MicroRNA-149 Regulates Proliferation, Migration, and Invasion of Pituitary Adenoma Cells by Targeting ADAM12 and MMP14*

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[Abstract] Objective: Pituitary adenomas (PAs) can adapt an aggressive phenotype by invading adjacent brain structures with rapid cellular proliferation. Previous studies demonstrated that excessive expression of metalloproteases ADAM12 and MMP-14 is instrumental for the active proliferation and invasiveness of PA cells *in vitro* and of tumors *in vivo*. However, the mechanisms regulating ADAM12 and MMP-14 expression in PAs remain unclear. **Methods:** Target gene prediction and transcriptomic profiling of invasive *vs*. noninvasive human PA samples were performed to identify miRNA species potentially involved in the regulation of ADAM12 and MMP14. For cellular analyses of miRNA functions, two mouse PA cell lines (AtT20 and TtT/GF) were transfected with miR-149-3p and miR-149-5p, respectively. The effects of miR-149 (3p and 5p) on expression levels of ADAM12 and MMP14 were determined by Western blotting followed by an analysis of proliferation and colony formation assays, scratch migration assays, and invasion assays. **Results:** A significant downregulation of miRNA-149 was observed in invasive *vs*. noninvasive PA (0.32 *vs*. 0.09, $P<0.0001$). In AtT-20 and TtT/GF mouse PAs cells, transfection of mimic miRNA-149 (3p and 5p) caused a significantly reduced cell proliferation and matrigel invasion, whilst the effect on cell migration was less pronounced. Both strands of miRNA-149 (3p and 5p) markedly reduced protein levels of ADAM12 and MMP-14 by at least 40% in both cell lines. **Conclusion:** This study proved that the invasiveness of PA cells is, at least partly, regulated by miRNA-149-dependent expression of ADAM12 and MMP-14.
Key words: pituitary adenoma; microRNA; miRNA-149; ADAM12; MMP-14; invasion

Pituitary adenoma (PA) is the second most common intracranial benign tumor. According to a recent survey, it accounts for about 10%–15% of all intracranial tumors and has even been identified in 27% of non-selected autopsies[1–3]. PAs are usually benign and slow-growing. However, about 35% of them are invasive pituitary adenomas (IPAs)[4], which, like malignant tumors, invade structures adjacent to the pituitary gland, such as the cavernous sinus, thereby wrapping internal carotid arteries. All these complications lead to a failure in total resection of IPAs during surgery, which in turn causes rapid recurrence and unfavorable prognosis. Therefore, understanding the molecular mechanisms of IPAs concomitant with identifying novel biomarkers and therapeutic targets is instrumental to improving the efficacy of IPA treatment. In previous studies comparing the molecular mechanisms underlying PA invasiveness, two major proteases, the matrix metalloprotease MMP-14 and a member of the ADAM family of proteases, ADAM12, were identified as critical proteins that determine invasiveness of PAs[5]. Clinically, these proteases are highly correlated with invasive PAs, and their regulation is dependent on estrogens since anti-estrogens or selective estrogen receptor modulators (SERMs) reduce PA invasiveness and, in particular, the expression levels of MMP-14 and ADAM12[6, 7].

Despite these findings, the precise mechanism of regulating the invasiveness of PA at the genomic level

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remains unclear. One such level could be the presence of miRNAs (miR) as short, endogenous, and highly conserved non-coding RNAs that contain approximately 19–25 nucleotides and bind to 3′-untranslated regions (3′-UTRs) of the target mRNA, which could lead to mRNA degradation[8]. MiRNAs can also act as tumor suppressor genes or oncogenes in various tumors, manipulating the cell cycle, cell proliferation, differentiation, and cell apoptosis in various cancers[9]. Various studies have recently confirmed that miRNAs participate in different types of PAs. For instance, miR-205-5p inhibits tumor cell proliferation and migration by directly targeting CBX1[10]. In addition, miR-26a and miR-106b facilitate the invasiveness of PAs[11, 12], and miR-200c inhibits apoptosis of PA cells by targeting the PTEN/Akt signaling pathway[13].

Notably, a recently published transcriptomic analysis revealed a strong downregulation of miR-149 in invasive PAs (approx. 5-fold lower in invasive compared to noninvasive PAs)[14], suggesting the possibility that this miR might regulate the translation of invasion-related proteins such as MMP-14 and ADAM12. Indeed, by using TargetScan (http://www.targetscan.org/vert_72/ Version:7.2), we predicted miR-149 to be a potential regulator of ADAM12 and MMP-14 by targeting 3′-UTRs of MMP-14 and ADAM12, respectively (table S1). Highly related to carcinomas, miR-149 was shown to inhibit tumor cell proliferation, migration, and invasion in renal cell carcinoma[15], bladder cancer[16], gastric cancer[17], and colorectal cancer[18]. Human miR-149 has two different mature sequences, which are either miR-149-3p (miRbase, Accession # MIMAT0004609, Sequence: AGGGAGGGCGGGGCUGUGC) (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MIMAT0004609) or miR-149-5p (miRbase, Accession # MIMAT0000450, Sequence: UCUGGCUCCGUGUCUUCACUCCC) (http://www.mirbase.org/cgibin/mirna_entry.pl?acc=MIMAT0004609). Both strands of miR-149 (3p and 5p) can inhibit tumor cell proliferation, migration, and invasion in carcinomas by targeting different downstream genes, i.e., by targeting AKT2 in oral squamous cell carcinoma[19]. Moreover, double strands of Pre-miR-149 inhibit cancer cell migration and invasion by targeting FOXM1 in renal cell carcinoma[15]. In bladder cancer, miR-149-3p impedes the proliferation, migration, and invasion of cancer cells by targeting S100A4[16].

Given the differential expression levels of miR-149 in invasive *vs*. noninvasive PAs, we attempted to investigate whether miRNA-149 could regulate proteases MMP-14 and ADAM12 in PA cells. To address this, we used two mouse pituitary adenoma cell lines: AtT-20 and TtT/GF, to investigate the role of miR-149-3p and miR-149-5p in PA cell proliferation, migration, invasion, and the potential regulation of MMP14 and ADAM12.

1 MATERIALS AND METHODS

1.1 Ethical Statement

Before conceiving this study, ethical approval was obtained from local authorities (Ethics Committee, Medical Faculty of Marburg University, file number 185/11 and amendment) following the Helsinki Criteria. Informed written consent was obtained from all patients, and their data were pseudonymized.

