregulated in glioma cell lines, U87MG and A172, compared to that in normal brain tissues (Fig. 1b). Taken together, these results demonstrated miR-383 was downregulated in human glioma.

Upregulation of miR-383 inhibits glioma cell invasion

As shown in Fig. 1b, we found that miR-383 expression levels become further lower in glioma grade IV, which is mainly characterized with its highly invasive abilities. We hypothesized that miR-383 might have effects on the glioma invasion. We then transfected glioma cell line U87MG with hsa-miR-383 mimic and examined the effects of miR-383 on cell invasion (Fig. 2a). As shown in Fig. 2b, an transwell matrix penetration assay showed that miR-383 upregulation significantly reduced the invasive ability of U87MG cells, to about 16.7 % of control (Fig. 2b).

Inhibition of miR-383 promotes glioma cell invasion

We further examined the effect of miR-383 inhibition on glioma cell invasion. Consistent with abovementioned results, transwell matrix penetration assays showed that miR-383 suppression dramatically promoted the invasive behavior of A172 when compared with that of control cells (Fig. 3a, b). Collectively, our results suggest that miR-383 plays a suppressive role in glioma invasion.

miR-383 regulates IGF1R/AKT signaling and MMP2 expression

We further investigated the underlying molecular mechanism that might be responsible for the miR-383-mediated repression of cell invasion. IGF1/IGF1R signaling pathway is found frequently hyperactivated in glioma[11, 21], and

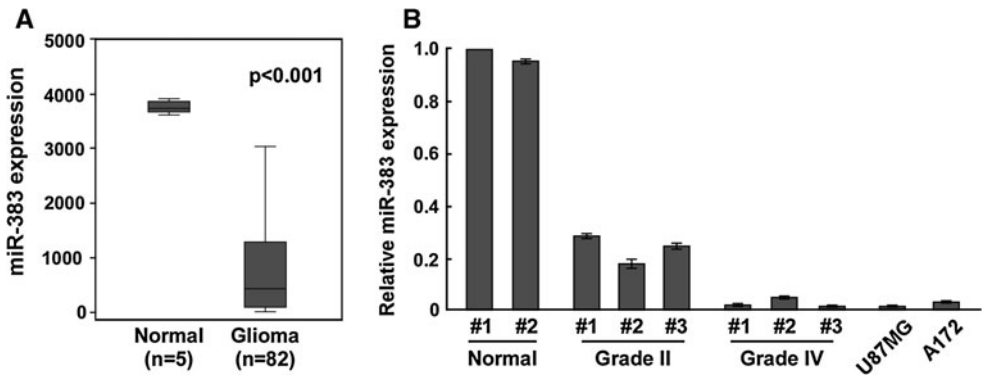

Fig. 1 miR-383 was downregulated in human glioma tissues and cell lines. **a** Analysis of a published high-throughput microarray data (GSE25631) revealed that miR-383 was downregulated in glioma patient tissues ($n = 82$), compared with that in noncancerous brain tissue ($n = 5$). **b** Real-time PCR analysis of miR-383 expression in 2 normal brain tissues, 3 grade II and 3 grade IV glioma tissues as well as two glioma cell lines, U87MG and A172. Transcript levels were normalized by *U6* expression. Each bar represents the mean of three independent experiments. $^*p < 0.05$

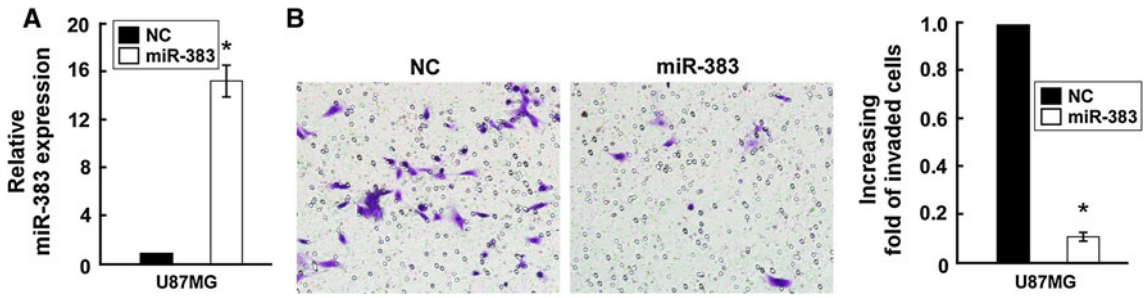

Fig. 2 Upregulation of miR-383 in U87MG inhibits cellular invasive ability. **a** Real-time PCR analysis of miR-383 expression in U87MG cells transfected with negative control (*NC*) and miR-383 mimic. **b** Representative micrographs (*left*) and quantification (*right*) of invaded cells were analyzed using the Transwell matrix penetration assay. Each bar represents the mean of three independent experiments. $*p < 0.05$

