RESEARCH PAPER

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TFF3 promotes pituitary tumor cell migration and angiogenesis *via* VEGFA

Xiaoan Wang[1], Han Qin[2]*

[1] Department of Neurosurgery, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China,
[2] Department of Neurosurgery, General Hospital, Central Theatre, PLA, Wuhan, China,
** Email: hqin9599@163.com*

Pituitary adenoma is a typical adult primary brain tumor and 35% of pituitary adenomas are invasive. The enhancement of angiogenesis is essential for the spread and invasiveness of invasive pituitary adenoma. Thus, it is urgent to uncover the mechanism and relevant biomolecular targets for the therapy and prognosis of pituitary adenomas. The HP75 cells were transfected with si-NC, si-TFF3, pcDNA, and pcDNA-TFF3 to investigate the effects of TFF3 on the proliferation, migration and invasion of pituitary tumor cell. The protein level of TFF3 and HIF-1α/VEGFA was determined by western blot. The transwell migration assay and wound healing assay were used to investigate the influence of TFF3 on the cell migration and invasion of HP75 cells. The tumor angiogenesis was determined by tube formation assay. The proliferation of HP75 cells was assessed by using MTT assay and colony-forming unit assay. The cell proliferation rate was separately enhanced and reduced remarkably in TFF3 overexpression group and si-TFF3 group. TFF3 could modulate the proliferation, migration and invasion ability of HP75 cells. Furthermore, TFF3 may play a oncogenic role in HP75 cells. Overexpression of TFF3 enhanced the number of branching points and network formation in HP75 cells, suggesting the TFF3 had positive effects on cell angiogenesis. These results also disclosed a novel relationship between TFF3 expression and the activation of the HIF-1α/VEGFA signaling pathway. In summary, this study uncovered new insight into the mechanisms of TFF3's anti-tumor activities in pituitary adenoma cells by investigating its effects on HIF-1α/VEGFA signaling pathway regulation.

Key words: TFF3, HP75 cells, pituitary adenoma, angiogenesis, VEGFA

INTRODUCTION

Pituitary adenoma is a typical adult primary brain tumor, constituting at least 15% of intracranial neoplasms (Lithgow et al., 2019). The anterior pituitary consists of multiple hormone producing cell types including thyrotropin, gonadotrophs, somatotrophs, lactotrophs, and corticotrophs, all of which can cause tumors, resulting in the heterogeneous group of neoplasms encompassed by the pituitary adenomas (Van Goor et al., 2001). The storage of genetic mutations in pituitary adenomas leads to downstream oncogenic change such as sustained angiogenesis, proliferation, growth suppression evasion, cell death resistance, and invasion (Melmed, 2011; Marques et al., 2017). More than 40% of pituitary adenomas are cellularly mutated, which can actively invade surrounding structures, including brain tissue, sphenoid sinus, clivus, orbital, and cavernous sinus (Johnston et al., 2014; Wang et al., 2018). Cell infiltration and angiogenesis of pituitary adenomas is the leading cause of incomplete tumor resection and postoperative recurrence (Di Ieva et al., 2014). Therefore, it is urgent to uncover the mechanism and relevant biomolecular targets for the therapy and prognosis of pituitary adenomas.

Tumor neovascularization and vascular remodeling are the basic processes of pituitary adenoma growth and invasion (Turner et al., 2003; Ilie et al.,