1.2 Patients and Tumor Characteristics

PAs were collected from patients undergoing surgery in the Department of Neurosurgery, University Hospital Marburg. In total, 7 women and 13 men with a mean age of 51.5 years (range 15–84 years) at the time of surgery were included in miRNA analysis. Based on intraoperative findings as described earlier[5], 10 invasive and 10 noninvasive cases were selected according to the Knosp classification, which accounts for invasion of the cavernous sinus as a measure of invasiveness (i.e., with Knosp grades 0–2 were considered noninvasive, whereas grades 3 and 4 were considered invasive). Cavernous sinus invasion was not observed to correlate with gender, patient age, tumor size, or hormone secretion type (data not shown).

1.3 Cell Culture

Two mouse cell lines, AtT-20 (ATCCTM-CCL 89), purchased from the American Type Culture Collection (ATCC, Germany), and TtT/GF cells, provided by Dr. Nicolai Savaskan (Erlangen University, Germany), were used in this study as described earlier[5]. Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Biochrom, Germany), sodium pyruvate (1 mmol/L; Biochrom), and nonessential amino acids (1×; Biochrom) and were incubated at 37°C in a humidified atmosphere of 5% CO_2.

1.4 Cell Transfection

AtT-20 cells were seeded 4×10^5/well, and TtT/GF seeded 3×10^5/well in 6-well plates. After 24 h, miR-149-3p mimic (Syn-mmu-miR-149-3p, Qiagen, Hilden, Germany), miR-149-5p (mimic Syn-mmu-miR-149-5p, Qiagen) and mimic control were respectively transfected into cells by mixing with Lipofectamine™ RNAiMAX Transfection Reagent (13778075, Invitrogen, USA) and OPTI-MEM (31985062, Thermo Fisher Scientific, USA) following the manufacturers' instructions. After 48 h, cells were washed 1× with PBS, detached with trypsin (Gibco), centrifuged at 500 r/min for 3 min, and resuspended for further use. Transfection efficiency was detected by quantitative real-time polymerase chain reaction.

1.5 Proliferation Assay

A total of 2000 cells were seeded in a 96-well plate with 100 μL medium and cultured at 37°C. After 24, 48, and 72 h, 50 μL of CellTiter-Glo® (Promega,

Germany) was added to each well after shaking for 10 min and incubated for another 20 min at room temperature in the dark. The absorbance (*A*) at 450 nm of experimental wells was measured using a FLUOstar OPTIMA Microplate Reader (BMG Labtech, Germany).

1.6 Colony Formation Assay

Totally, 1000 cells/well of each cell line were cultured in 6-well plates for two weeks after transfection of respective miRNA mimics. After that, cells were washed with PBS once, fixed with 10% formalin (1 mL/well) for 15 min, then washed with PBS again, followed by staining with 0.1% crystal violet (w/w in water) solution for 30 min at room temperature. The number of colonies with more than 50 cells was counted under the light microscope.

1.7 Quantitative Real-Time Polymerase Chain Reaction

Both miRNA and total RNA were extracted by miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen) following the manufacturer's instruction. MicroRNA and RNA quantification was performed using a NanoPhotometer NP80 (Implen, Germany). MicroRNAs were reverse-transcribed by miScript Ⅱ RT kit (Qiagen), and cDNA was obtained using the RNA to cDNA Ecodry™ Premix Kit (Takara Bio Europe, France) according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) analyses were performed in triplicates using the Precision FAST MasterMix with ROX (Primer Design, UK). QuantiTect primers for detecting RNA encoding mouse MMP-2, MMP-9, MMP-14, and ADAM12 were obtained from Qiagen. We used XS13 as a housekeeping gene with primers (fwd-TGGGCAAGAACACCATGATG, rev-AGTTTCTC-CAGCTGGGTTG) purchased from Microsynth (Balgach, Switzerland). MiScript SYBR Green PCR kit (Qiagen) was used to analyze the miRNA expression, and RNU6 (Qiagen) was used as a housekeeping gene. A StepOnePlus™ qPCR system (Thermo Fisher Scientific, USA) was employed for the RT-PCR. The relative gene expression was calculated by $2^{-\Delta\Delta CT}$ methods.

1.8 Antibodies, Protein Extraction, and Western Blotting

Rabbit polyclonal anti-MMP-14 antibody was purchased from Abcam (Cambridge, UK), and rabbit polyclonal anti-ADAM12 antibody from Proteintech (Rosemont, USA). Anti-β-tubulin (Novus Biologicals) served as a loading control, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G from Abcam was used as a secondary antibody in a 1:2000 dilution.

Total protein was extracted from the cells (AtT20: 5×10^5, TtT/GF: 2×10^5) with radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH=7.4, Sigma), 150 mmol/L NaCl, 1% nonyl phenoxy-polyethoxylethanol (NP-40, Sigma), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS, SERVA, Germany), 10 mmol/L phenantroline, 10 mmol/L ethylenediaminetetraacetic acid (EDTA, Sigma)] containing phosphatase inhibitors (Pierce™ Phosphatase Inhibitor Mini Tablets, Thermo Fisher Scientific), and protease inhibitors (Pierce™ Protease Inhibitor Mini Tablets, EDTA free, Thermo Fisher Scientific, USA). In a 6-well plate, 200 μL RIPA buffer per well was added to the cells and incubated for 15 min on ice. Shearing of the DNA was accomplished by a 25 G needle to resuspend the samples, which were incubated for another 30 min on ice. The lysates were then centrifuged (12 000 r/min, 15 min, 4°C), and the resulting supernatant, i.e., the total cellular protein, was stored at –80°C. Lysates were boiled for 5 min in Laemmli buffer (60 mmol/L Tris-HCl, pH=6.8; 2% SDS; 10% Glycerol; 5% β-mercaptoethanol; 0.01% bromophenol blue). A bicinchoninic acid assay (Thermo Fisher Scientific, USA) was used to determine protein concentrations.

Equal amounts of lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membranes. To block any unspecific binding, membranes were immersed and gently shaken in 5% non-fat, dried milk in Tris-buffered saline Tween-20 [TBST; 50 mmol/L Tris, pH=7.5; 150 mmol/L NaCl; 0.1% Tween-20 (CARL ROTH, Germany)] for 1 h, after which they were incubated with primary antibodies at 4°C overnight. After three washing steps with TBST, blots were incubated with the secondary antibody for 1 h. After another washing step, signals were detected with Western Bright Chemiluminescence Substrate Sirius (Biozym Scientific, Germany) and quantified by Image J.

