Regulation of miR-375 and Sonic hedgehog on vascular endothelial growth factor in preeclampsia rats and its effect on trophoblast cells

Running title: VEGF regulated by miR-375 and SHH pathway

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Abstract

**Purpose:** To study the mechanism of vascular endothelial growth factor (VEGF) regulated by miR-375 and Sonic hedgehog (SHH) signaling pathways on the characteristics of trophoblast cells (TCs) in preeclampsia (PE) rats.

**Methods:** Female (100) Wistar rats in rut were mated with males (50) at a ratio of 2:1, and 60 pregnant rats were assigned to a normal group (Nor, n=10) and a model group (Mod, n=50) in a random manner. Rat TCs of placental villus were isolated and were divided into 8 groups. The related indicators in each group were detected, respectively.

**Results:** In contrast to the NC group, miR-375 expression greatly elevated in the miR-375 mimic group (mimic group); in the mimic and cyclopamine groups, VEGF, proliferating cell nuclear antigen (PCNA), N-cadherin and Bcl-2 expression, cell proliferation, S phase cells, and migration and invasion ability were significantly decreased and E-cadherin and Bax expression, G1 phase cells and apoptosis rate were increased significantly, but in Over expressing-VEGF (ov-VEGF) group, the above indicators presented contrary results (all \(P<0.05\)). In contrast to the mimic group, the expression of SHH, VEGF, PCNA, N-cadherin and Bcl-2, cell proliferation, S phase cells and the invasion and migration activity in the miR-375 mimic + oe-VEGF group were evidently increased.

**Conclusions:** Over-expressing miR-375 can inhibit the expression of VEGF, thus slow down the invasion and proliferation of TCs in PE rats, which is realized by regulating SHH signal pathway.

**Keywords:** miR-375, Sonic hedgehog signaling pathway, Vascular endothelial growth factor, Preeclampsia, Trophoblast cells

Introduction

Preeclampsia (PE) is a malady specific to pregnant women with a prevalence rate of 7%-12% worldwide [1]. As one of the most common pathological complications of pregnancy, PE, characterized by hypertension and proteinuria, is the primary cause of pregnancy-related death,
causing a series of problems in mothers and fetuses [2]. Without proper and timely prevention, PE may progress to a more serious stage, eclampsia, which is accompanied by seizures and other serious injuries, including hemolysis, elevated liver enzymes, platelet syndrome, ischemic stroke or hemorrhagic, and acute respiratory distress syndrome [3]. PE is a serious hypertensive disorder of pregnancy featured with increased levels of ceramide, apoptosis and autophagy, and
increased trophoblast cell death [4]. Some studies have found increased apoptosis rate in villous trophoblast cells (TCs) in PE [5].

MicroRNAs (miRNAs) are non-coding RNAs, 22 nucleotides or so in size, which are able to negatively affect and silence target gene binding with the 30 untranslated regions of mRNA [6]. Increasing number of reports have proved that miRNAs affect various important cellular processes including proliferation, differentiation, and migration [7]. According to earlier studies, miRNAs affect the development of PE by regulating multiple targets or signal pathways. For example, the expression of microRNA-34a, miRNA-31-5p and miR-134 is significantly up-regulated in the placenta of preeclampsia patients [8-10]. Recent studies have shown that miR-375 in neuropathic mice was up regulated. [11]. However, the mechanism of miR-375 in occurrence and development of PE remains unclear. Sonic hedgehog (SHH) is a morphogen important for the development of mammalian embryos. In the post-embryonic phase, SHH is important for maintaining steady-state processes such as angiogenesis and cardiac repair [12]. SHH is secreted to ectoderm as monomer or multimer and exerts its cellular function through molecules located in the primary cilium. Once SHH binds to its 12-transmembrane receptor, Patched1, it releases Smoothened (SMO), which is a 7-transmembrane receptor and part of the G-protein coupled receptor superfamily. SMO will transduce the SHH signal through the cytoplasm, thereby activating the family of zinc finger transcription factor including GLI1, GLI2, and GLI3 (to a lesser extent). The GLI transcription factor recognizes the GLI binding locus in DNA and stimulates transcription of target genes such as PTCH1 and GLI1 via negative and positive feedback loops, respectively [13-15]. Mutations in different transcription factors of SHH signaling and dysregulation of their target genes will cause a variety of maladies including basal cell nevus syndrome and basal cell carcinoma [16]. To date, few studies have reported the function of the SHH axis in PE. Vascular endothelial growth factor (VEGF) is pivotal for networks and angiogenesis of vessels [17]. VEGF signals by its cognate receptor, and the kinase activity of VEGF receptor 2 is the key to trophoblast invasion [18]. EG-VEGF is not only an
important marker and protein in embryo implantation, but also a critical factor in endocrine control of placentation development in the third trimester of pregnancy, moreover, VEGF is a specific gene of the SHH axis[19]. In the present research, we found a targeting relation between miR-375 and VEGF via bioinformatics prediction. Therefore, we speculate that miR-375 and SHH signaling pathway is crucial for the biological characteristics of TCs in preeclampsia rats.
Materials and methods

