

GDF15 Promotes Corneal Neovascularization and Retinoblastoma Cell Progression via AKT/ERK Signal Pathway

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Primary research

Keywords: growth differentiation factor 15, corneal neovascularization, retinoblastoma, AKT/ERK, HIF-1 α , SDF-1

Posted Date: August 11th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-50656/v1>

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Abstract

Objective: We performed this study to investigate the role of growth differentiation factor 15 (GDF15) in corneal neovascularization and retinoblastoma cell progression and the potential mechanisms.

Methods: Human retinal endothelial cell (HREC) and retinoblastoma cell line (Y79, RB116 and WERI-Rb1) were treated with recombinant human (rhGDF15, 50 ng/mL) and transfected with small interfering RNA, si-GDF15. Cell migration and proliferation were detected by Scratch assay and CCK8 assay. We performed flow cytometry to measure apoptosis. Tube formation assay for in vitro angiogenesis measurement was used. RT-PCR and western blot were applied to detect the angiogenesis-related factors expression and the level of p-AKT(Ser473), p-AKT(Thr308), AKT, p-ERK1/2(Thr202/Tyr204), and ERK1/2. Tumor growth curve was used after subcutaneous injection of Y79 cells in nude mice and rhsi-GDF15 injection into the tail vein during 21 days.

Results: In retinoblastoma cell, rhGDF15 promoted Y79, RB116 and WERI-Rb1 cell migration and suppressed the level of apoptosis. The knock-down of GDF15 significantly inhibited cell migration and promoted apoptosis. In HREC, rhGDF15 increased the tube formation, the level of HIF-1 α and SDF, and cell migration, compared with control group, and si-GDF15 played the opposite role. The level of p-AKT(Ser473), p-AKT(Thr308), AKT, p-ERK1/2(Thr202/Tyr204), and ERK1/2 were increased by rhGDF15 in both HREC and retinoblastoma cell lines, contrary to the results of GDF15 knock-down.

Conclusion: The downregulation of GDF15 can inhibit corneal angiogenesis and the migration of retinoblastoma cells and promote their apoptosis, which could be dependent on the AKT/ERK pathway.

Introduction

Each year, more than 1.4 million people were threaten by visual impairment or even blindness originating from corneal neovascularization (CNV), across all stratifications of age [1]. Therefore, it is critical for clinical treatment to inhibit corneal neovascularization. Corneal transparency partly depends on the balance between angiogenesis and anti-angiogenesis related essentials. The corneal stroma could be intruded by abnormal neovessels once the balance fails, which usually induces the damages of normal structures and the neovascularization [2]. Corneal neovascularization may arise from various reasons, comprising degenerative, infectious and inflammatory factors, etc., leading to the pathological angiogenesis [3, 4]. Hence, current and emerging therapies focus on preventing angiogenesis and attenuating growing vasculature [5].

Vascular endothelial growth factor (VEGF) has been known as one of the most common angiogenetic factors among the pro-angiogenesis pathways [6]. Tyrosine kinases activation participates in the function of VEGF dominantly and derivate corresponding studies of targeting VEGF [7]. Past literatures have indicated VEGF plays a crucial role in corneal angiogenesis in the animal and preclinical model [8–10]. Moreover, neovascularization can be blocked by anti-VEGF treatment by downregulating the proliferation and migration of endothelial cell [11]. Similarly, the mechanisms of tumor angiogenesis usually are

pointed to VEGF pathways, and inhibition of VEGF receptor in Y79 cell line could attenuate the retinoblastoma neovascularization through the downstream AKT signaling pathways [12]. Meanwhile, the inhibition of AKT/ERK has been demonstrated that could alleviate the retinoblastoma neovascularization to prevent the glioblastoma progression [13]. However, it remains ambiguous whether these signaling pathways related to pro-angiogenesis are associated with CNV.

Growth differentiation factor-15 (GDF15), one of the TGF- β superfamily members, exists in epithelial cells and is regulated in metabolic diseases, infection and inflammation [14]. Therefore, GDF15 is regarded as a potential factor in affecting diseases development [15]. There has been investigations showing GDF15 could promote angiogenesis through modulating p53/HIF-1 α signaling pathway in hypoxic human umbilical vein endothelial cells [16]. GDF15 is also explained as the signaling marker of breast cancer related angiogenesis involving the AKT/ERK pathway [17]. As has been noted, inflammation and infection could be associated with corneal neovascularization; additionally, AKT/ERK signaling may take part in retinoblastoma cell proliferation through angiogenesis. Thus, we hypothesized that GDF15 could play an analogous role as VEGF in the corneal neovascularization and retinoblastoma cell progression.

In this study, we would detect the function of GDF15 in corneal neovascularization and retinoblastoma cell in vitro using HREC and different cell lines of retinoblastoma. We compared the GDF15 with VEGF to explore the potential similar mechanisms. In vivo, we used nude mice to investigate the tumor growth after Y79 cells injection and further detect the proteins alternation.