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2019; Yang et al., 2019). Vascular endothelial growth factor A (VEGF-A) and its tyrosine kinase receptors such as vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) are involved in physiological and pathological angiogenesis (Lacal et al., 2018; Park et al., 2018; Ali et al., 2019). VEGFR-1 acts as a negative regulator through its ligand-binding domain and positively affects proliferation or migration through its tyrosine kinase activity (Stevens et al., 2019). VEGFR-1 has a high affinity for VEGF-A and acts as a negative regulator in angiogenesis by inhibiting pro-angiogenic signals to frame a key balance, which is essential for physiological vascular formation (Kearney et al 2004; Shibuya 2006). Current studies have reported that VEGFA was overexpressed in pituitary adenomas (He et al., 2017). Hypoxia-inducible factor-1α (HIF-1α) is an essential inducer of angiogenesis, regulating the expression of VEGF in pituitary adenomas and other human tumor cells (Zagzag et al., 2000; He et al., 2017). It has been confirmed that HIF-1α can activate VEGF (Ahluwalia et al., 2012). Trefoil factor 3 (TFF3) is a member of the trefoil factor family and is a secreted protein mainly expressed in the small and large intestines, which plays a critical role in maintaining mucosal integrity and repair (Okada et al., 2005). Recent research indicated that TFF3 was overexpressed in multiple solid tumors, including breast, endometrium, prostate, and other cancers, and can also trigger oncogenic cell growth, angiogenesis, invasion, and metastasis (Emami et al., 2004). Moreover, previous studies have indicated that TFF3 promoted the proliferation, migration and invasion of glioma cells through regulating the HIF-1α pathway (Diao et al., 2017). According to recent reports, TFF3 was highly expressed in pituitary adenoma tissues and knockdown of TFF3 promoted HP75 cell apoptosis. Therefore, this study aimed to investigate the effect of TFF3 on the proliferation, migration, invasion, and angiogenesis of pituitary adenomas and the underlying mechanisms.

METHODS

Cell culture

The HP75 cell line was obtained from American Type Culture Collection cell bank (ATCC, Manassas, VA, USA). The cells were incubated in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere with 5% CO2. The cells were seeded onto 6-well plates at a density of 1 × 105 cells/well, and. HP75 cells were divided into four groups including: si-NC (as control), si-TFF3, pcDNA, pcDNA-TFF3, which were used in all experiments. The cells were transfected with pcDNA3 vector carrying a full cDNA sequence encoding human TFF3, empty pcDNA3 vector, negative control siRNA (si-NC), or TFF3 siRNA (si-TFF3) with Lipofectamine® 2000 reagent following the manufacturer's protocol when the cell confluence reached to 80%–90%. Since transfection of si-NC does not affect the physiological state of HP75 cells, which can be served as control in this experiment.

Western blot

Total protein from each group was extracted and measured by western blot. Protein samples (20 μg protein/sample) were separated by 8% SDS-PAGE gel and then transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies against TFF3 (1:1000, Invitrogen, USA), HIF-1α (1:3000, Abcam, Cambridge, UK), VEGFA (1:2000, Abcam, Cambridge, UK), GAPDH (1:10000, Abcam, Cambridge, UK) overnight at 4°C, and subsequently incubated with secondary antibody for 1 h. The specific protein bands were visualized by enhanced chemiluminescence reagent (Bio-Rad, USA).

MTT assay

HP75 cells were seeded into 96-well plates at a density of 5×103 cells/well and incubated at 37°C for 24 h. After incubation, the reagent 3-(4,5-dimethylthiazol--2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma--Aldrich, St. Louis, MO) was added into the plate and incubated for 5 h at 37°C. The supernatant was removed and cells were mixed with DMSO (Sigma-Aldrich) to dissolve the formazan products. The absorbance was detected by a microplate reader (Tecan Group Ltd., Switzerland) at the wavelength of 570 nm.

Colony-forming unit assays

Colony-forming unit assays were used to analyze the proliferation of HP75 cells. The HP75 cells were seeded into a 12-well plate at a density of 1 × 104 cells/well and cultured with the medium containing 1.2% 1500-cps methylcellulose, 30% FBS, 1% bovine serum albumin, 0.1 uM 2-mercaptoethanol and 2 mM L-glutamine. The cells were incubated at 37°C for

7 days. Colonies were independently counted by two investigators under an inverted microscope. Aggregates consisting of more than 50 cells were counted as colonies.

For invasion assay of HP75 cells, Boyden chamber (Corning) with 8 mm pore were used as upper chambers in a 24-well. 5 × 104 cells suspended in serum free medium were added into upper chamber. Meanwhile, the medium containing 10% FBS was loaded into the lower chamber. Twenty-four hours later, the cells in the upper chamber were removed and the cells migrated on the bottom membrane were fixed with 4% paraformaldehyde for 30 min. The fixed cells were stained with haematoxylin solution for 5 min. Numbers of invaded cells were counted and photographed in four representative fields.