1.9 Transwell Invasion Assay

The 8 μm pore transwell inserts (Greiner Bio-One, Germany) were used with 24-well plates. The inserts were coated on the upper side with 50 μL Matrigel (Corning® Matrigel® Matrix, Basement Membrane Matrix Growth Factor Reduced, Corning Incorporated, Corning, USA). The Matrigel was solid after 1 h at 37°C under 5% CO_2. The transwell insert was then turned upside down, and 20 000 TtT/GF and 30 000 AtT-20 cells per well in 50 μL medium containing 0.5% FBS were seeded on the other side. After 4 h of adherence, the transwell insert was turned upside up again. To build an FBS gradient, 250 μL medium containing 20% FBS was applied on top of each Matrigel layer, while 750 μL medium containing 0.5% FBS was placed in each of the 24 wells. After 24 h allowed for invasion, cells were fixed with 4% paraformaldehyde (Sigma), treated with 0.3% octoxynol-9 (Triton™ X-100 buffer; Sigma), and stained with 4',6-diamidin-2-phenylindole

(DAPI; Sigma). Cells were counted in five randomly chosen viewing fields. The percentage of cells invading the Matrigel was determined.

1.10 Wound-Healing Assay

A total of 60 000 cells/well of AtT-20 and 30 000 cells/well of TtT/GF cells were seeded in a culture-insert (Ibidi culture-insert 2 well, Ibidi GmbH, Germany). After allowing the cells to attach overnight, we removed the culture insert and washed the cells with PBS to remove non-adherent cells. We then provided a fresh culture medium and photographed the plate every 6 h to capture the two different fields at each time point on each plate. The number of cells that migrated into the wound space was manually counted in three fields per well under a light microscope at 50× magnification. We then quantified the areas using image J analysis.

1.11 Statistical Analyses

Continuous variables are presented as the mean ± standard error of mean. One-way ANOVA was carried out for multiple comparisons using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, USA). Welch's *t*-test was a statistical test, and *P*-values ≤0.05 indicated a statistically significant difference.

2 RESULTS

Expression levels of miR-149-3p and miR-149-5p were initially determined by qPCR in PA tumor tissues. To address this issue, PA specimens collected after surgery were classified into noninvasive *vs*. invasive PA tumors (fig. 1). Changes between noninvasive and invasive tumors were highly significant ($P<0.0001$) for both miRNA 149 strands, although expression levels of miRNA-149-3p were higher than those of miRNA-149-5p. In contrast to tumor tissues, endogenous expression levels of miR-149-3p and miR-149-5p in PA cell lines AtT-20 and TtT/GF were comparable with mean CT values of 25 for miR-149-3p and 26 for miR-149-5p (fig. S1). To investigate the effect of miR-149 on cellular functions with regard to proliferation, migration, and invasion behaviors, mimic miRNAs for miR-149-3p and miR-149-5p were introduced into both cell lines by transfection (fig. 2). In comparison to untreated cells (NC) or scramble control (Control), cells transfected with mimic miR-149 showed an at least 100-fold higher expression of the respective miR-149 strands (fig. 2).

After transfection of mimic miR-149-3p and miR-149-5p, the proliferation of cells was monitored over 72 h (fig. 3A). In the presence of both miR-149 strands (3p and 5p, respectively), cell proliferation was reduced significantly in both cell lines (for 3p: $P<0.01$ in AtT-20, $P<0.05$ in TtT/GF cells; for 5p: $P<0.001$ in AtT-20 and $P<0.01$ in TtT/GF cells). In both cell lines, the strands were equally effective in reducing cell proliferation.

In addition, a similar effect of miR-149 on cell

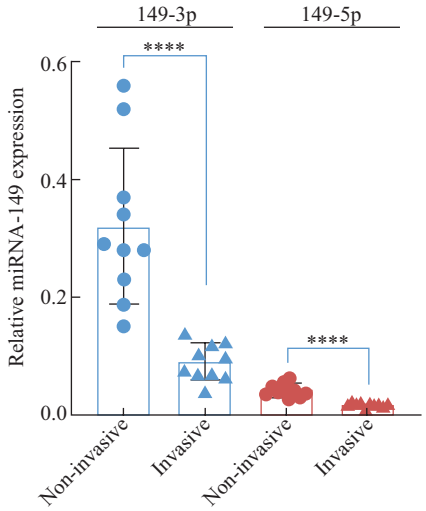

Fig. 1 miRNA 149-3p (blue) and -5p (red) expression in PA tumors separated by noninvasive (circles, $n=10$) *vs*. invasive (triangles, $n=10$) tumors based on the Knosp scale

Results are based on triplicates performed in 3 independent runs, and values are shown as mean values±SEM. Wilcoxon test was performed to evaluate statistical significance. $^{****}P<0.0001$

growth was observed by the evaluation of colony formation assays in both PA cell lines (fig. 3B). As a result, colony formation was significantly reduced by up-regulation of miR-149-3p and miR-149-5p ($P<0.05$ for 3p and $P<0.01$ in AtT-20 cells; $P<0.01$ for 3p and 5p in TtT/GF cells).

Moreover, cell migration was assessed in AtT-20 and TtT/GF cells (fig. 4A and 4B). Cell migration was significantly reduced after miR-149 up-regulation at 120 h for AtT-20 and 12 h for TtT/GF cells ($P<0.01$ for AtT-20 and $P<0.001$ for TtT/GF) (fig. 4). As even more relevant for invasive PA cells, cell invasion was evaluated in Matrigel after miR-149 mimic transfection (fig. 5). A significant reduction of invaded cells was shown with both mimic miRNA-149 strands and in both cell lines ($P<0.0001$ for AtT-20 cells and both mimics; $P<0.001$ for 3p and $P<0.0001$ for 5p in TtT/GF cells). This supports the notion that miRNA149 (3p and 5p) is a strong regulator of invasiveness in PA cells. Since ADAM12 and MMP-14 were previously identified to regulate PA cell invasiveness, the effect of mimic miRNA-149 transfection on the protein expression levels of ADAM12 and MMP-14 was investigated in AtT-20 and TtT/GF cells (fig. 6). The results showed that approximately 50% reduction of MMP-14 and ADAM12 expression were observed after miR-149 up-regulation. In particular, compared to miRNA-149-3p, miRNA149-5p exerted a stronger effect on the reduction of ADAM12 expression (fig. 6).

