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Original Article

Progranulin promotes colorectal cancer proliferation and angiogenesis through TNFR2/Akt and ERK signaling pathways

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Abstract: Progranulin (PGRN) has been shown to be involved in the process of inflammation, wound healing, and cartilage development; and its role in the progression of breast and ovarian cancer is also well established. However, the expression status of PGRN in colorectal cancers (CRCs) and its molecular mechanisms responsible for tumorigenesis have not been addressed so far. Herein, we demonstrated that PGRN was highly expressed and had clinical relevance with CRCs since its overexpression was associated with advanced stages of CRCs, poorer patients' prognosis, and increased expression of proliferation and angiogenesis markers. PGRN up-regulation significantly promoted the expression of Ki67 and vascular endothelial growth factor A (VEGF-A) as well as the growth rate in CRC cell lines, while PGRN down-regulation had the opposite effects. Strikingly, PGRN derived from CRCs could directly induce proliferation, migration, tubule formation, as well as VEGF-A expression in human umbilical vein endothelial cells (HUVECs). Furthermore, we provided mechanistic evidences that the regulation of Ki67 and VEGF-A expression by PGRN was mediated by tumor necrosis factor receptor 2 (TNFR2)/Akt and the ERK signaling pathways in both CRC cells and HUVECs. Taken together, these findings suggested that PGRN could promote proliferation and angiogenesis through TNFR2/Akt and ERK signaling pathways in CRCs, providing the new insight into the mechanism of PGRN in tumor proliferation and angiogenesis.

Keywords: Progranulin (PGRN), colorectal cancers (CRCs), proliferation, angiogenesis, tumor necrosis factor receptor 2 (TNFR2)

Introduction

Colorectal cancers (CRCs) are one of the major leading causes of cancer-related deaths in the world [1]. Whereas the overall survival rate of CRC patients has increased, the prognosis of those with advanced or metastatic disease remains poor [2]. Angiogenesis is one of the major hallmarks of cancer [3], which is involved in tumor development, progression, and metastasis [4]. Thus, understanding the molecular mechanism in carcinogenesis and identification of molecular events responsible for angiogenesis can benefit CRCs diagnosis and treatment.

Progranulin (PGRN), also known as granulin-epithelin precursor, acrogranin, proepithelin, and GP88/PC-cell derived growth factor, is a secreted glycoprotein composed of 7.5 repeats of cysteine-rich motif [5, 6]. It is expressed in immune cells [7], neurons [8], epithelia cells [7, 9], and chondrocytes [10]; and could mediate wound healing, neurodegeneration, and cartilage development [6-10]. Besides, high levels of PGRN could predict the poor prognosis in ovarian cancer [11], bladder cancer [12], and glioblastoma [13]. In addition, it is also reported that PGRN could act as a growth factor which could stimulate proliferation, migration, and confer chemoresistance in many types of can-

Table 1. Primary antibodies used in immunohistochemistry and western blotting

Name	Company
PGRN	Enzo life science, Farmingdale, NY, USA
Ki67	abcam, Cambridge, MA, USA
CD34	Maixin, Fuzhou, China
Bcl-2	cell signaling technology, CST, Danvers, MA, USA
VEGF-A	Proteintech Group Inc., Chicago, USA
VEGF-C	Abgent, San Diego, CA, USA
FGF-1	Abnova, CA, USA
phospho-ERK1/2	Epitomics, Burlingame, CA, USA
ERK1/2	Cell Signaling Technology, Danvers, MA, USA
phospho-Akt	Cell Signaling Technology, Danvers, MA, USA
Akt	Cell Signaling Technology, Danvers, MA, USA
phospho-P38	Cell Signaling Technology, Danvers, MA, USA
P38	Cell Signaling Technology, Danvers, MA, USA
GAPDH	Proteintech Group, Inc., Wuhan, China

cers including breast cancers [14-16], ovarian cancers [11, 17, 18], liver cancers [19, 20], and bile duct cancers [21]. However, the expression and function of PGRN in CRCs remains unclear.