The motility of HP75 cells was measured by wound-healing assay. The HP75 cells were seeded at a density of 6 × 104 cells per well in 6-well plates. When the cells reached to 80–90% confluence, the sterile 200 µL tips were used to scratched and cells were incubated under standard conditions for 24 h. After cells were washed for several times, the process of cell recovery was captured at 0 and 24 h using a phase contrast microscope.

Tube formation assay

The *in-vitro* tube formation assay was performed by using an angiogenesis assay kit (ECM625, Sigma-Aldrich). The Human Umbilical Vein Endothelial Cells (HUVECs) were seeded onto Matrigel in 24 well plates at a density of 5 × 104 cells/well and pre-incubated for 30 min at 37°C for cell attachment. Culture supernatants was collected from each group of transfected HP75 cells. The pre-coating HUVECs were co-cultured with the HP75 cell's supernatants for 24 h. The average tube length and the number of branch points was calculated in 5 random microscopic fields with a computer-assisted inverted light microscope. The ImageJ with the angiogenesis analyzer plugin was used for thed quantification of tube networks.

Statistics

All experiments were obtained in triplicate to ensure the reproducibility and analyzed using GraphPad Prism Software 6.0 (GraphPad Software, La Jolla, USA). All data are presented as the mean ± SEM. One-way analysis of variance (ANOVA), and t-tests were used to make a comparison. A statistically significant difference was defined as $p<0.05$.

RESULTS

TFF3 promoted the proliferation and migration of pituitary tumor cells

To investigate the effects of TFF3 on pituitary tumor cell proliferation, the HP75 cells were transfected with si-NC, si-TFF3, pcDNA, and pcDNA-TFF3, and the protein level of TFF3 was determined by western blot (Fig. 1). The western blot analysis demonstrated a remarkably higher protein expression of TFF3 in pcDNA-TFF3 group, anda suppressed expression of TFF3 in si-TFF3 group (Fig. 1A). To further determine the impact of TFF3 on pituitary tumor cell proliferation, the cell's function was assessed by using MTT assay and colony-forming unit assay (Fig. 1B). Compared with the si-NC transfected cells, an obvious reduction of cell proliferation was detected in HP75 cells transfected with si-TFF3. Conversely, the cell proliferation rate was enhanced remarkably in pcDNA-TFF3 group. Similarly, the colony-forming unit count was significantly declined in si-TFF3 group compared to si-NC group (Fig. 1C). Furthermore, pcDNA-TFF3 transfection could reverse the effect of si-TFF3 transfection on HP75 cells.

To further investigate the influence of TFF3 on the cell migration and invasion in HP75 cells were tested *via* transwell migration assay and wound healing assay. After 24 h of cultivation, the results of wound healing showed the wound width of migrated cells which transfected with si-TFF3 was significantly less than transfected with si-NC (Fig. 2A). The results of transwell invasion assay also indicated that the cell invasive ability in HP75 cells was attenuated when cells were transfected with si-TFF3 compared with the si-NC group (Fig. 2B). In contrast, the enhanced wound width of migrated cells in HP75 cells were observed in the pcDNA-TFF3 group; meanwhile, the invasive ability of pituitary tumor cells was remarkably raised as compared with the pcDNA group (Fig. 2A, B). Taken together, these results indicate that TFF3 could modulate the proliferation, migration and invasion ability of HP75 cells. TFF3 may play a regulated oncogenic role in HP75 cells.

TFF3 induced tumor angiogenesis and regulated HIF-1α/VEGFA signaling pathway in HP75 cells

To evaluate the role of TFF3 on tumor angiogenesis, the tube formation assay was performed. The HP75 cells were seeded onto growth factor-reduced Matrigel to avoid induction of tube formation by growth factors. As shown in Fig. 3, the si-TFF3 transfected group