3 DISCUSSION

This study was initiated to assess the functional

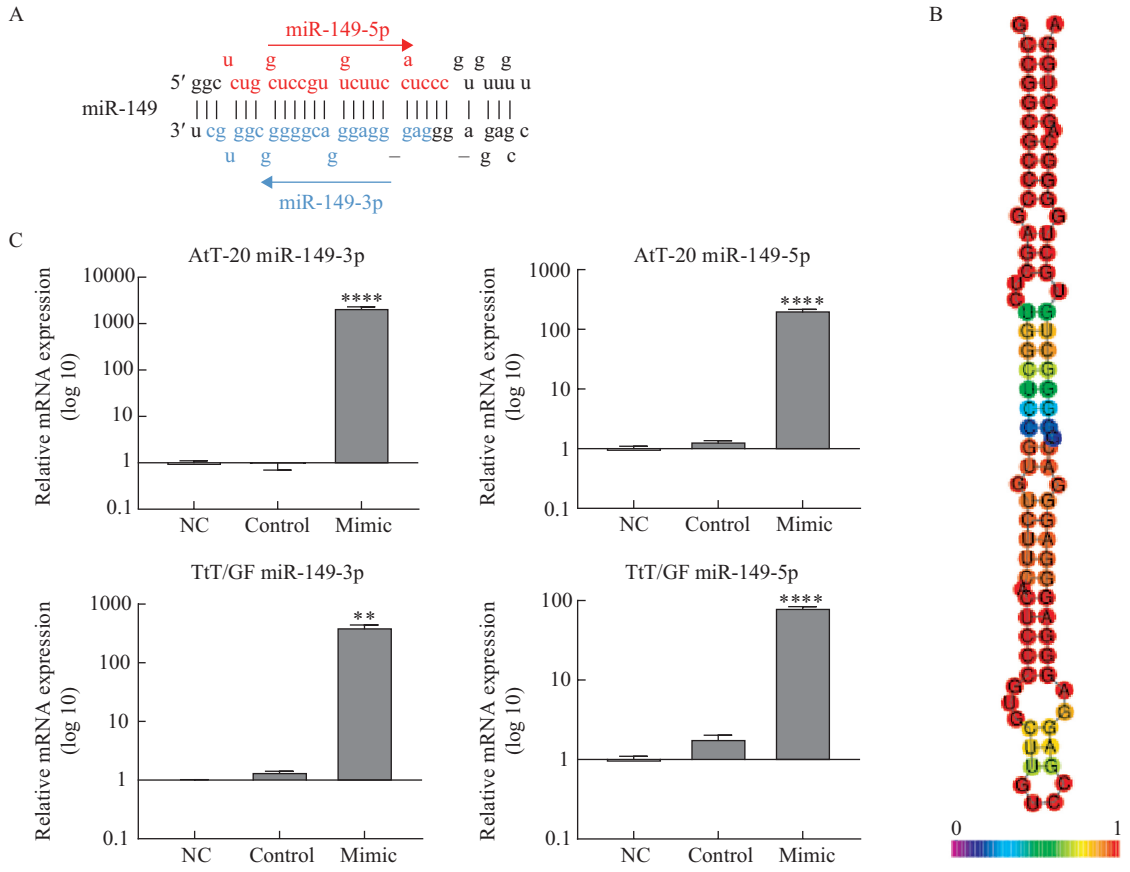

Fig. 2 Sequence of miR-149 and detection of mimics after transfection of AtT-20 and TtT/GF cell lines

A: The sequence of miR-149-3p and -5p is shown in red (miR-149-5p) and blue (miR-149-3p); B: predicted centroid secondary structure of miR-149[26]; C: detection of mimic miR-149-3p and -5p in AtT-20 and TtT/GF cells 24 h after transfection with the respective mimics. Values are provided as mean±SEM. Three independent experiments were performed, and expression levels were determined in triplicates. $^{**}P<0.01$, $^{****}P<0.0001$