Angiogenesis, a process of neovascular formation from pre-existing blood vessels, is critical for development and tumorigenesis. PGRN was firstly reported to increase capillary size and number after tissue wounding in 2003 [22], suggesting its important role in angiogenesis. Elevated PGRN level was found to be associated with increased vascular endothelial growth factor (VEGF) expression and higher microvessel density (MVD) in breast cancer [23] and esophageal squamous cell carcinoma [24]. It is suggested that a connection between PGRN expression and tumor angiogenic behavior may exist, but the mechanism by which PGRN regulates tumor angiogenesis remains unclear.

PGRN can stimulate multiple intracellular pathways to promote cell proliferation, migration and transformation, including the extracellular regulated kinase (ERK) signaling pathway, phosphatidyl inositol 3 kinase (PI3K) pathway or the adhesion/motility pathway [25-27], which may vary due to its different combination with different receptors. Recently, a direct binding of PGRN with tumor necrosis factor receptor (TNFR) was reported in chondrocytes, bone marrow-derived macrophages and splenocytes [10, 28]. PGRN exhibited high affinity of TNFR, preferentially for TNFR2, which inhibited the TNFα-mediated neutrophil activation in acute lung injury [29] and inflammatory response in rheumatoid arthritis in mice [10]. But whether TNFR is required for mediating the function of PGRN in tumor development and which signaling pathways would be involved in tumor angiogenesis are still elusive.

In the present study, the effects of PGRN on CRC proliferation and angiogenesis as well as its underlying molecular mechanism were investigated. We demonstrated that PGRN was highly expressed in CRCs and promoted proliferation and angiogenesis through TNFR2/Akt and ERK signaling pathways.

Materials and methods

Patients

On the basis of approval of the review board and ethics committee, primary colorectal tumors and corresponding normal colorectal tissues were collected between 2006 and 2012 in Jinan Central Hospital Affiliated to Shandong University. No patients received chemotherapy, radiotherapy or immunotherapy before surgery. Follow-up data were summarized on December 31th, 2014, with a median follow-up of 50 months. Tumor tissues were classified according to the AJCC Cancer Staging Manual [30].

Immunohistochemistry

Sections from tumors were cut into 3 μm thickness and incubated with primary antibodies as shown in **Table 1**. Normal mouse or rabbit IgG instead of primary antibodies was used as negative control. Then the sections were incubated with HRP goat anti-rabbit/mouse IgG polymer (Maixin, Fuzhou, China). Finally, the analysis was performed by two independent investigators simultaneously. The percentage of stained cells was recorded at 400× magnification in at least 5 random fields. The proportion score represented the fraction of positive staining tumor cells (0 = none; 1 = less than 25%; 2 = 26-75%; 3 = greater than 75%). The intensity score represented the average staining intensity (0 = none; 1 = weak; 2 = intermediate; 3 = strong). The expression level of Ki67 was evaluated using the proportion score directly, and the

Table 2. Primers used in RT-PCR

	Forward	Reverse
PGRN	5'-ATCTTTACCGTCTCAGGGACTT-3'	5'-CCATCGACCATAACACAGCAC-3'
Ki67	5'-AGAAGAAGTGGTGCTTCGGAA-3'	5'-AGTTTGCGTGGCCTGTACTAA-3'
Bcl-2	5'-GGGGTCATGTGTGTGGAGAG-3'	5'-GAAATCAAACAGAGGCCGCA-3'
GAPDH	5'-AGAAGGCTGGGGCTCATTTG-3'	5'-AGGGGCCATCCACAGTCTTC-3'

expression score of VEGF-A and PGRN was calculated as the product of proportion score and intensity score (ranging from 0 to 9). Score ≥4 was classified as high expression, <4 as low expression. Microvessel density (MVD) was determined by CD34 immunoreactivity and quantified as described previously [31].