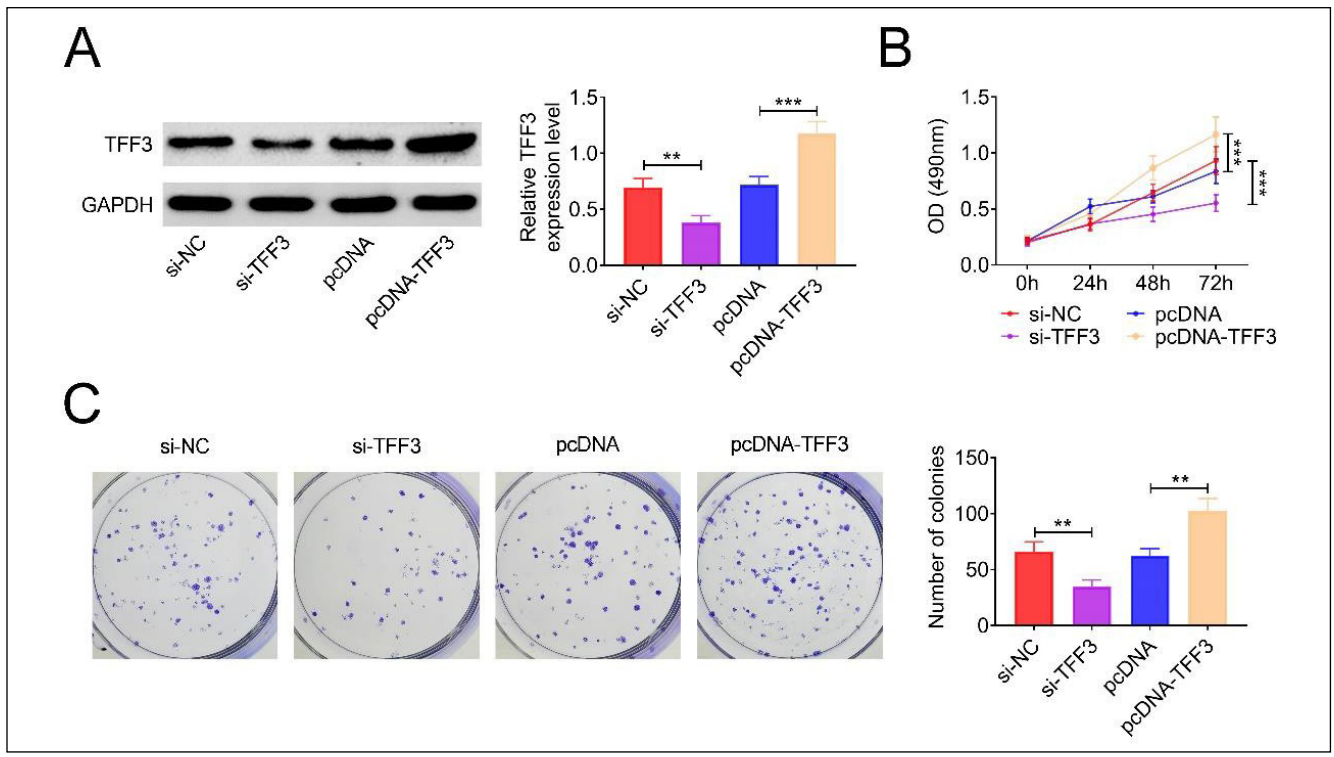

Fig. 1. Effect of TFF3 on cell proliferation of pituitary tumor cells. (A) Western blot was performed to examine the expression level of TFF3. (B) MTT assay was used to explored the effect of si-TFF3 and pcDNA-TFF3 transfection on proliferative potential of HP75 cells. (C) Cell proliferation is measured by colony-forming unit assay. ***p<0.005 *vs.* si-NC or pcDNA. **p<0.01 *vs.* si-NC or pcDNA. Data are expressed as mean ± SEM.