Materials And Methods

Animals

Approval of animals experiments was obtained from Guidelines for the Ethics Care of Laboratory Animals of Shandong Provincial Third Hospital. We injected Y79 cells into nude mice to form subcutaneous tumors, and subsequently with administration of rhsi-GDF15 (0.1 mg/kg) [18] via coccygeal veins. Twenty days later, we sacrificed the mice were through cervical dislocation and harvested tumors. We applied precision balances to measure tumor weights and calculated tumor volumes as length \times width \times width/2 [19].

Cell culture and transfection

HRECs were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in endothelial growth medium (C1556; Sigma–Aldrich, St. Louis, MO) plus penicillin–streptomycin and 10% fetal bovine serum (FBS). HRECs were cultured on dishes coated by 10mg/ml of fibronectin. Y79, RB116 and WERI-Rb1 (ATCC) were cultured in RPMI1640 medium supplemented with 10% FBS [20]. Si-GDF15 was obtained from Hollybio Biotechnology Company (Shanghai, China) and transfected into cells by lipofectamine 3000 (Invitrogen) as past studies described [21].

Si-GDF15-F: AGUCUUUGGCUAACAAGUCAU

Si-GDF15-R: GACUUGUUAGCCAAAGACUGC

Real-time PCR

As manufacturer's protocol described, we applied TRIzol (Invitrogen) to extract total RNA from cells of different groups. Transcriptor First Strand cDNA Synthesis Kit (Roche, USA) was applied to reversely transcript 1 µg RNA into cDNA for cDNA synthesis. We used FastStart Universal SYBR Green Master (ROX) and ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) to conduct qPCR after mixing the specific primers and 20 ng cDNA. The primer sequences applied in our study were as follows:

GAPDH-primer-F: CTCTGCTCCTCCTGTTCGAC

GAPDH-primer-R: CGACCAAATCCGTTGACTCC

HIF-1α-primer-F: GAACGTCGAAAAGAAAAGTCTCG

HIF-1α-primer-R: CCTTATCAAGATGCGAACTCACA

SDF-1-primer-F: ATTCTCAACACTCCAAACTGTGC

SDF-1-primer-R: ACTTTAGCTTCGGGTCAATGC

Scratch assay

We further assessed cell migration with scratch assay. In brief, we plated HRECs or retinoblastoma cells onto 6-well plates and preserved them to 90% confluence. As described previously, subsequently we scratched the cell monolayers with a 100 µl pipette tip to establish a non-cell regions [22]. Cells were washed by phosphate buffer saline three times and subsequently incubated in DMEM (mixing 4% FBS) for 48h. We applied ImageJ software (National Institutes of Health, Bethesda, MA, USA) to photograph the non-cell areas and measure the rate of the closed space.

CCK8 assay

We applied Cell Counting Kit-8 assay to evaluate the cell viabilities of HRECs. According to previous studies, growing cells (3×10^3 /ml, 50 µl) were inoculated on a 96-well plate and cultured at room temperature for 72 h. After the beginning of incubation, we added WST-8 Solution (10 µl/well) to every well each 24 hours. After incubation at 37°C for another 4 hours, we added DMSO (200 µl) to every well, and measured the optical density at 460 nm using a microplate reader to evaluate the cell viability with the different conditions [21].

Flow cytometry and apoptosis detection

We applied the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Jingmei Biotech Co., Ltd., Beijing, China) to evaluate the role of rhGDF15 or GDF15 downregulation in the apoptosis process. The FACScan flow cytometer (Accuri™ C6; BD Biosciences) was used to assess the apoptosis rates; Apoptotic cell rate = the late apoptosis rate + the early apoptosis rate (the sum of right upper quadrant - percentage of late apoptotic cells and right lower quadrant - percentage of early apoptotic cells).

Tube formation assay

The conditioned mediums of HRECs or transfected with si-GDF15 (48h subsequent to transfection). We added 1×10^5 HRECs into the collected conditioned mediums and then put them in a 24-well plate. We applied the bright-field microscope to detect the results of tube formation 24h subsequent to culture.

Western blot

We extracted the total proteins by centrifugation from different tissues or cells samples. The lysate proteins (50 μ g) were separated through SDS-PAGE and then transferred to the nitrocellulose membranes. Nitrocellulose membranes were sealed by 5% skim dry milk for 2h and subsequently incubated by various primary antibodies. The protein bands were incubated by horseradish peroxidase-conjugated antibodies and detected by enhanced chemiluminescence reagent. The p-AKT (Ser473, 1:1000, dilution), p-AKT (Thr308, 1:1000, dilution), AKT (1:1000, dilution), p-ERK1/2 (Thr202/Tyr204, 1:1000, dilution), ERK1/2 (1:1000, dilution), HIF-1 α (1:1000, dilution), SDF-1 (1:1000, dilution) were obtained from Abcam (Cambridge, MA, USA).

Statistical analysis

We analyzed all data as the mean \pm standard deviation (SD). The statistical significances were assessed by Student's t test or ANOVA using SPSS 21.0 (IBM SPSS, USA). $P < 0.05$ could be regarded as significant difference.

Results

GDF15 significantly inhibits proliferation and induces caspase-dependent apoptosis in retinoblastoma cells.