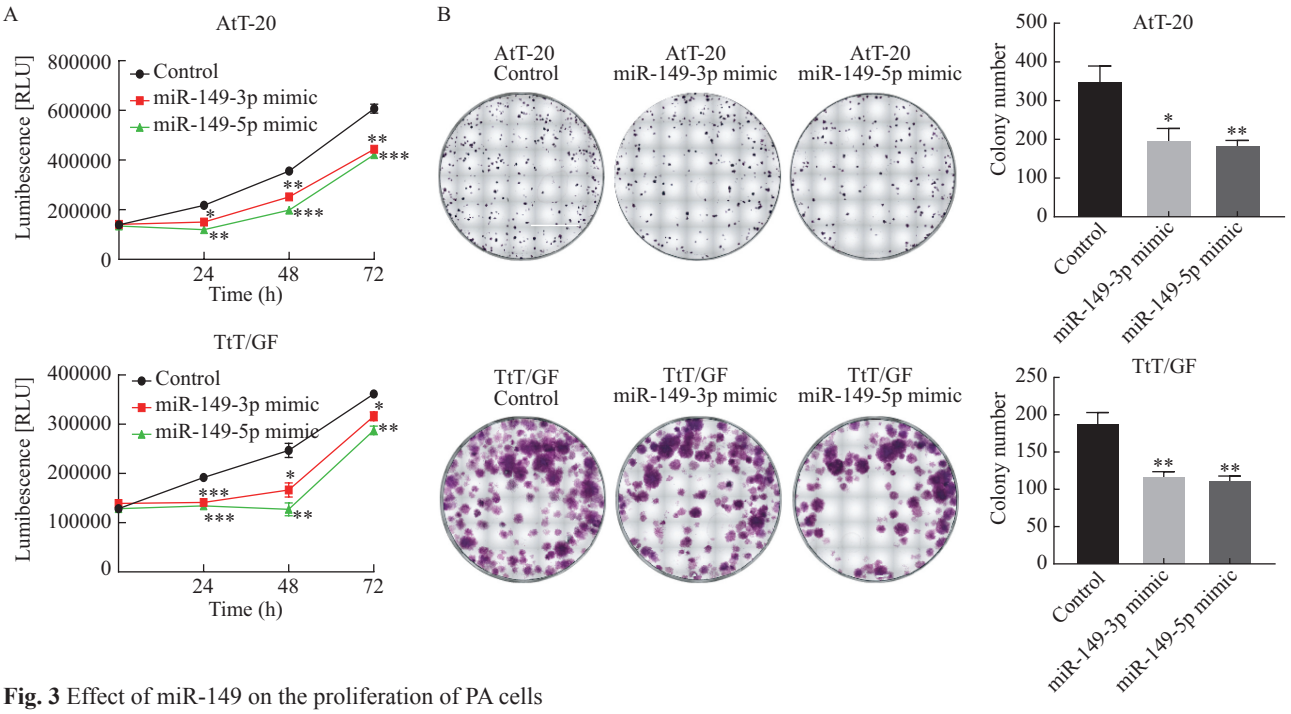

Fig. 3 Effect of miR-149 on the proliferation of PA cells

A: After transfection of mimic miR-149-3p and -5p, AtT-20 and TtT/GF cells were monitored for proliferation over 72 h; B: To assess the long-term effects of miR-149 on PA cells, colony formation assays were performed with AtT-20 and TtT/GF cells. Staining with crystal violet revealed fewer colonies in miR-149 mimic transfected cells. Note that the effects of both strands on proliferation were similar. Colonies with more than 50 cells were counted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Scale bar: 10 mm

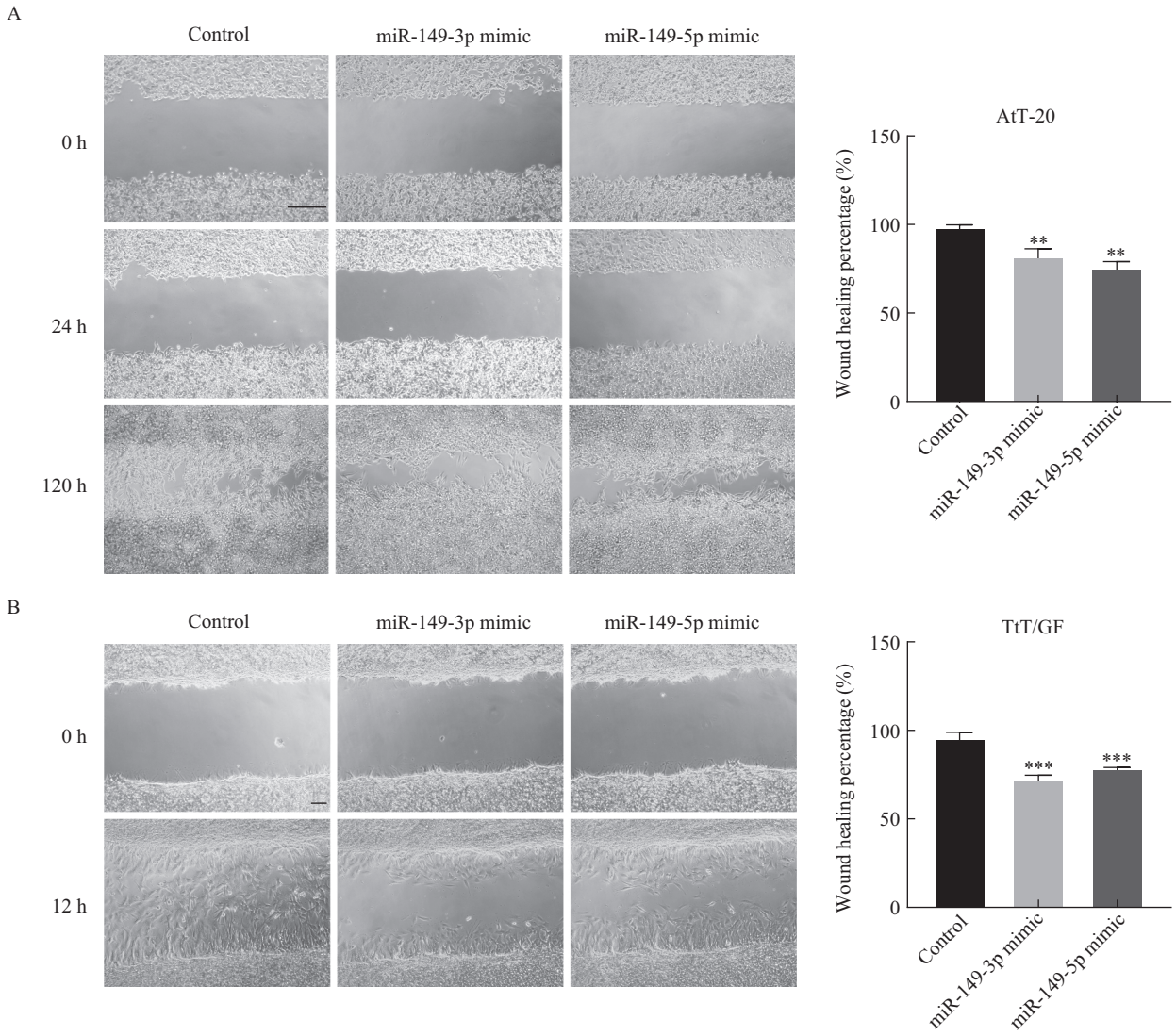

Fig. 4 Analysis of cell migration in AtT-20 (A) and TtT/GF (B) cells due to miR-149 mimic expression
The number of cells that invaded the gap was determined in 5 randomly chosen viewing fields and determined after 12, 24, 72, and 120 h. A: AtT-20 cells migrated very slowly, and quantitative analysis was performed after 120 h; B: TtT/GF cell migration was determined after 12 h. For both cell lines, quantification was performed in three independent experiments. Values are given as mean±SEM and Welch's *t* test was performed to evaluate statistical significance. $^{**}P<0.01$, $^{***}P<0.001$. Scale bar: 50 μm