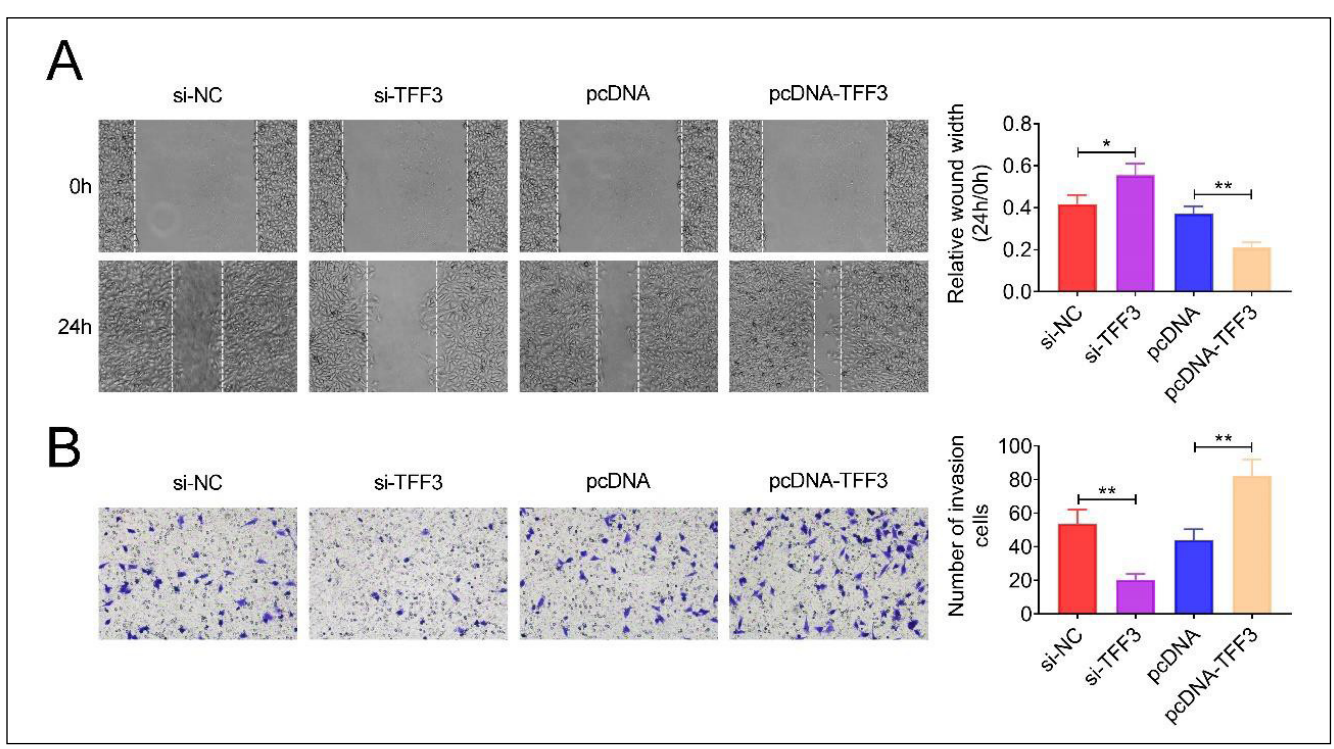

Fig. 2. Effect of TFF3 on cell migration and invasion of HP75 cells. (A) Wound healing assay was used to explore the migratory potential of TFF3-silenced or TFF3-overexpressed HP75 cells. (B) HP75 cells migration induced by TFF3 silence or overexpression was assessed by using transwell co-cultured system (8 μm pore size). **p<0.01 *vs.* si-NC or pcDNA. *p<0.05 *vs.* si-NC or pcDNA. Data are expressed as mean ± SEM.

showed significantly decreased tube branching points and network formation compared with the si-NC group. Compared with the pcDNA group, treatment of pcDNA-TFF3 was able to enhance the number of branching points and network formation in HP75 cells, suggesting the TFF3 exhibited positive effects on cell angiogenesis (Fig. 3).

A recent study indicated that TFF3 was involved in cancer progression through potential target genes and may modulate several solid tumor growths through regulating HIF-1α signaling pathway. In order to clarify the correlation between the expression level of TFF3 and the activation of the HIF-1α/VEGFA signaling pathway, the si-TFF3 and pcDNA-TFF3 were transfected into HP75 cells. The change of cell protein expression level was shown in Fig. 4. The activation of the HIF-1α/VEGFA signaling pathway was attenuated when TFF3 gene was silenced. The results showed that the protein expression level of HIF-1α and VEGFA were raised when TFF3 was overexpressed (Fig. 4). These results disclosed a novel relationship between TFF3 expression and the activation of the HIF-1α/VEGFA signaling pathway.