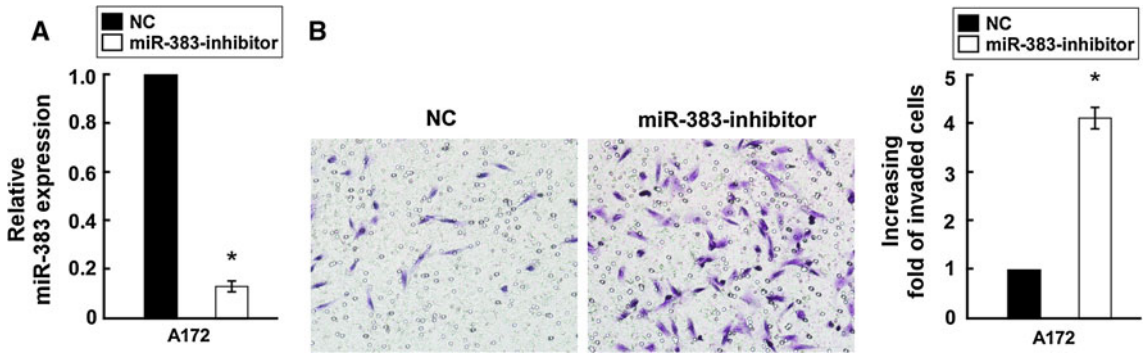

Fig. 3 Inhibition of miR-383 in A172 promotes cell invasion. **a** Real-time PCR analysis of miR-383 expression in A172 cells transfected with negative control (*NC*) and miR-383 inhibitor. **b** Representative micrographs (*left*) and quantification (*right*) of invaded cells were analyzed using the Transwell matrix penetration assay. Each bar represents the mean of three independent experiments. $*p < 0.05$

activation of IGF1/IGF1R signaling pathway would induce cell invasion via activation of AKT signaling and upregulation of MMP2 [14–16]. We then examined whether miR-383 regulated the IGF1R, p-AKT and MMP2 expression. As shown in Fig. 4a, Western blotting analysis revealed that IGF1R, p-AKT and MMP2 were significantly decreased in the miR-383-overexpressing cells, but increased in the miR-383 inhibited cells. Consistently, real-time PCR analysis indicated that MMP2 was transcriptionally regulated by miR-383. Therefore, our results suggest that miR-383 downregulation might induce cell invasion via activation of IGF1R/AKT signaling following upregulation of MMP2 expression.

miR-383 directly targets IGF1R

As a matter of fact, when we used the miRanda tool for miR-383 target prediction, we found that IGF1R is the potential target of miR-383 (Fig. 5a). Importantly, Western blotting analysis showed that ectopic expression of miR-383 dramatically decreased, but inhibition of miR-383 increased, IGF1R protein expression in U87MG and A172 cell lines (Fig. 4a). To examine whether miR-383-induced IGF1R downregulation was mediated by the 3-untranslated region (3′UTR) of IGF1R, we subcloned the IGF1R 3′UTR fragment, containing the miR-383-binding site, into pGL3 dual luciferase reporter vectors. As shown in Fig. 5b, overexpression of miR-383 decreased and inhibition of miR-383 increased the luciferase activity of the IGF1R-3′UTR. However, neither miR-383 overexpression nor inhibition altered the luciferase activity of the IGF1R-3′UTR-mutant, containing point mutations in the miR-383-binding seed region of IGF1R-3′-UTR (Fig. 5c), suggesting that miR-383 specifically targets the 3′-UTR of IGF1R. Collectively, our results demonstrate that IGF1R is a *bona fide* target of miR-383.