consequences of different expression levels of the tumor suppressor miRNA-149 between noninvasive and invasive PAs. As a prerequisite for analyses of miRNA-149, we provided evidence that miRNA-149-3p is expressed in significant amounts in PA tissue and is downregulated in invasive PAs. In contrast, the expression levels of miRNA-149-5p in PA tissues are very low and might not be relevant for controlling invasiveness *in vivo*, although it forms the guide strand. By using miRNA mimics in PA cell lines, we provided evidence that both strands of miR-149 can regulate MMP-14 and ADAM12 expression, both highly associated with the invasive behavior of PAs[3–5]. Based on expression levels, only miRNA-149-3p as the passenger strand might be the relevant strand for pathophysiological regulation of ADAM12 and MMP-14 in PA tumors, which is in accordance with the prediction in miR target prediction database (https://mirdb.org). Silencing of *Adam12* and *Mmp14* by miRNA-149-3p is likely to be achieved by RNA degradation, as lower mRNA levels for both proteases were found as a consequence of mimic miRNA transfection in PA cell lines. Several lines of evidence verified the regulation of MMP expression by miR-149. For instance, Wang *et al* demonstrated a negative modulation of MMP2 and MMP9 expression by miR-149 in colon carcinoma[20]. In contrast, miR-149 downregulation caused MMP-9 overexpression[21], suggesting that this miRNA controls the expression of invasion-related proteases. This relationship seems to be common in diverse pathologies. For instance, in cerebral ischemia, MMP expression is increased, and they are a target of miR-149-5p so that in the middle cerebral artery occlusion (MCAO) model, the use of miR-149-5p mimic can reduce the extent of neurological defects and tissue damage by decreasing

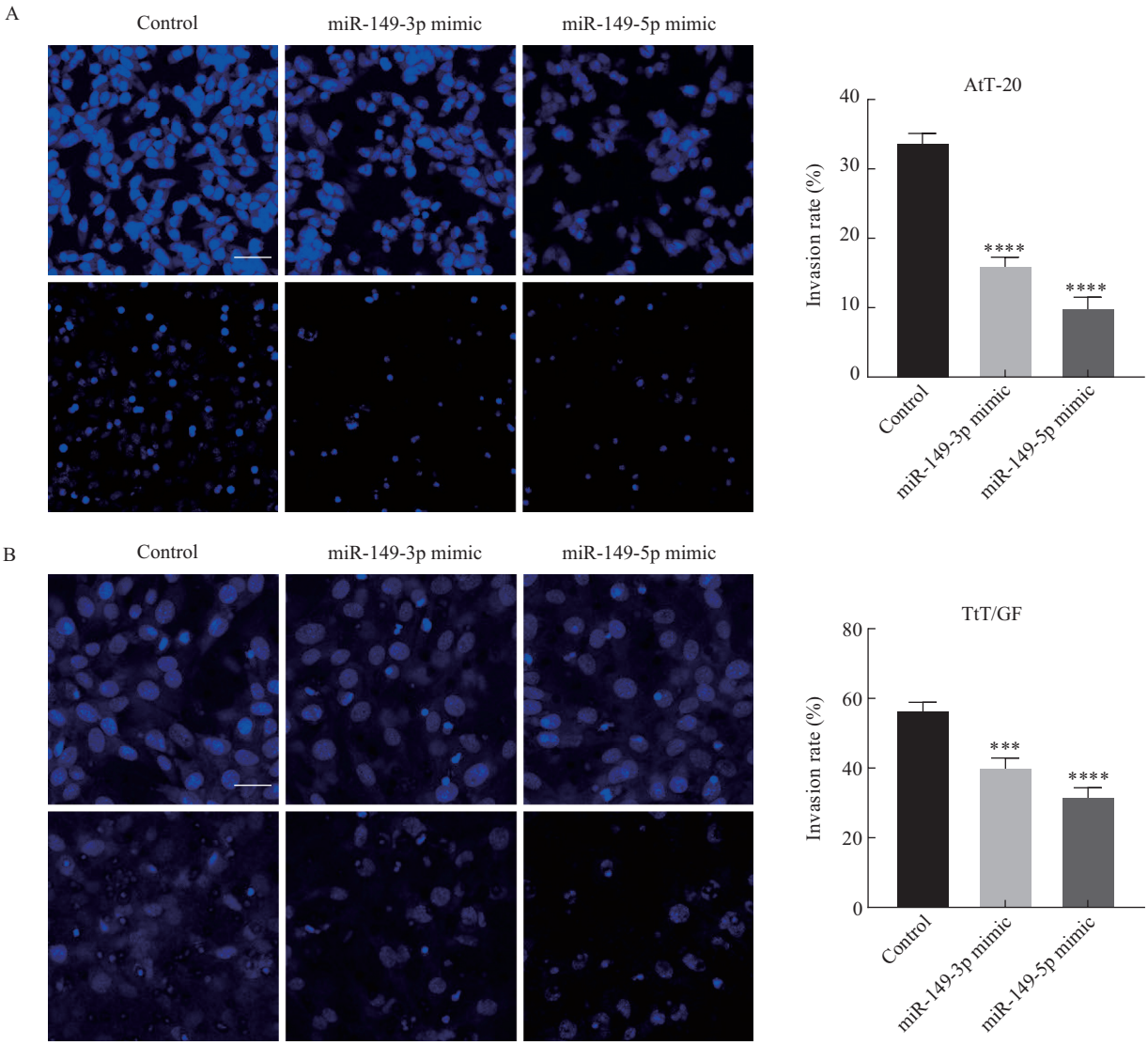

Fig. 5 Effect of miR-149 mimic expression on cell invasiveness in AtT-20 and TtT/GF cell lines
A and B: representative images of DAPI staining to depict uninvaded (upper row) and invaded (bottom row) AtT-20 (A) and TtT/GF (B) cells. Quantification of invasion rate (given as ratio of invaded to uninvaded cells) is based on three independent experiments and provided as percentage. Values are given as mean±SEM. Welch's *t* test was performed to evaluate statistical significance. $^{***}P<0.001$, $^{****}P<0.0001$. Scale bar: 50 μm

the level of MMP2 and MMP9[22]. Another study demonstrated that in the MCAO model, miR-149-5p significantly decreased the expression of MMP9[23]. In U251 GBM cells, overexpression of miR-149 inhibits expression of MMP2, thereby suppressing proliferation and invasion of GBM via AKT1 signaling; however, in this work, no distinction is made between both strands of miRNA-149[24]. Since proliferation is affected by mimic miRNA 149, one of the most important target genes of miRNA-149-3p deduced from the miRNA target database is AKT1, so we can hypothesize that this particular pathway could be affected in PA cells by downregulating AKT1. Concerning MMP-2 and MMP-9, we could not detect their downregulation in PA cells (data not shown), suggesting that gene regulation by miR-149 might be cell-specific.