Animals

Clean grade adult Wistar rats (200g to 225g) were fed at room temperature and they could obtain the water and food freely. Female (n=100) Wistar rats in rut were mated with males (n=50) at a ratio of 2:1. The secretions of rats are examined daily and the time of discovery of sperm was defined as the beginning of pregnancy. Sixty pregnant rats were assigned to two groups: normal group (Nor, n=10) and model group (Mod, n=50), in a random manner. Rats in the Mod were subcutaneously injected with L-nitro-arginine methyl ester at 125 mg/(kg·d) since the 15th day of pregnancy to the 22th day, while those in the Nor were subcutaneously given with normal saline. The systolic blood pressure was measured on days 7, 14, 18, 19, and 20 of gestation. If systolic blood pressure was significantly increased, PE rat model was successfully established.

Rats were sacrificed after childbirth, 1% pentobarbital sodium (250 mg/kg) were injected into the abdomen of rats CO2 died of euthanasia by inhalation, and the uterus placenta was taken to isolated chorionic trophoblast tissues for primary cell isolation and culture. Approval from the medical ethic committee of The First Affiliated Hospital of Xinxiang Medical University had been obtained prior to the study. This study has been approved by the animal experimental ethics committee of The First Affiliated Hospital of Xinxiang Medical University. All animal experiments were performed at laboratory of Xinxiang Medical University.

Isolation, culture and transfection of TCs from placental villus tissue

The chorionic trophoblast tissues were cut and trypsinized to obtain the cell suspension, which was then centrifuged, cleaned thrice using PBS, and resuspended by RPMI1640 culture medium (Gibco, United States) having 10% fetal bovine serum (FBS, Gibco, United States) and routine antibiotics. After being incubated in a 5% CO2 incubator at 37 degrees centigrade, the cells were in the morphology of epithelioid-like cells and spread in a sheet-like manner under the inverted microscope.
TCs grown in log phase were seeded in 6-well culture plates with serum free RPMI1640 medium at 1*10^5/well. Then, rat TCs were assigned to the Nor (TCs from normal rat), Mod (TCs from model rat), NC group (TCs from model rats transfected with NC, 50nM), miR-375 mimic group (mimic group, TCs from model rats transfected with miR-375 overexpression vector, 50nM), cyclopamine group (TCs from model rats treated with SHH signaling pathway specific inhibitor cyclopamine, 5μM) [20], oe-VEGF Group (TCs from model rats transfected with VEGF overexpression vector, 50nM), miR-375 mimic+oe-VEGF group (mimic+oe-VEGF group, TCs from model rats treated through miR-375 overexpression vector (50nM) and VEGF overexpression vector (50nM)) and cyclopamine+oe-VEGF group (TCs from model rats treated with cyclopamine and transfected with VEGF overexpression vector). Lipofectamine 2000 was used for the transfection of vectors according to the instructions. The medium was substituted by complete RPMI1640 after 6 hour transfection, and the cells were obtained for later study after transfection for 48h.

**Dual luciferase reporter (DLR) system assay**

Firstly, the binding locus was explored via the bioinformation prediction website (www.targetscan.org) between miR-375 and VEGF. The targeting correlation of miR-375 with VEGF was next investigated by the DLR system assay. The VEGF DLR vector (PGL3-VEGFwt) and the mutants (PGL3-VEGFmut) that bind to the miR-375 binding locus were constructed, respectively. The Rellina plasmid and the above two were co-injected into HEK293T cells with the miR-375 plasmid and the NC plasmid, respectively. After 24 hours of transfection, DLR assays were performed. The cells of each group were pyrolysed, followed by 1 minute centrifugation at 12,000 rpm to collect, the supernatant. The luciferase activity (LU activity) was determined by the DLR kit (Promega, USA) according to the instruction of kit. Briefly, the lysed cell specimen (10μL) and firefly luciferase working solution (100μL) were added into EP tubes, and then the firefly LU activity was determined. Afterwards, 100μL Renilla luciferase working solution was put in the tubes and the LU activity was detected again. Relative LU activity = firefly LU activity / renilla LU activity.
qRT-PCR