Cell lines and cell culture

Human colon epithelial cell line FHC and human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Manassas, VA, USA), and colorectal cancer cell lines SW480, SW1116 and HT29 were all obtained from the Institutes of Biochemistry and Cell Biology (Shanghai, China) which originated from ATCC (Manassas, VA, USA). All cell lines were cultured in media according to manufacturer's instructions.

Transfection

Cells of Colorectal cancer cell line SW1116 or HT29 were transfected using HP X-treme GENE HP Reagents (Roche, Basel, Switzerland) by p-GPU6/GFP/Neo/PGRN or shRNA control (Shanghai GenePharma Co. Ltd, Shanghai, China) to interfere PGRN expression, while pEZ-M61/GFP/PGRN or vector control (Gene Copoeia, Guangzhou, China) was used to upregulate PGRN expression. Transfection procedures were performed according to the manufacturer's instructions. The supernatant was collected as conditioned medium (CM). Total mRNA and protein of cells were extracted for further study.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from cells was extracted by Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNAs were synthesized using Prime Script RT-PCR Kit (TaKaRa, Dalian, China) as protocol. Then RT-PCR was performed using the primers shown in **Table 2**. All experiments were performed in a minimum of three replicates.

Protein extraction and western blotting

Equal total protein was separated by SDS-PAGE and transferred to PVDF membrane (Millipore Corp., Billerica, MA, USA). The membrane was incubated with primary antibodies (**Table 1**) and horseradish peroxidase-conjugated secondary antibody (Proteintech Group, Inc, Wuhan, China). Then the protein on the membrane was detected by chemiluminescence solution (Millipore Corporation, Billerica, MA, USA), the band intensity was measured by image J software.

Enzyme-linked immunosorbent assay (ELISA)

Secretion of PGRN and VEGF-A were detected using PGRN ELISA Kit (Cusabio life science, Wuhan, Hubei, China) and VEGF-A ELISA Kit (Cusabio life science, Wuhan, Hubei, China) according to the manufacturer's instructions. The experiment was repeated at least three times.

Cell proliferation assay

Cell proliferation assays were performed using the MTT assay (Beijing solarbioscience & technology co. ltd., Beijing, China) following the manufactures' protocol. Briefly, CRC cells which have been treated with PGRN shRNA or vector were plated in 96-well plate. Cell viability was measured at 24, 48, 72, 96, and 120 hours. While HUVECs were plated for 24 hours, then the medium was replaced by CM from SW1116 or endothelial cell culture medium (ECM) with different concentrations of recombinant PGRN (rPGRN, Sino Biological Inc., Beijing, China) (0 ng/ml and 64 ng/ml). 48 hours later, MTT assay was performed.

Cell apoptosis assay

SW1116 cells transfected with PGRN shRNA or vector were harvested at 72 hours after transfection, and incubated in the mixture of 195 μl Annexin V-FITC binding buffer and 5 μl Annexin V-FITC using an Annexin V-FITC/PI Staining Kit (Medical and Biological laboratories co. ltd., Japan). Then followed by incubation with mixture of 190 μl Annexin V-FITC binding buffer and 10 μl PI, cytometry assay was performed.

Migration assay of HUVECs

Cell migration activity was assessed using Boyden chambers (BD Biosciences, Bedford,