We examined the effects of recombinant human GDF15 factor (rhGDF15, 50ng/mL) on the migration of Y79, RB116 and WERI-Rb1 cell lines. The results showed that the rhGDF15 promoted the migration of human retinoblastoma cells (Fig.1A, $P < 0.01$). Y79, RB116 and WERI-Rb1 cell lines were treated with rhGDF15, 50ng/ml. The apoptosis was detected by flow cytometry, and the rhGDF15 inhibited the apoptosis of human retinoblastoma cells (Fig. 1B, $P < 0.01$). As Fig. 1C showed, the role of si-GDF15 transfection on the migration of human retinoblastoma cells were examined and si-GDF15 inhibited the migration of human retinoblastoma cells ($P < 0.01$). Moreover, flow cytometry results demonstrated that

the apoptotic levels of human retinoblastoma cells increased significantly after si-GDF15 transfection (Fig. 1D, $P<0.01$).

GDF15 promotes corneal neovascularization.

HRECs were inoculated with matrix glue and treated with rhGDF15 (rhGDF15,50ng/ml) [23] and rhVEGF factor (rhVEGF,100ng/ml, as positive controls for promoting angiogenesis) [24] respectively. After 6 hours of treatment, the tube formation in control group, rhVEGF group and rhGDF15 group were detected. The results show that rhGDF15 and rhVEGF promote tube formation remarkably (Fig. 2A, $P<0.01$).

Subsequent of 24h treatment, the levels of angiogenesis related factors HIF-1 α and SDF-1 were detected by RT-PCR. The results of control group, rhVEGF group and rhGDF15 group showed that rhGDF15, rhVEGF promoted the increase of HIF α and SDF-1 (Fig. 2B, $P<0.01$). The protein levels of angiogenesis related factors HIF-1 α and SDF-1 were detected by western blot. The results of control group, rhVEGF group and rhGDF15 group showed that rhGDF15 and rhVEGF promoted the increase of HIF α and SDF-1 protein levels (Fig. 2C, $P<0.01$).

Moreover, tube formation was detected after si-GDF15 transfection treatment. The results showed that rhVEGF promoted tube formation, while si-GDF15 transfection inhibited tube formation (Fig. 2D, $P<0.01$). Likewise, RT-PCR and western blot results showed rhVEGF promoted the increase of HIF-1 α and SDF-1, while si-GDF15 transfection inhibited the expression of HIF-1 α and SDF-1 (Fig. 2E-F, $P<0.01$, $P<0.01$).

GDF15 promotes wounding migration of human endothelial cells.

After HRECs cells were treated with rhGDF15 or transfected with si-GDF15 for 24 hours, the ability of cell migration was detected by scratch assay. The results showed that rhGDF15 increased cell migration significantly (Fig. 3A, $P<0.01$), while knock-down GDF15 inhibited cell migration (Fig. 3C, $P<0.05$), compared with either control group or rhVEGF group. The CCK8 assay results showed rhGDF15 increased the cell viability (Fig. 3B, $P<0.01$) and knock-down GDF15 decreased cell viability significantly (Fig. 3D, $P<0.05$).

GDF15 promotes corneal neovascularization by regulating ERK/AKT pathway.

The protein levels of p-AKT (Ser473), p-AKT (Thr308), AKT, p-ERK1/2 (Thr202/Tyr204) and ERK1/2 in HRECs cells were detected by western blot after 24 hours of rhGDF15 or rhVEGF. As Fig. 4A showed, rhGDF15 and rhVEGF caused the increased expression of p-AKT (Ser473), p-AKT (Thr308) and p-ERK1/2 (Thr202/Tyr204) significantly ($P<0.01$), while knock-down GDF15 decreased the proteins level of HIF-1 α and SDF-1, compare with either control group or rhVEGF group (Fig. 4B, $P<0.01$).

Knock-down GDF15 inhibits retinoblastoma growth in mice.

After subcutaneous injection of Y79 cells and caudal vein injection of rhsi-GDF15 in nude mice, the tumor formation curve within 21 days was shown as Fig. 5A. The results showed that rhsi-GDF15 inhibited

tumor growth (volume and weight, $P < 0.01$). The western blot of tumor nodules was used to detect the protein levels of p-AKT (Ser473), p-AKT (Thr308), AKT, p-ERK1/2 (Thr202/Tyr204) and ERK1/2. The results showed that knock-down GDF15 inhibited the expression of p-AKT (Ser473), p-AKT (Thr308) and p-ERK1/2 (Thr202/Tyr204) significantly (Fig. 5B, $P < 0.01$). Meanwhile, knock-down GDF15 significantly decreased the proteins levels of HIF-1 α and SDF-1 (Fig. 5C, $P < 0.01$).