Total RNA of cells was obtained in each group by Trizol (Cat. No. 16096020, Thermo Fisher Scientific, New York, United States) after 48 hours of transfection. Reverse transcription was conducted to synthesize cDNA via the TaqMan MicroRNA Assays Reverse Transcription Primer (Thermoscientific, USA). SYBR® PremixExTaq™II Kit (BOSTER Biological Technology Co., Ltd., Wuhan China) was employed for fluorescent quantitative PCR detection. The following components were added in sequence: 25 μL SYBR® PremixExTaq™II (2×), 2 μL PCR upstream and downstream primers, 1 μL ROXReferenceDye (50×), 4μL DNA template, and 16 μL ddH₂O, followed by a FQ-PCR in ABIPRISM® 7300 (Prism® 7300, Detie Experimental Equipment Co., Ltd., Nanjing, China) under 95 degrees centigrade for 10min, 95 degrees centigrade for 15s, 60 degrees centigrade for 30s (32 cycles), and 72 degrees centigrade for 1min. ΔCt=CT(target gene)-CT(internal reference), ΔΔCt=ΔCt(exp group)-ΔCt(Con group). With U6 as internal reference for miR-375 and GAPDH for other genes, $2^{-ΔΔCt}$ represents the relative level of every gene of interest. See Table 1 for primers.

Western blot (WB)

At 48 h after transfection, the cells were cleaned through pre-cooled PBS thrice, and their total protein was obtained through RPPA lysate supplemented by PMSF (R0010, solarbio, Beijing, China) and protein level was determined via a BCA kit (thermo, USA). The samples were put with the loading buffer and subjected to a 10 min water bath. Subsequently, each protein sample (50 μg) was loaded, electrophoresed at 70 V for 3h and moved to a PVDF membrane (ISEQ00010, Millipore, Billerica, Massachusetts, United States) at compliance voltage of 150mA, followed by a 2h sealing via 5% skim milk at indoor temperature,. Afterwards, after being cleaned by TBST solution, the membranes were respectively incubated with primary antibodies, rabbit antimouse VEGF (ab32152, 1:5,000, Abcam, UK), SHH (ab19897, 1 μg/mL, Abcam, UK), proliferating cell nuclear antigen (PCNA)(ab29, 1 μg/mL, Abcam, UK), E-cadherin (ab76055, 1:1,000, Abcam, UK), N-cadherin (ab76057, 1:1,000, Abcam, UK), Bax (ab32503, 1:1,000, Abcam, UK), Bcl-2 (ab182858, 1:1,000, Abcam, United Kingdom), GAPDH
(ab22555, 1:2,000, Abcam, United Kingdom) overnight at 4 degrees centigrade. Subsequently, the membranes were cleaned by TBST solution 3 times, 6 min/time, followed by 2 h incubation with secondary antibody HRP-labeled goat anti-rabbit IgG antibody (Zhongshan Biotechnology Co., Ltd., Beijing, diluted 1:5,000) at indoor temperature. At last, the membranes were developed by ECL fluorescence assay kit (Cat. No. BB-3501, Ameshame, United Kingdom) and photographed via Bio-Rad image analysis system (BIO-RAD, United States) after washing through TBST solution again. The results were analyzed by Image J software, and the relative protein expression = the gray value of target protein band/that of GAPDH protein band.

**MTT**

After 48 hours of transfection, the cells were harvested through conventional digestion and transferred to 96-well plates at 6×10^3 cells/well. Each group had 6 duplicative wells. Then, 20 μL of 5 mg/mL MTT solution (Gibco, USA) was put at 24 h, 48 h and 72 h, separately, followed by 4 h incubation in the dark. Subsequently, DMSO (100 μL) was put in each well, and the absorbance of each well at 495 nm was detected by an enzyme-linked immunosorbent detector (NYW-96M, Noah Instrument Co., Ltd., Beijing, China). The curve for cell viability was drawn (time point; the x-axis; optical density: the y-axis).