DISCUSSION

Pituitary adenoma is a typical adult primary brain tumor that comprises 10–25% of intracranial neoplasm, and about 35% of pituitary adenomas are invasive (Yang and Li, 2019). Numerous studies have demonstrated that the enhancement of angiogenesis is essential for the spread and invasiveness of invasive pituitary adenoma (Turner et al., 2000; Liu et al., 2017). The angiogenesis of tumors is related to vascular endothelial cell tube-like structure formation and extracellular matrix remodeling. The angiogenesis in the tumor is a complex dynamic progression that is associated with vascular basement membrane remodeling and endothelial matrix degradation (Quintero-Fabián et al., 2019). Compared with non-invasive pituitary adenomas, the invasive ones have more vascular angiogenesis. TFF3 is a member of the trefoil peptide family with a trefoil and C-terminal dimerization domain. Recent research has shown that TFF3 has modulated essential function of oncogenesis, including cell survival metastasis, proliferation, invasion, and cell apoptosis (Meyer zum Büschenfelde et al., 2004; Perera et al., 2015). Previous studies reported that TFF3 could induce angiogenesis in breast tumors and promoted cell invasion in gastrointestinal cancer cells (Guleng et al., 2012; Lau et al., 2015). However, the potential role of TFF3 in pituitary adenomas is so far unknown and is worthy of further investigation. The present research focused on the effect of TFF3 on pituitary tumor cell proliferation and identified TFF3 as an oncoprotein in pituitary adenomas. This study revealed the expression of TFF3 in pituitary adenomas, blockade of which can be used as a novel strategy to ameliorate tumor angiogenesis in an *in vitro*

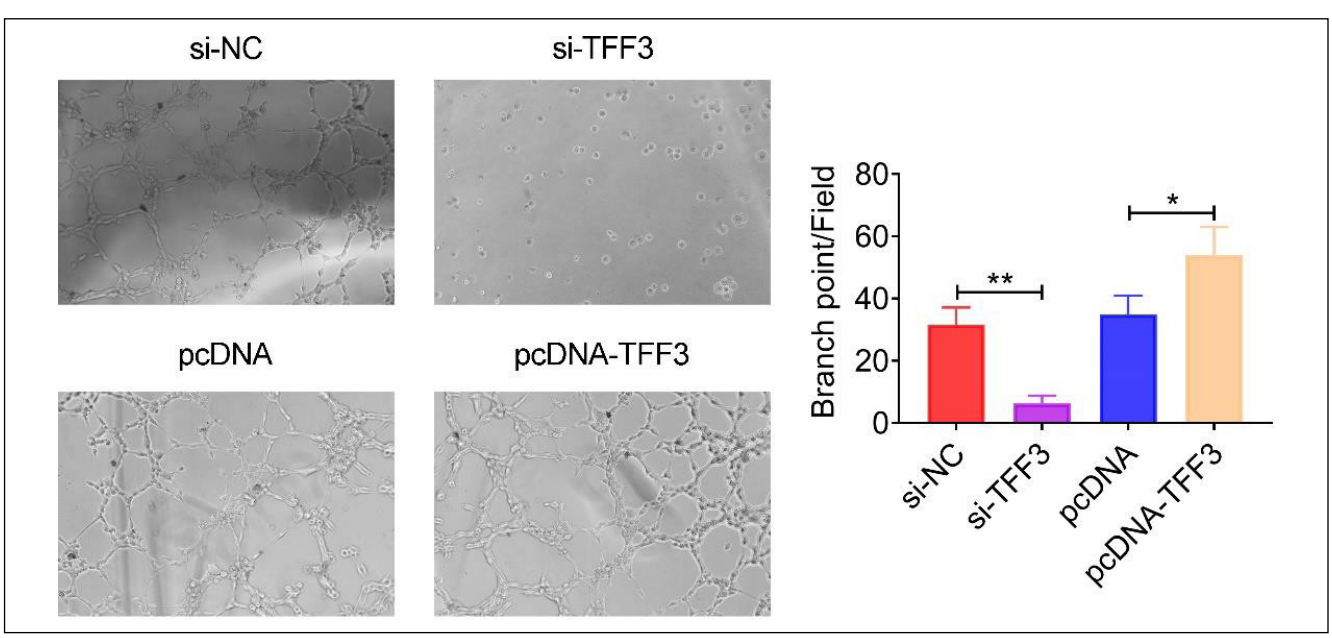

Fig. 3. Results of tube formation assay in HP75 cells. Analysis of tumor angiogenesis ability of pituitary tumor cells transfected with si-TFF3 and pcDNA-TFF3. **p<0.01 *vs.* si-NC or pcDNA. *p<0.05 *vs.* si-NC or pcDNA. Data are expressed as mean ± SEM.