Discussion

The evidence of corneal neovascularization was found in 20% of transplant corneal specimens. Meanwhile, it was reported that 12% of patients with this alternation got vision loss [25, 26]. Therefore, corneal neovascularization has gradually become a developing concern of public health. *Aydin et al.* has revealed that combined therapy including anti-VEGF agents was highly effective in blocking corneal neovascularization targeted mechanisms maintaining angiogenesis [27]. Meanwhile, the efficiency of decreasing neovascularization in human CNV has been investigated with non-side effects [28]. Likewise, other angiogenesis inhibitor, angiostatin has been indicated that it could reduce the corneal vascularization significantly in rats [29]. Thus, we attempted to explore the novel reliable therapy targeted pro-angiogenesis and anti-angiogenesis as the direction of VEGF. In our current study, we demonstrated that rhGDF15 could promote corneal vascularization as rhVEGF in HRECs and inhibition of GDF15 was expected to become the potential therapy for CNV for the first time. Moreover, GDF15 played an critical role in the viability and migration of human retinoblastoma cells, which is consistent with the mechanisms of pro-growth in HRECs. In vitro (HRECs and retinoblastoma cell lines) and vivo, we all detected the activation of AKT/ERK signaling pathways with HIF-1 α and SDF-1 expression. This potentially suggested a definite association between CNV and retinoblastoma (RB).

On the one hand, GDF15 has been widely known for the activity in apoptosis and inflammation pathways [30]. In solid tumors and stromal tumors, there were studies reporting the overexpression of GDF15 [31, 32]. Recent investigations indicated that GDF15 could get involved in the progression of tumors and endothelial cells through pro-angiogenesis, which was dependent on the AKT and ERK intracellular signaling cascades [23, 33, 34]. Considering that the effect of pro-angiogenetic factors on corneal neovascularization and retinoblastoma, we confirmed the expression and functions of AKT/ERK CNV and RB without precedent. On the other hand, HIF-1 α could promote migration to SDF-1 in tumor cells [35]. Interestingly, the cells migration was not increased by the upregulation of SDF-1 [36]. Here, we observed the augment both in HIF-1 α and SDF-1, which might be the result of SDF-1 receptors. Meanwhile, the level of VEGF was found to be upregulated by HIF-1 α [22], hence we hypothesises that the increase of HIF-1 α and SDF-1 were only associated with GDF15 upregulation instead of it results.

Our study still have limitations required discussion, despite the unique discovery. Firstly, we didn't establish the CNV animal model to perform in-vivo experiments for the effect of GDF on angiogenesis. Secondly, we did not confirm the definitive link between CNV and RB, even if these two diseases have similarity in mechanisms as previously described. Thirdly, we did not investigate the upstream and downstream of AKT/ERK thoroughly, and how AKT/ERK was associated with HIF-1 α and SDF-1, despite

that we detected the corresponding pathways expression. In addition, there are no studies indicating that the safety of rhGDF in human so that our study we did not have the data from individuals. Thus we expected the future further explorations.

Conclusion

Summarily, this current study revealed that rhGDF15 could promote corneal vascularization and human retinoblastoma cells migration, which is could be dependent on the AKT/ERK signaling pathways through angiogenesis role. GDF15 could be a promising target to exploring the better management for CNV.

Declarations

Ethics approval and consent to participate

Approval of animals experiments was obtained from Guidelines for the Ethics Care of Laboratory Animals of Shandong Provincial Third Hospital.

Consent for publication

Consent for publication was obtained from all authors.

Competing interests

The authors declare that there is no conflict of interest to disclose.

Funding

No funding received for this study.

Authors' contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Shi-liang Cheng, Chun-guang Li. Experiment: Wen Zheng, Miao Yu, Meng Li, Xin-feng Liu, Fang-fang Li. Analysis, interpretation of data, and drafting of the manuscript: Wen Zheng, Shi-liang Cheng, Chun-guang Li. Critical revision of the manuscript for important intellectual content: all authors.

Availability of Data and Materials

All primary data and materials presented in this study are available from the corresponding author upon reasonable request.

Acknowledgements

No other issues.