**Flow cytometry**

At 48 h after transfection, the cells were harvested and cleaned through PBS thrice, followed by 20 minutes of 2,000 rpm centrifugation. The supernatant was removed, and the cell concentration was changed to 1×10^7/mL with PBS. Then, pre-cooled 75% ethanol (1 mL) was put in and the specimens were stood at 4 degrees centigrade for 1 h, followed by 1,200 r/min centrifugation for 5 minutes and thrice cleaning by PBS. After then, Rnase A (120 μL, Siemo, United States) was put in the samples in the dark and cultured at 37 degrees centigrade for 40 min, followed by mixing with PI dye solution (500 μL, Sigma, USA) and incubating at 4 degrees centigrade in the dark for 30 min. Cell cycle (red fluorescence) was determined through flow
cytometry (Beckman Coulter, United States) at 488 nm.

At 48 h after transfection, the cells were trypsinized with trypsin (thromo, USA) without EDTA, and put in a flow tube, followed by 30 minutes of 2,000 rpm centrifugation, and the supernatant was removed. Then, the cells were subjected to 20 minutes of 2,000 rpm centrifugation after being cleaned through pre-cooled PBS thrice, and the supernatant was taken out again. Annexin-V-FITC/PI dye solution containing HEPES buffer, Annexin-V-FITC, as well as PI (50:1:2) were prepared under the instruction of Annexin-V-FITC Apoptosis Detection Kit (Sigma, United States). Subsequently, the dye solution (100μL) was put in the sample, and cultured at indoor temperature for 15 minutes, followed by addition of HEPES buffer (1mL) and mix with the sample through shaking. Apoptosis was determined by flow cytometer at 488 nm excitation wavelength.

**Transwell assay**

The Transwell chamber (Shanghai Kelton Bio, China) was placed in a 96-well plate, and the upper chamber was coated with Matrigel gel (Shanghai Qianchen Biotechnology, China) at 1:8 dilutions and dried at indoor temperature. After being trypsinized, the cells were cleaned with PBS thrice and resuspended in RPMI1640. The cell concentration was changed to 1*10^5 cells/mL and the suspension (300μL) was put in the upper compartment, and RPMI1640 (500 μL) having 10% FBS was put in the lower compartment. After 24 h routine culturing, the Transwell insert was taken out and the extra cells in the upper compartment were gently cleaned off through a cotton swab. After 20 minutes of immobilization via 4% paraformaldehyde (Regen Bio, Beijing, China), the cells were dyed through 0.5% crystal violet solution (Solebao Bio, Beijing, China) for 10 min and cleaned with PBS thrice. Five randomly selected fields (200×) were photographed under an inverted microscope and the transmembrane cells were quantified.

**Statistical analyses**
All data were analyzed through SPSS21.0. The quantitative data were represented as the mean ± standard deviation. One-way ANOVA and Tukey post-Hoc test were employed for inter-group comparison. \( P < 0.05 \) indicates a remarkable difference.

**Results**

**miR-375 targets and negatively regulates the expression of VEGF gene**

Specific binding loci between miR-375 and VEGF were predicted through the bioinformation prediction site microrna.org (http://www.microrna.org/microrna/home.do) (Figure. 1a).

According to the DLR system assay, the LU activity of Wt-VEGF in the mimic group was greatly weaker than that in the NC group (\( P < 0.05 \)). The LU activity of the Mut-VEGF presented insignificant difference between the two groups, indicating miR-375 could target the negative regulation of VEGF gene (\( P > 0.05 \), Figure. 1b).

**MiR-375, SHH and VEGF expression in cells from each group**

To investigate how miR-375 and SHH signaling pathways affect the VEGF gene expression and play a role on the biological characteristics of TCs in preeclampsia rats, we detected the mRNA and protein levels of miR-375, SHH and VEGF by qRT-PCR and WB assays (Figure. 2). It came out that compared with the Nor, miR-375 was up-regulated in the other groups, while SHH and VEGF were down-regulated (\( P > 0.05 \)); in contrast to the Mod, no remarkable difference was seen in the gene expression in the NC group (\( P > 0.05 \)); the VEGF expression in the mimic group and the cyclopamine group greatly decreased, while it greatly elevated in the oe-VEGF group (\( P < 0.05 \)). The VEGF expression in the mimic + oe-VEGF group was much above that in the mimic group (\( P < 0.05 \)), and the VEGF expression in the cyclopamine+oe-VEGF group was also above that in the cyclopamine group (\( P < 0.05 \)). MiR-375 was remarkably up-regulated in the mimic group, and SHH was remarkably down-regulated in the cyclopamine group (both
Cell proliferation in different groups