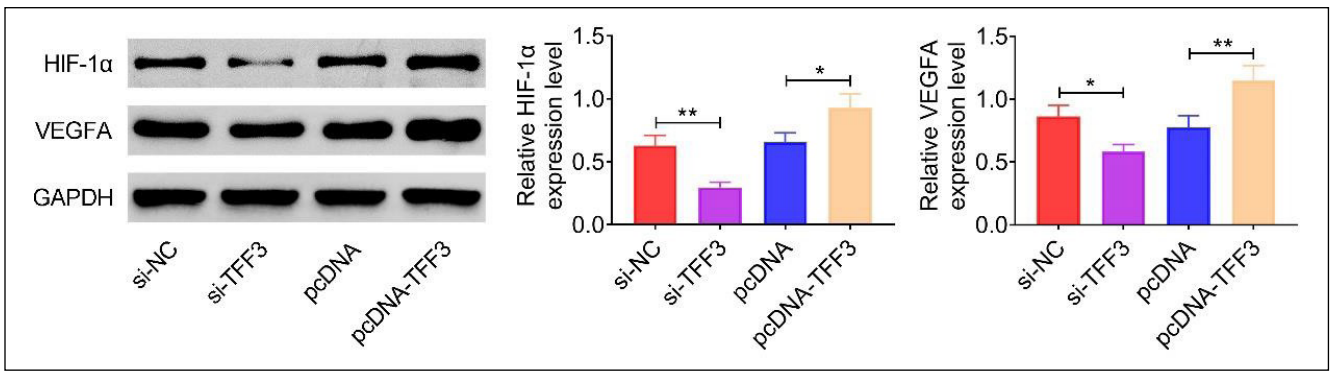

Fig. 4. TFF3 overexpression positively correlated with activation of HIF-1α/VEGFA signaling pathway in HP75 cells. Western blot analysis of proteins expression in the HIF-1α/VEGFA signaling pathway after si-TFF3 and pcDNA-TFF3 transfection. **p<0.01 *vs.* si-NC or pcDNA. *p<0.05 *vs.* si-NC or pcDNA. Data are expressed as mean ± SEM.

model. The results in this study indicated that TFF3 could modulate the proliferation, migration and invasion ability of HP75 cells. Moreover, TFF3 overexpression enhanced the number of branching points and network formation of HP75 cells, suggesting that TFF3 had positive effects on cell angiogenesis. In consistent with our research, accumulating evidence found that TFF3 was upregulated in cancer cell lines and clinical samples and its genomic suppression remarkably reduced the migration and invasion of tumor cells.

In addition, there are numerous pieces of evidence supporting that the function of TFF3 was mediated by multiple signaling pathways, as well as the HIF-1α signaling pathway in gastric cancer, nuclear factor kappa B pathways in a rat intestinal epithelial cell line, phosphatidylinositol-3-kinase-AKT in colonic cancer cells, and mitogen-activated protein kinase in gastric cell lines (Baus-Loncar et al., 2004; Lin et al., 2013; Diao et al., 2017). Among them, the enhaced expression of TFF3 was often accompanied by a enhancement in HIF-1α protein expression and they were co-localized in the nucleus. In human pituitary adenoma, HIF-1α was reported to regulate VEGF expression and had the anti-apoptotic function (Abdel-Latif et al., 2020). Furthermore, in invasive pituitary adenoma, the HIF-1α and VEGFA were upregulated, confirming that HIF-1α was a key factor in regulating the expression of multiple target genes that drived abnormal proliferation and invasion during tumor progression. Numerous researches indicated that VEGF-related pathways were involved in pituitary adenomas treatment (Yamada et al., 2003). Compared to the patients with glioma who expressing high levels of TFF3 gene, patients with a decreased level of TFF3 had a remarkably prolonged survival time. The interesting phenomenon was that the expression level of HIF-1α and VEGFA protein were increased when TFF3 was overexpressed. As suggested by this study, TFF3 expression was positively correlated with both the expression of HIF-1α and VEGFA protein.

CONCLUSION

In summary, this study uncovered new insight into the mechanisms of TFF3's anti-tumor activities in pituitary adenoma cells by investigating its effects on HIF-1α/VEGFA signaling pathway regulation.

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