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Figures

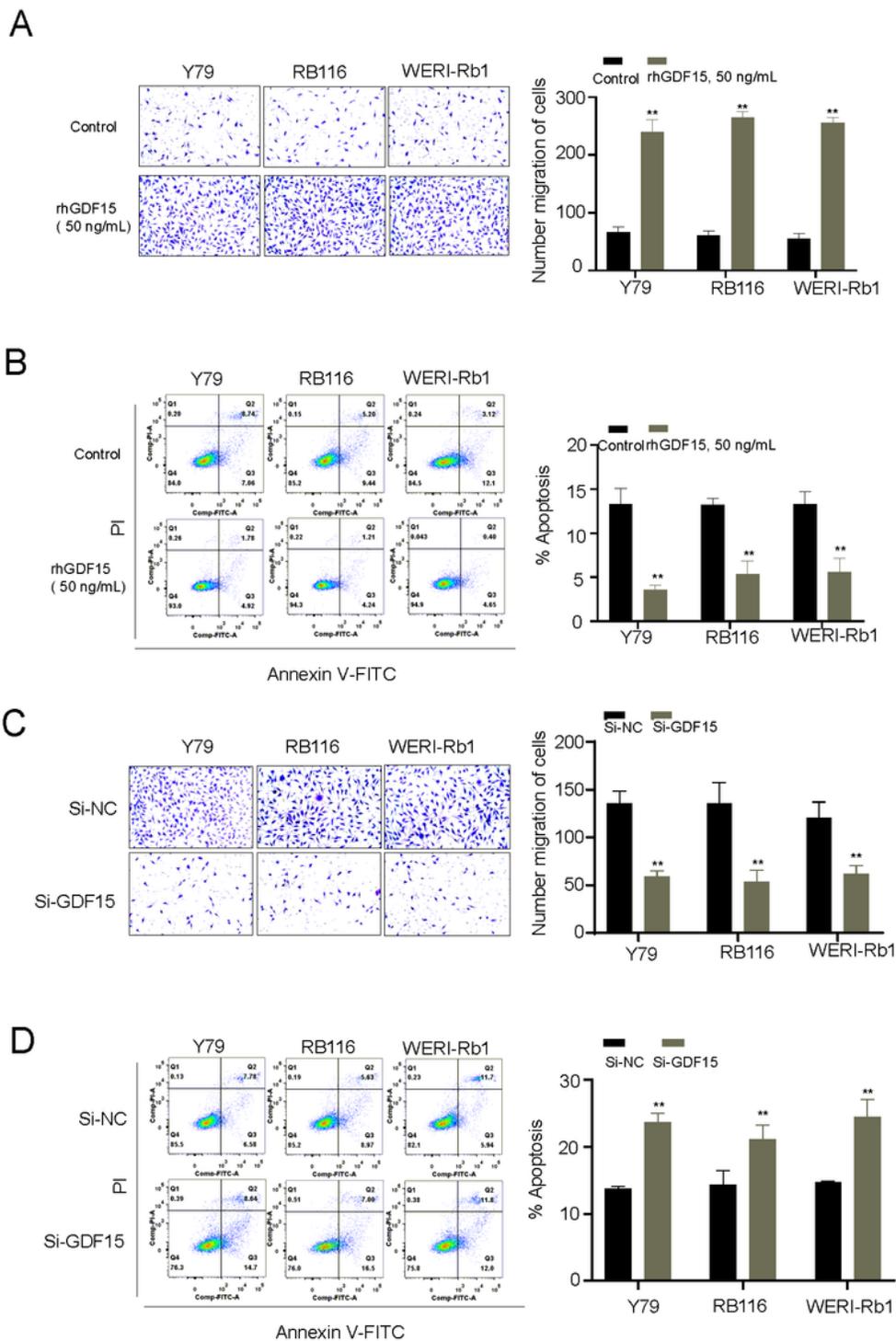


Figure 1

The role of rhGDF and si-GDF15 on the cell migration and apoptosis of Y79, RB116 and WERI-Rb1. A. The number of migration cells in three cell lines between control group and rhGDF15 group; B. Flow cytometry results for apoptosis rate in Y79, RB116 and WERI-Rb1 between control group and rhGDF15 group; C. The number of migration cells in three cell lines between si-NC group and si-GDF15 group D. Flow cytometry

results for apoptosis rate in Y79, RB116 and WERI-Rb1 between si-NC group and si-GDF15 group. * means $P < 0.05$; ** means $P < 0.01$.