The MTT assay showed that the cell proliferation in all groups elevated with time (Figure 3). In comparison with the Nor, the cell proliferation in the rest was down-regulated ($P > 0.05$). In contrast to the Mod, the proliferation showed no remarkable difference in the NC group, but decreased in the mimic and cyclopamine groups and raised in the oe-VEGF group ($P < 0.05$). The mimic+oe-VEGF group showed stronger proliferation of TCs than the mimic group ($P < 0.05$), and the cyclopamine+oe-VEGF group also showed stronger proliferation of TCs than the cyclopamine group ($P < 0.05$).

Cell cycle of cells in different groups

The cell cycle in each group was explored through flow cytometry (Figure 4). It came out that compared with the Nor, there were evidently more G1-phase cells in the rest groups and remarkably less S-phase cells ($P < 0.05$). The cell phase was insignificantly different between the Mod and NC group ($P > 0.05$); the mimic and cyclopamine groups presented much more G1-phase cells and much less S-phase cells (both $P < 0.05$), while the oe-VEGF group had contrary results. The mimic + oe-VEGF group presented remarkably less G1-phase cells and much more S-phase cell that the mimic group (both $P < 0.05$). The cyclopamine+oe-VEGF group presented remarkably less G1-phase cells and remarkably more S-phase cells than the cyclopamine group (both $P < 0.05$).

Apoptosis in different groups

Apoptosis was determined via flow cytometry (Figure 5) and it came out that the rest groups showed greatly higher apoptosis rate of TCs than the Nor ($P > 0.05$). In contrast to the Mod, the apoptosis rate presented insignificant difference in the NC group ($P > 0.05$), but was greatly elevated in the mimic and cyclopamine groups and remarkably lowered in the oe-VEGF group.
The mimic + oe-VEGF group presented greatly lower apoptosis rate than that of the mimic group ($P < 0.05$), and cyclopamine + oe-VEGF group showed greatly lower apoptosis rate than the cyclopamine group ($P < 0.05$).

**Cell invasion in different groups**

Transwell assay was employed for cell invasion ability detection in each group (Figure 6). It came out that other groups showed remarkably less invasive cells than the Nor ($P > 0.05$). In contrast to the Mod, and the number of invasive cells presented no remarkable difference in the NC group ($P > 0.05$), but it was greatly increased in the mimic and cyclopamine groups, and obviously increased in the oe-VEGF group ($P < 0.05$). The mimic+oe-VEGF group showed much more invasive cells than the mimic group ($P < 0.05$), and the cyclopamine+oe-VEGF group also showed much more invasive cells than the cyclopamine group ($P < 0.05$).

**mRNA and protein levels of PCNA, E-cadherin, N-cadherin, Bcl-2 and Bax in each group of cells**

To investigate how miR-375 and SHH signaling pathways affect the VEGF gene expression and play a role on the biological characteristics of TCs in preeclampsia rats, we detected the mRNA and protein levels of PCNA, E-cadherin, N-cadherin, Bcl-2, and Bax via qRT-PCR and WB, respectively (Figure 7). It was turned out that in comparison with the Nor, the mRNA and protein levels of PCNA, N-cadherin, Bcl-2 decreased, while those of E-cadherin and Bax increased in other groups ($P < 0.05$). In contrast to the Mod, no obvious difference was seen in the mRNA and protein level of each indicator in the NC group ($P > 0.05$); the mRNA and protein levels of PCNA, N-cadherin and Bcl-2 were down-regulated, while those of E-cadherin and Bax were up-regulated in the mimic and cyclopamine groups, and these indicators showed an opposite trend in oe-VEGF group ($P < 0.05$). The mimic+oe-VEGF group presented higher mRNA and protein levels of PCNA, N-cadherin and Bcl-2 and lower mRNA and protein levels
of E-cadherin and Bax than the mimic group ($P < 0.05$). The cyclopamine + oe-VEGF group presented higher mRNA and protein levels of PCNA, N-cadherin and Bcl-2 and lower mRNA and protein levels of E-cadherin and Bax than the cyclopamine group ($P < 0.05$).