IGF1R expression is critical for miR-383 downregulation-induced cell invasion

We further investigated the role of IGF1R expression in miR-383 downregulation-induced cell invasion. As shown in Fig. 6, we found that the miR-383 inhibition-induced cell invasion was abolished by IGF1R downregulation. Therefore, our results suggest that IGF1R plays important role in miR-383-mediated cell invasion.

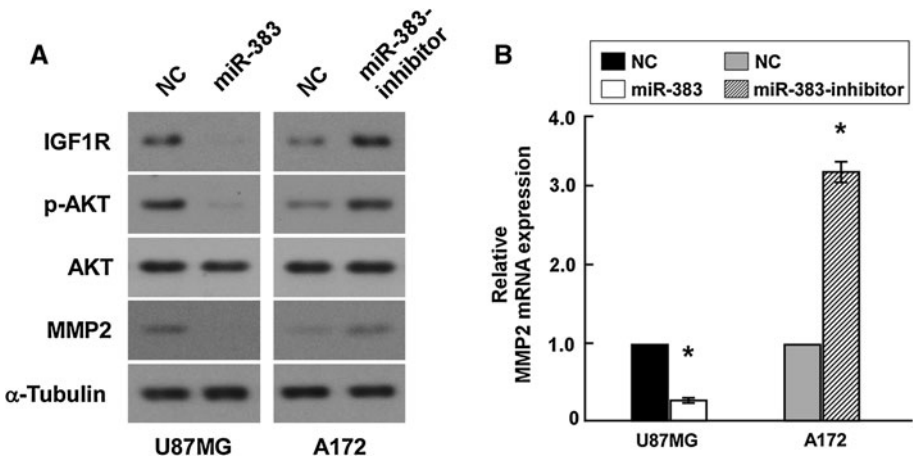

Fig. 4 miR-383 regulates IGF1R/AKT signaling and MMP2 expression. **a** Western blotting analysis of IGF1R, p-AKT, AKT and MMP2 expression in miR-383-transduced or miR-362-inhibited cells. α-Tubulin served as the loading control. **b** Relative mRNA expression of MMP2 in indicated cells assessed by real-time PCR. *GAPDH* was used as a loading control. Each bar represents the mean of three independent experiments. $*p < 0.05$

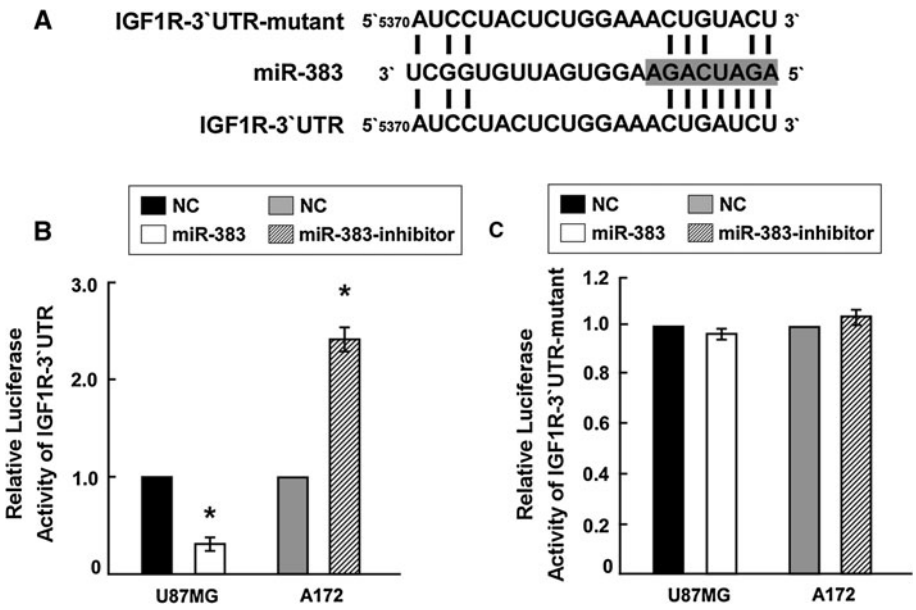

Fig. 5 miR-383 suppresses IGF1R expression via directly targeting IGF1R- 3′UTR. **a** Predicted miR-383 target sequence in the 3′UTR of IGF1R (IGF1R-3′UTR) and mutant containing two mutated nucleotides in 3′-UTR of IGF1R (IGF1R-3′UTR-mut). **b** Luciferase assays of pGL3-IGF1R-3′UTR reporter in miR-383-transduced or miR-383-inhibited cells. **c** Luciferase assays of pGL3-IGF1R-3′UTR-mutant reporter in miR-383-transduced or miR-383-inhibited cells. Each bar represents the mean ± SD of three independent experiments. $*p < 0.05$