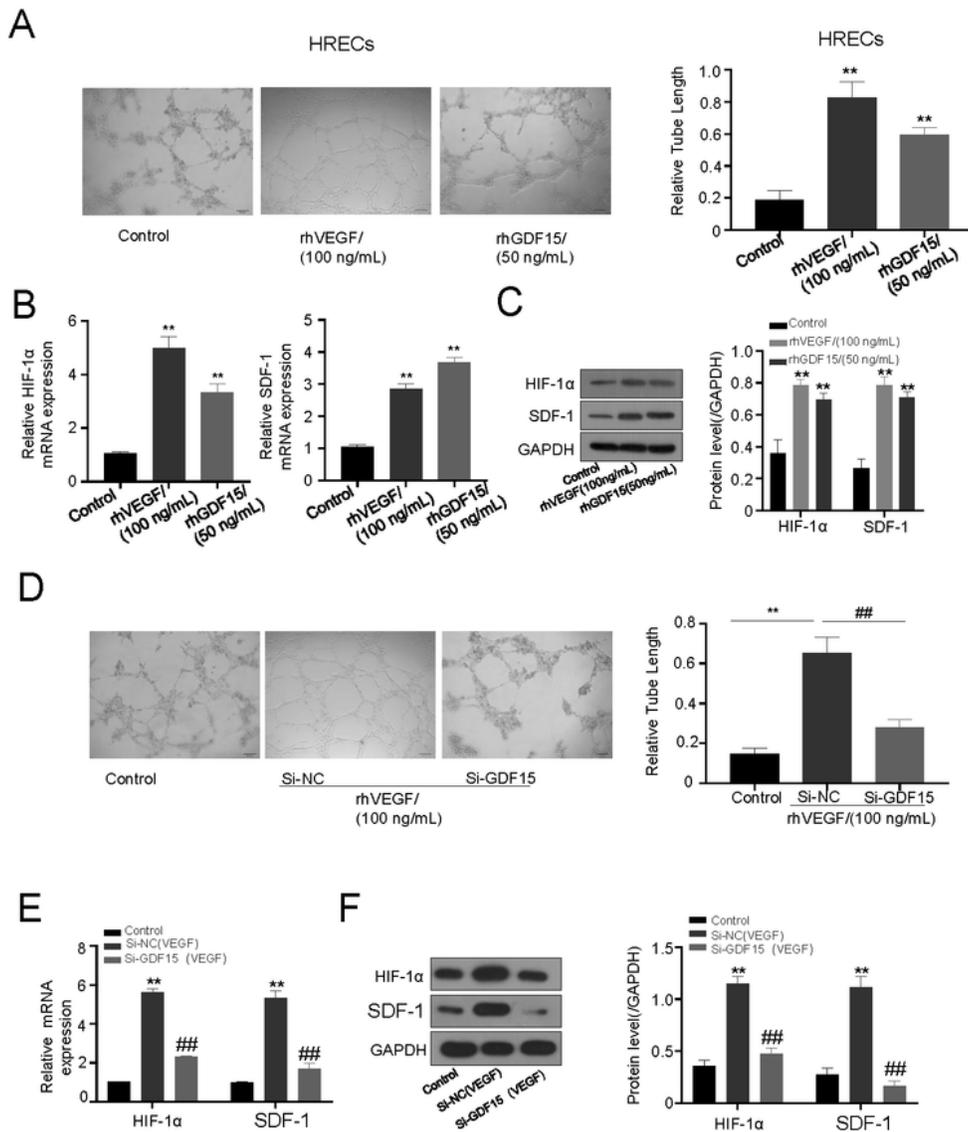


Figure 2

The effect of rhGDF and si-GDF15 on the angiogenesis of HRECs. A. The results of tube formation of HRECs among the control group, rhVEGF group and rhGDF15 group; B. The results of RT-PCR for HIF-1 α and SDF-1 among the control group, rhVEGF group and rhGDF15 group; C. The results of western blot for

HIF-1 α and SDF-1 among the control group, rhVEGF group and rhGDF15 group; D. The results of tube formation of HRECs among the control group, si-NC group and si-GDF15 group; E. The results of RT-PCR for HIF-1 α and SDF-1 among the control group, si-NC group and si-GDF15 group; F. The results of western blot for HIF-1 α and SDF-1 among the control group, si-NC group and si-GDF15 group * means P <0.05; ** means P<0.01.