**Discussion**

Currently, up-regulation of miR-375 has been observed in a variety of diseases such as adrenal disorders, diabetes and ionizing radiation [21, 22]. The SHH pathway is strongly involved in tumor progression, metastasis, and angiogenesis. Recent reports have revealed that abnormal activation of the hedgehog axis is positively correlated with the overall survival of several clinicopathological features, lymphatic metastasis, breast carcinoma, and lung carcinoma [23]. The glycoprotein of the hedgehog family, as a signaling molecule, strongly affects the progression and cell cycle. SHH is the sole hedgehog protein with expression in the central nervous system (CNS) of adult mammals. The developmental effects of SHH signaling have been well documented, but only several reports have addressed the function of SHH in the CNS of adult mammals [24]. The disturbance of placental angiogenesis is a key feature of the pathogenesis of pre-eclampsia. The process of angiogenesis is caused by various angiogenic indicators including VEGF and placental growth factor and its membrane-bound receptors, which are the most widely studied angiogenic factors during placental development [25, 26].

In our study, we found an up-regulation of miR-375 and a down-regulation of SHH and VEGF in TCs of model rats. Previously study has proved that VEGF supplementation can treat pre-eclampsia [27]. Therefore, we transfected VEGF over-expression vector to rat TCs and found that after the transfection, the proliferation-related factor (PCNA) and invasion-related factor (N-cadherin) was up-regulated, cell proliferation and invasion ability was enhanced, cell cycle progression was promoted, while apoptosis rate was decreased, indicating that overexpression of VEGF could improve pre-eclampsia by promoting the growth of rat embryonic TCs and inhibiting its apoptosis, which was in line with the results of previously literature.

It has been uncovered that the SHH axis is abnormally activated in human lung cancer tissues, and the highly expressed SHH ligand initiates the SHH signaling pathway, leading to the transcription of Gli1 to active the target gene VEGF, so as to affect the regulation of tumorigenesis and development and
promote the proliferation of tumor blood vessels [28]. The SHH signaling pathway takes a part in the survival, proliferation and repair of endothelial cells [29]. We predicted that VEGF has a targeting relationship with miR-375 through the website, and the literature also showed that miR-375 has inhibitory effects in various diseases [30]. Our results verified that miR-375 could negatively affect the VEGF expression and over-expressing miR-375 or inhibiting the SHH signaling pathway can inhibit trophoblast cell proliferation, invasion and promote apoptosis, but VEGF over-expression can partially reverse the inhibition effect.

**Conclusion**

This study has not elucidated the exact molecular mechanisms by which miR-375 and SHH signaling pathways regulate VEGF, and the related regulatory networks of miR-375 in pre-eclampsia. To further clarify the above issues, we will conduct in-depth research in this direction in the future. In conclusion, Over-expressing miR-375 can inhibit the expression of VEGF, thus slow down the invasion and proliferation of TCS in PE rats, which is realized by regulating SHH signal pathway. It is expected to be a potential therapeutic target.

**Author contribution statement**

All authors contributed significantly to this work. Yifang Xu for the data analysis and manuscript writing, Yanfang Ren for the data collection and analysis, protocol development and manuscript writing, Jianguo Wen, Yuanyuan Wang, Yongjie Jiang for the data collection.

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**Conflict of Interest:**
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Table:

Table 1. Primer sequence

| Genes   | Sequences                                      |
|---------|-----------------------------------------------|
| miR-375 | F: 5'-CCAGCGTGTTTGTTCGTTTC-3'                 |
|         | R: 5'-AGCAGGTCGGAGTTATTC-3'                  |
|         | F: 5'-CAAGCCACCATAGGAGAGAT-3'                |
| VEGF    | R: 5'-TTTTTGCCAGGAACATTTACACGTC-3'           |
|         | F: 5'-GGCGGAGGGGACCATTCTCTC-3'               |
| SHH     | R: 5'-CCCCTCGCGCTCGCTACAGAT-3'               |
|         | F: 5'-TTTGAGGCACGCCGATCC-3'                  |
| PCNA    | R: 5'-GGAGACGTAGACGAGGTCCAT-3'                |
|         | F: 5'-GCTCGCTGAACTCCTCTGA-3'                 |
| E-cadherin | F: 5'-TATGGTGTTGGTGATGACTGA-3'              |
|         | R: 5'-TCGCCGACCACATACA-3'                    |
| N-cadherin | F: 5'-ATGCCTTTGTGGAACATATGAGC-3'            |
|         | R: 5'-CGGTGCTAGTGGACTACAGA-3'                |
| Bcl-2   | F: 5'-GGTATGCCGAAGAGTGAATGC-3'               |
|         | R: 5'-GGTATGCCGAAGAGTGAATGC-3'               |
| Bax     | F: 5'-GATGGGGCCGAGACACTCG-3'                 |
|         | R: 5'-GGTCCCTTCGGCACACA-3'                   |
| U6      | R: 5'-GAGCTATTCGCCAGAGGAGA-3'                |
| GAPDH   | F: 5'-GGTGCCGAGTTGAGTGC-3'                   |
|         | R: 5'-CCCAGCATGAAGGTAGAGGT-3'                |