Discussion

The key findings of the current study provide new insight into the tumor suppressor role of miR-383, the downregulation of which contributes to IGF1R signaling and glioma cell invasion. We found that miR-383 directly repressed IGF1R expression, leading to upregulation of p-AKT and MMP2, and IGF1R expression was demonstrated to be critical for the miR-383 downregulation-induced cell invasion.

The IGF1R is a multifunctional membrane-associated tyrosine kinase, which can be activated by its ligands IGF-I, IGF-II and insulin. Constitutive activation of the IGF1R signaling is observed in many different cancers, including lung, breast, prostate and glioma cancers [8–13]. It was demonstrated that IGF-I, IGF-II and insulin contributed to the hyperactivation of IGF1R signaling [12, 13]. Meanwhile, the IGF1R protein itself was frequently found to be overexpressed in cancers, however, its regulatory mechanism remains unclear in gliomas. Herein, we suggest a microRNA-mediated mechanism, by which downregulation of miR-383 leads to IGF1R overexpression in gliomas.

miR-383 has been found to be downregulated in human testicular embryonal carcinoma and medulloblastomas [22, 23]. Lian J et al. [22] found that miR-383 directly targeted interferon regulatory factor-1 (IRF1). Downregulation of miR-383 inactivated the pRb signaling and promoted testicular germ cell tumor. Li and colleagues reported that miR-383 was downregulated in human medulloblastoma, and its downregulation directly upregulated peroxiredoxin 3 to promote cell growth [23]. Consistently, we newly find that miR-383 is downregulated in glioma and its downregulation promotes cell invasion. Recently, Yin M et al. [24] reported that miR-383 is one of the most downregulated miRNA in TGF-β1-treated mouse ovarian granulosa cells (GC). Moreover, they found that the transcription factor steroidogenic factor-1 (SF-1) specifically bound to the promoter and directly transactivated

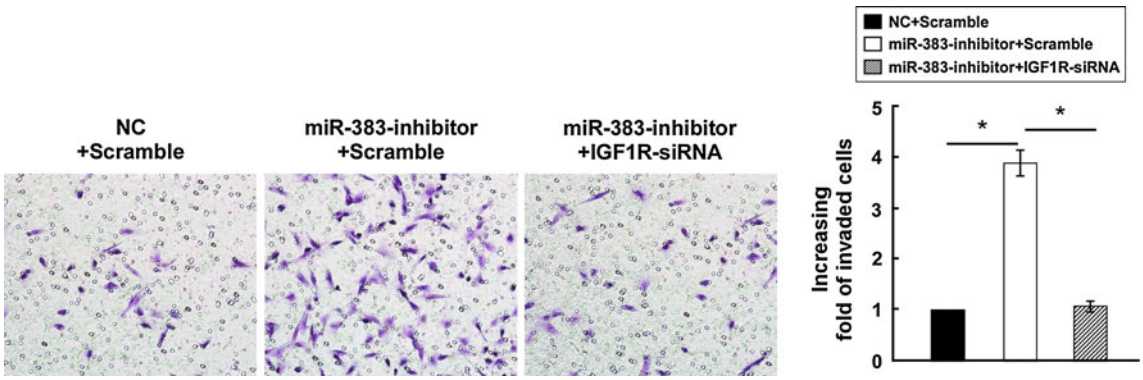

Fig. 6 IGF1R expression is critical for miR-383 downregulation-induced cell invasion. Representative micrographs (*left*) and quantification (*right*) of transwell matrix penetration assay indicated the invasive ability of indicated cells transfected with miR-383 inhibitor, or miR-383 inhibitor plus IGF1R-siRNA

miR-383, functioning to promote estradiol release from mouse ovarian granulosa cells by targeting RBMS1. It remains still unclear about the downregulation mechanism of miR-383 in cancers. Whether it is caused by SF-1, or other transcription factors, or the methylation of miR-383 promoter, remains to be investigated.

Conflict of interest None.

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