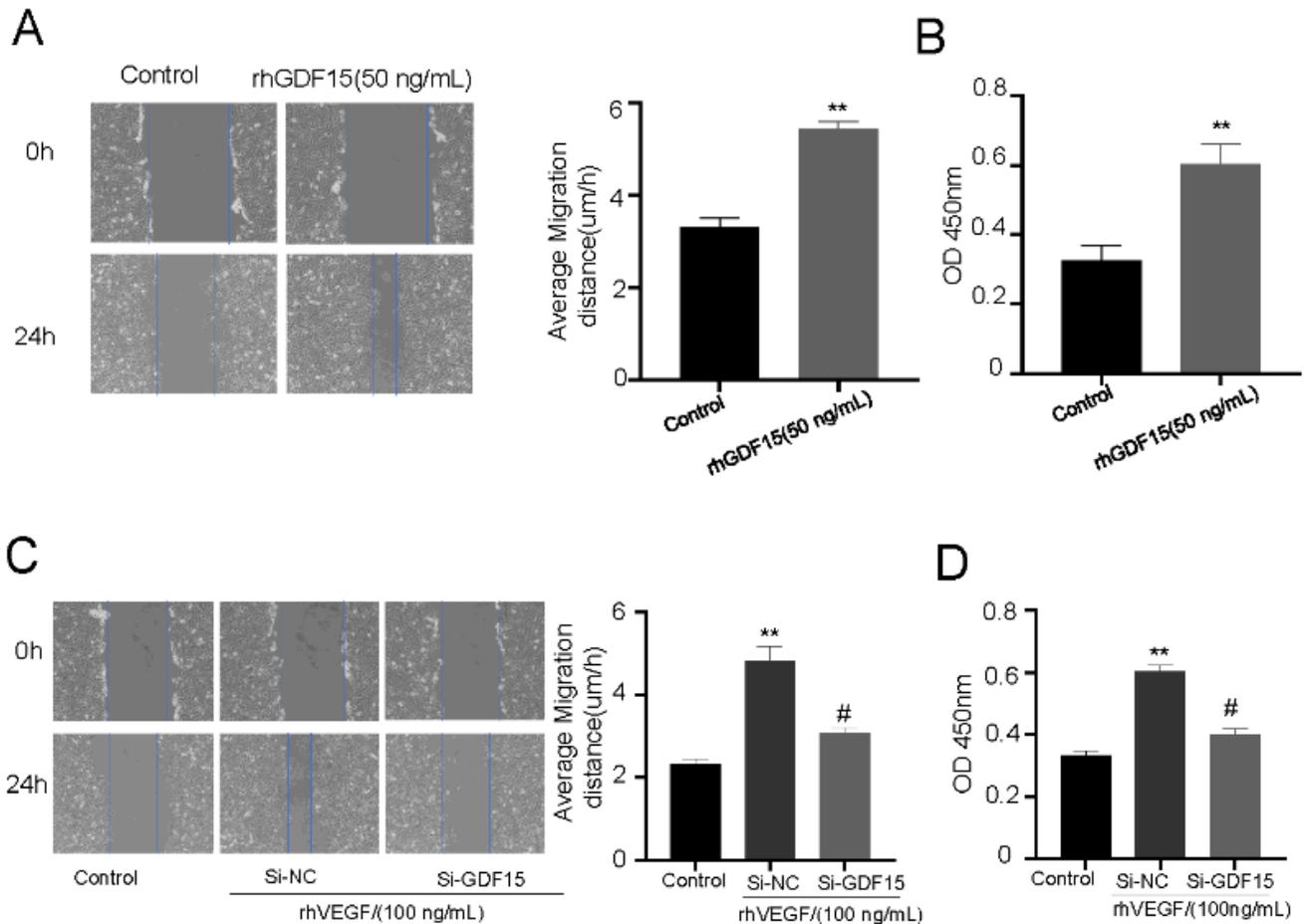


Figure 3

The role of rhGDF and si-GDF15 on the cell migration and viability of HRECs. A. The results of scratch assay between the control group and rhGDF15 group after 24 h; B. The OD value of the control group and rhGDF15 group; C. The results of scratch assay among the control group, si-NC group and si-GDF15 group after 24 h; D. The OD value of the control group, si-NC group and si-GDF15 group. * means P <0.05; ** means P<0.01.

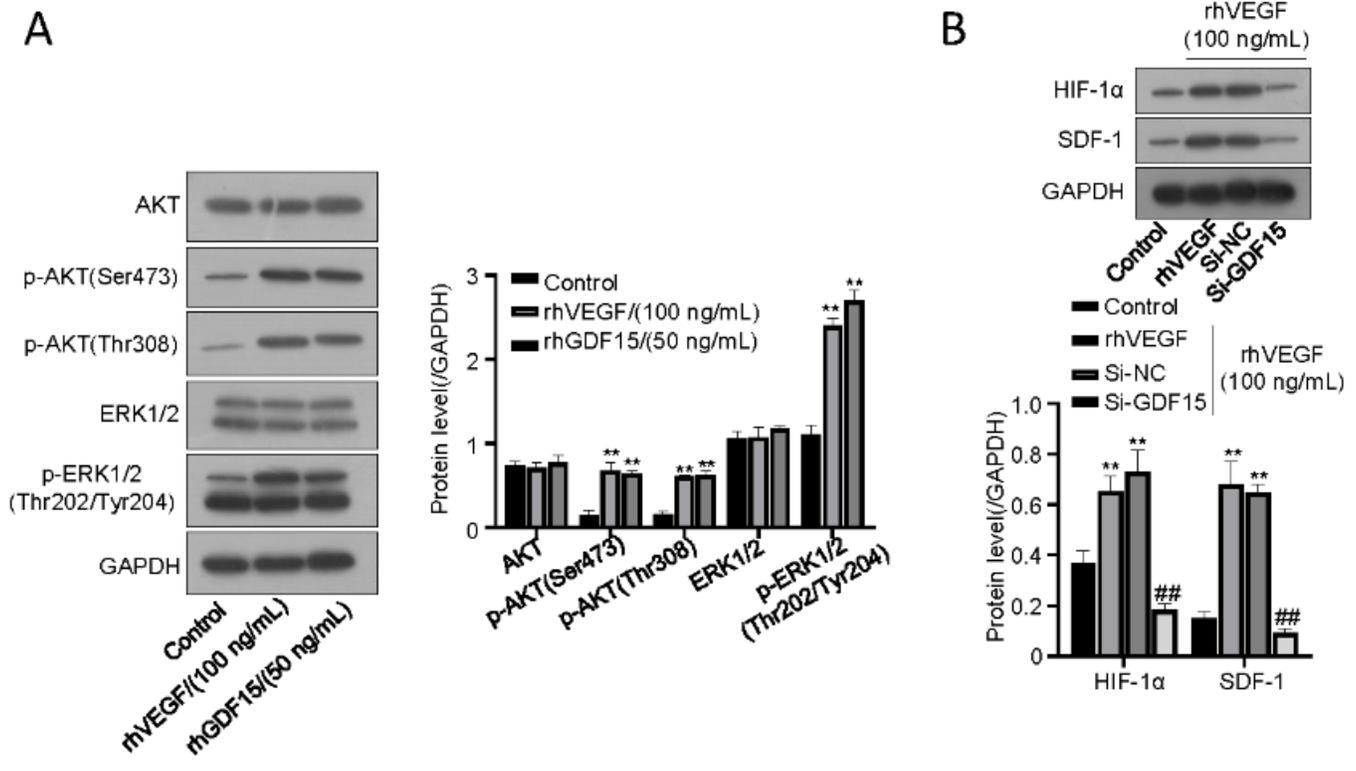


Figure 4

The effect of rhGDF, rhVEGF and si-GDF15 on the AKT / ERK pathway. A. The results of western blot in p-AKT(Ser473), p-AKT(Thr308), AKT, p-ERK1/2(Thr202/Tyr204), and ERK1/2 among the control group, rhVEGF group and rhGDF15 group; B. The results of western blot in HIF-1α and SDF-1 among the control group, rhVEGF group and HIF-1α and SDF-1. * means $P < 0.05$; ** means $P < 0.01$.