VEGF, vascular endothelial growth factor; SHH, Sonic hedgehog; PCNA00, proliferating cell nuclear antigen; F, forward; R, reverse.
Figure 1. miR-375 targetedly and negatively affects the VEGF gene expression. a, sequence of the 3'-UTR region in which miR-375 binds to VEGF; b, DLR assay detects luciferase activity.

*P<0.05 vs. the NC group.
Figure 2. The expression of miR-375, SHH and VEGF in different groups. a, the mRNA levels of miR-375 and SHH, VEGF in different groups; b, the protein levels of miR-375 and SHH, VEGF in different groups; c, statistical diagram of protein levels of SHH and VEGF in different groups. *P<0.05 vs. the Nor; †P<0.05 vs. the Mod; ‡P<0.05 vs. the NC group; ‡‡P<0.05 vs. the mimic group; ‡‡‡P<0.05 vs. the cyclopamine group; ‡‡‡‡P<0.05 vs. the oe-VEGF group; ^P<0.05 vs. the mimic + oe-VEGF group.
Figure 3. Cell proliferation ability in different groups.

*P<0.05 vs. the Nor; \#P<0.05 vs. the Mod; \&P<0.05 vs. the NC group; $P<0.05$ vs. the mimic group;

*%P<0.05 vs. the cyclopamine group; \@P<0.05 .vs. the oe-VEGF group.
Figure 4. Cell cycle in each group.

a, the cell cycle in different groups were determined via flow cytometry. b, the cell cycle distribution in different groups. *P<0.05 vs. the Nor; #P<0.05 vs. the Mod; &P<0.05 vs. the NC group; $P<0.05 vs. the mimic group; %P<0.05 vs. the cyclopamine group; @P<0.05 vs. the oe-VEGF group.
Figure 5. Apoptosis in each group.

a, the apoptosis in different groups was determined via flow cytometry; b, apoptosis rate in different groups. *P<0.05 vs. the Nor; †P<0.05 vs. the Mod; ‡P<0.05 vs. the NC group; §P<0.05 vs. the mimic group; ¶P<0.05 vs. the cyclopamine group; ‰P<0.05.VEGF vs. the oe-VEGF group.
Figure 6. Transwell cell invasion assay to determine the cell invasive activity in different groups.

a, the cell invasive ability was detected via Transwell assay (200×); b, the statistical graph of the number of invasive cells in different groups. *P<0.05 vs. the Nor; †P<0.05 vs. the Mod; ‡P<0.05 vs. the NC group; §P<0.05 vs. the mimic group; ¶P<0.05 vs. the cyclopaamine group; ©P<0.05 vs. the oe-VEGF group.
Figure 7. mRNA and protein levels of PCNA, E-cadherin, N-cadherin, Bax and Bcl-2 in cells of different groups. a, mRNA levels of PCNA, E-cadherin, N-cadherin, Bax and Bcl-2 in cells of different groups; b, representative protein bands of PCNA, E-cadherin, N-cadherin, Bax and Bcl-2 in cells of each group; c, results of protein levels in different groups. *P<0.05 vs. the Nor; †P<0.05 vs. the Mod; ‡P<0.05 vs. the NC group; §P<0.05 vs. the mimic group; ¶P<0.05 vs. the cyclopamine group; ‖P<0.05 vs. the oe-VEGF group.
rno-miR-375/Vegfa Alignment

3’ agugcgucggcuuugCUUGUuU 5’ rno-miR-375

38:5’ gaccaagaagauagAAACAAa 3’ Vegfa

mirSVR score: 0.1430
PhastCons score: 0.6935