As a limitation of our study, we cannot wholly explain the biological relevance of miRNA-149 on effects apart from invasion since miRNA-149-3p has 1871 predicted target genes. However, AKT1 is one of the primary targets that could be highly relevant for controlling proliferation. Another limitation is that the mouse PA cell lines do not match the human PA tumor tissue, so the effect of miRNA-149-5p, although detectable in PA cell lines, has no pathophysiological function, as the expression levels are very low. Here, human PA cell lines as tumor models, still lacking in PA research, could be valuable tools to solve this discrepancy.

In our study, we also observed that ADAM12, an active protease involved in growth regulation by shedding of EGF ligands such as HB-EGF[25], is significantly downregulated by miR-149 as predicted by target sequence analysis with complementary seed

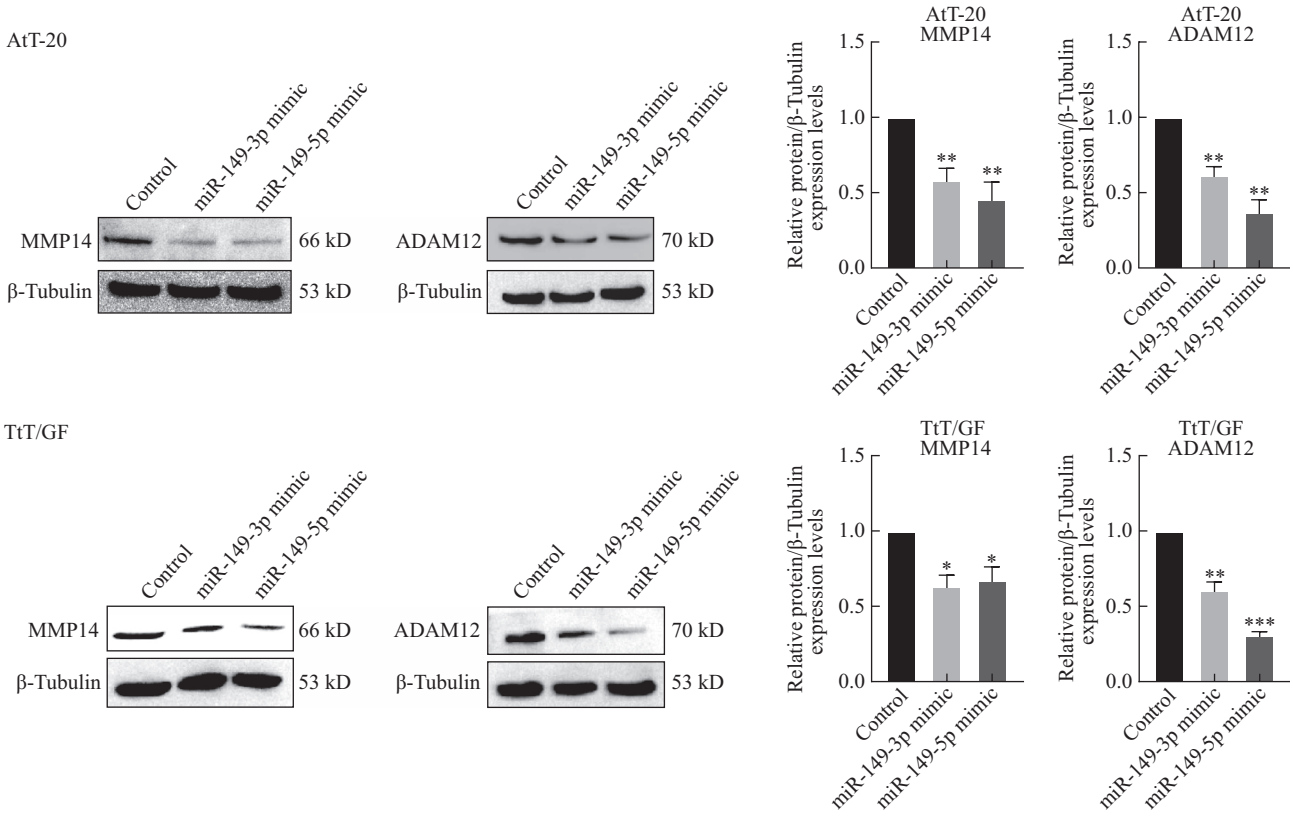

Fig. 6 Effect of miR-149 on expression levels of MMP-14 and ADAM12 in AtT-20 and TtT/GF cells
Cells were lysed, and lysates were subjected to Western blot analysis 48 h after mimic transfection. In both cell lines and with both miR-149 mimics, protein expression of MMP-14 and ADAM12 was reduced. Quantification is based on three independent experiments, and values are given as mean±SEM. Welch's *t* test was performed to evaluate statistical significance. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

sequences in the 3′ region of the mouse *Adam12* and the human *ADAM12* gene. Interestingly, both strands of miRNA-149, 3p, and 5p, are effective in regulating ADAM12 with a preference for miR-149-5p in both cell lines. For MMP-14, both strands of miR-149 were similarly effective in downregulating its expression levels. Similar results were obtained for other tumor entities, such as renal cell carcinoma[15]. In PA tumors, the main regulatory function of miRNA-149-3p on ADAM12 and MMP-14 expression levels might be orchestrated by higher expression levels of miRNA-149-3p, determining their invasive behavior[5]. Since SERMs were previously shown to regulate MMP-14 and ADAM12 in PA cells[5], we analyzed a potential regulation of miR-149 by SERMs which could indicate a direct involvement of SERMs in miR regulation. While SERMs could reduce expression levels of ADAM12 and MMP-14, we did not detect any changes in miR expression upon treatment of PA cells with SERMs such as Clomiphene (fig. S2), suggesting that the SERM effects are not mediated by miR-149.

While the functional consequences of miR-149 expression for the invasive behavior of PAs are clearly demonstrated here, it remains to be established whether miR-149 could be a diagnostic marker for the malignancy of pituitary adenomas, i.e., by localization of miR-149 in extracellular vesicles.