A

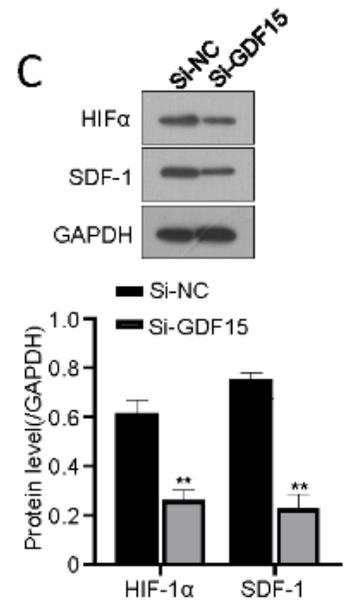
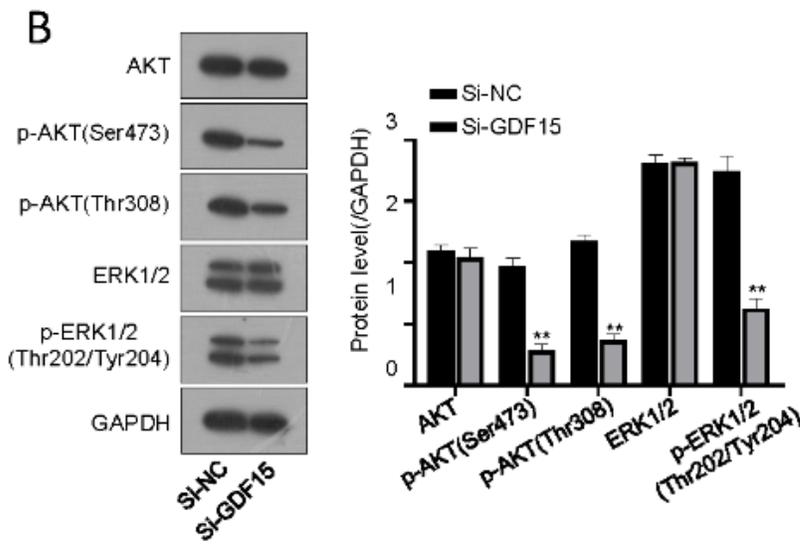
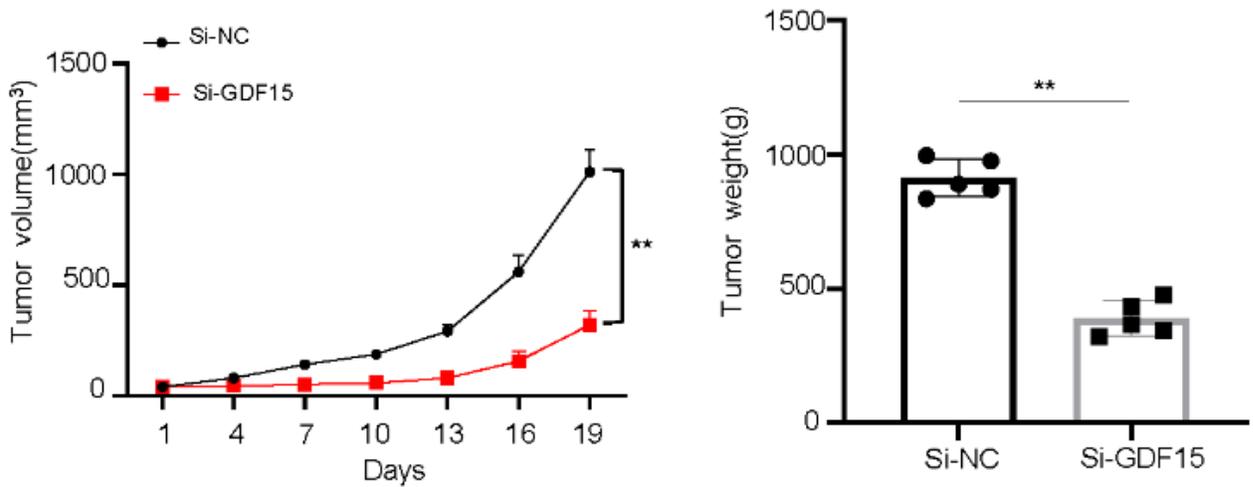


Figure 5

Knock-down GDF15 inhibits retinoblastoma growth in nude mice. A. The results of tumor growth within 21 days between si-NC group and si-GDF15 group; B. The results of western blot in p-AKT(Ser473), p-AKT(Thr308), AKT, p-ERK1/2(Thr202/Tyr204), and ERK1/2 between si-NC group and si-GDF15 group; C. The results of western blot in HIF-1α and SDF-1 between si-NC group and si-GDF15 group; D. The results of RT-PCR in HIF-1α and SDF-1 between si-NC group and si-GDF15 group. * means P < 0.05; ** means P < 0.01.