In conclusion, here we show that both strands of miR-149 (3p and 5p) regulate the expression levels of ADAM12 and MMP14 in pituitary adenoma tumors and PA cell lines, thereby determining their invasive behavior. Our findings have implications for clinical applications, as these can contribute to the identification of novel biomarkers and therapeutic approaches for PAs.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Authors Jörg W. Bartsch and Ting LEI are members of the Editorial Board for Current Medical Science. The paper was handled by other editors and has undergone rigorous peer review process. Authors Jörg W. Bartsch and Ting LEI were not involved in the journal's review of, or decisions related to, this manuscript.

REFERENCES

1 Ezzat S, Asa SL, Couldwell WT, *et al*. The prevalence of pituitary adenomas: a systematic review. Cancer, 2004,101(3):613-619
2 Asa SL, Ezzat S. The pathogenesis of pituitary tumours. Nat Rev Cancer, 2002,2(11):836-849
3 Lithgow K, Batra R, Matthews T, *et al*. Management of endocrine disease: visual morbidity in patients with pituitary adenoma. Eur J Endocrinol, 2019,181(5): R185-R197
4 Scheithauer BW, Kovacs KT, Laws ER, Jr., *et al*. Pathology of invasive pituitary tumors with special reference to functional classification. J Neurosurg, 1986,65(6):733-744
5 Wang J, Voellger B, Benzel J, *et al*. Metalloproteinases ADAM12 and MMP-14 are associated with cavernous sinus invasion in pituitary adenomas. Int J Cancer, 2016,139(6):1327-1339
6 Hannen R, Steffani M, Voellger B, *et al*. Effects of anti-estrogens on cell invasion and survival in pituitary adenoma cells: A systematic study. J Steroid Biochem Mol Biol, 2019,187(3):88-96
7 Zhang Z, Bartsch JW, Benzel J, *et al*. Selective estrogen receptor modulators decrease invasiveness in pituitary adenoma cell lines AtT-20 and TtT/GF by affecting expression of MMP-14 and ADAM12. FEBS Open Bio, 2020,10(11):2489-2498
8 Mikhaleva LM, Davydov AI, Patsap OI, *et al*. Malignant transformation and associated biomarkers of ovarian endometriosis: a narrative review. Adv Ther, 2020,37(6):2580-2603
9 Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov, 2017,16(3):203-222
10 Hu A, Zhang Y, Zhao X, *et al*. CBX1 is a direct target of miR-205-5p and contributes to the progression of pituitary tumor. Pharmazie, 2019,74(3):154-156
11 Yu C, Li J, Sun F, *et al*. Expression and Clinical Significance of miR-26a and Pleomorphic Adenoma Gene 1 (PLAG1) in Invasive Pituitary Adenoma. Med Sci Monit, 2016,22(5):101-108
12 Zheng Z, Zhang Y, Zhang Z, *et al*. Effect of miR-106b on Invasiveness of Pituitary Adenoma via PTEN-PI3K/AKT. Med Sci Monit, 201,23(3):1277-1285
13 Liao C, Chen W, Fan X, *et al*. MicroRNA-200c inhibits apoptosis in pituitary adenoma cells by targeting the PTEN/Akt signaling pathway. Oncol Res, 2013,21(3):129-136
14 Zhang C, Qian Y, Qiao Y, *et al*. Analysis of whole genome-wide microRNA transcriptome profiling in invasive pituitary adenomas and non-invasive pituitary adenomas. Chin Neurosurg J, 2019,5(1):1-7
15 Okato A, Arai T, Yamada Y, *et al*. Dual Strands of Pre-miR-149 Inhibit Cancer Cell Migration and Invasion through Targeting FOXM1 in Renal Cell Carcinoma. Int J Mol Sci, 2017,18(9):1969
16 Yang D, Du G, Xu A, *et al*. Expression of miR-149-3p inhibits proliferation, migration, and invasion of bladder cancer by targeting S100A4. Am J Cancer Res, 2017,7(11):2209-2219
17 Cao D, Jia Z, You L, *et al*. 18β-glycyrrhetinic acid suppresses gastric cancer by activation of miR-149-3p-Wnt-1 signaling. Oncotarget, 2016,7(44):71960-71973
18 Ruan Z, Deng H, Liang M, *et al*. Downregulation of long non-coding RNA MAFG-AS1 represses tumorigenesis of colorectal cancer cells through the microRNA-149-3p-dependent inhibition of HOXB8. Cancer Cell Int, 2020,20:511
19 Shen Q, Zhu H, Lei Q, *et al*. MicroRNA-149-3p inhibits cell proliferation by targeting AKT2 in oral squamous cell carcinoma. Mol Med Rep, 2021,23(3):172
20 Wang AL, Li Y, Zhao Q, *et al*. Formononetin inhibits colon carcinoma cell growth and invasion by microRNA-149-mediated EphB3 downregulation and inhibition of PI3K/AKT and STAT3 signaling pathways. Mol Med Rep, 2018,17(6):7721-7729
21 Hentati-Kallel M, Le Jan S, Bernard P, *et al*. Histone deacetylases meet microRNA-associated MMP-9 expression regulation in glucocorticoid-sensitive and -resistant cell lines. Int J Oncol, 2017,50(2):717-726
22 Ghasemloo E, Oryan S, Bigdeli MR, *et al*. The neuroprotective effect of MicroRNA-149-5p and coenzymeQ10 by reducing levels of inflammatory cytokines and metalloproteinases following focal brain ischemia in rats. Brain Res Bull, 2021,169(4):205-213
23 Yan Z, Deng Y, Zou Y, *et al*. Analysis of regulatory effect of miR-149-5p on Sphingosine-1-phosphate receptor 2 of pericytes and its neuroprotective molecular mechanism after acute cerebral ischemia reperfusion in rats. Bioengineered, 2021,12(1):3348-3357
24 Pan SJ, Zhan SK, Pei BG, *et al*. MicroRNA-149 inhibits proliferation and invasion of glioma cells via blockade of AKT1 signaling. Int J Immunopathol Pharmacol, 2012,25(4):871-881
25 Kodama T, Ikeda E, Okada A, *et al*. ADAM12 is selectively overexpressed in human glioblastomas and is associated with glioblastoma cell proliferation and shedding of heparin-binding epidermal growth factor. Am J Pathol, 2004,165(5):1743-1753
26 Lorenz R, Bernhart SH, Höner Zu Siederdissen C, *et al*. ViennaRNA Package 2.0. Algorithms Mol Biol, 2011,6:26

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