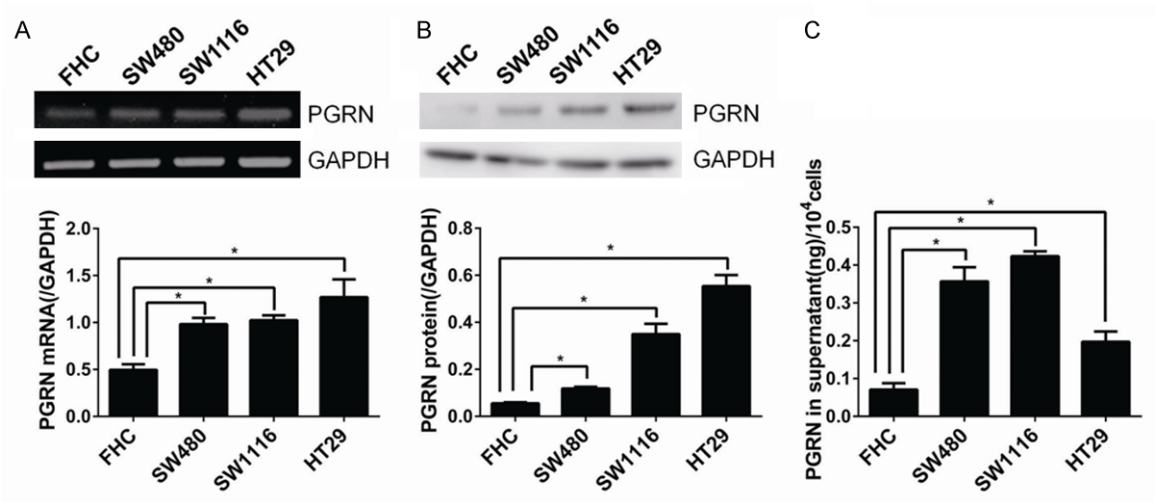

Figure 1. PGRN expression in CRC cell lines. CRC cell lines (SW480, SW1116, and HT29) exhibited significantly increased PGRN levels as compared to normal cells (FHC) at both mRNA and protein level by RT-PCR (A), Western Blotting (B), and ELISA (C). *P < 0.05, compared to normal cells.

MA, USA). HUVECs in ECM without FBS were plated in the upper chambers, CM from SW1116 cells transfected with PGRN shRNA/vector or ECM with different concentrations of rPGRN (0 ng/ml and 64 ng/ml) supplemented with 3% FBS were plated in the lower chamber. After 6 h, the cells that moved to the lower surface of polycarbonate membrane were stained with 0.1% crystal violet and counted at five random 200× fields to get the sum.

Tubule formation assay

HUVECs were plated in Matrigel (BD Science, Sparks, MD, USA) precoated 48-well plate at 3×10^4 cells/well with CM from SW1116 cells transfected with PGRN shRNA/vector or ECM with different concentration of rPGRN (0 ng/ml and 64 ng/ml, respectively). The tubular structures were quantified by manual counting at three random 100× fields to get the sum. The experiment was repeated three times.

Statistical analysis

The statistical analysis was performed using SPSS 11.0 software. The associations between the expression of PGRN and clinical parameters, Ki67 and VEGF-A were analyzed using the χ^2 test or the Student two-tailed t test. Survival curves were drawn using the Kaplan-Meier method and compared by means of the log-rank test. $P < 0.05$ was considered significant.

Results

PGRN is highly expressed in CRCs and associated with a poor prognosis of CRC patients

We first evaluated the expression of PGRN at both mRNA and protein levels in human normal cell line (FHC) and CRC cell lines (SW480, SW1116, and HT29) by RT-PCR, Western Blotting, and ELISA. CRC cell lines exhibited significantly increased PGRN levels as compared to normal cells (**Figure 1**).

Then we assessed PGRN expression in CRC specimens by immunohistochemistry assay. The clinicopathological characteristics of 77 CRC patients were shown in **Table 3**. As shown in **Figure 2A**, strongly positive staining of PGRN was observed in CRC tissues, but faint staining in adjacent normal colorectal cells. The higher expression of PGRN was associated with higher CRCs TNM staging and more lymph node metastasis ($P = 0.021$ and 0.046, respectively, **Table 3**). Using the Kaplan-Meier method, the Disease-free survival (DFS) of high-PGRN expression CRC patients was lower compared with the low-PGRN cases ($P = 0.042$, **Figure 2B**). The overall survival (OS) curves revealed an unfavorable prognosis for the high-PGRN group compared to the low-PGRN group although the difference did not reach statistical significance ($P = 0.086$, data not shown).

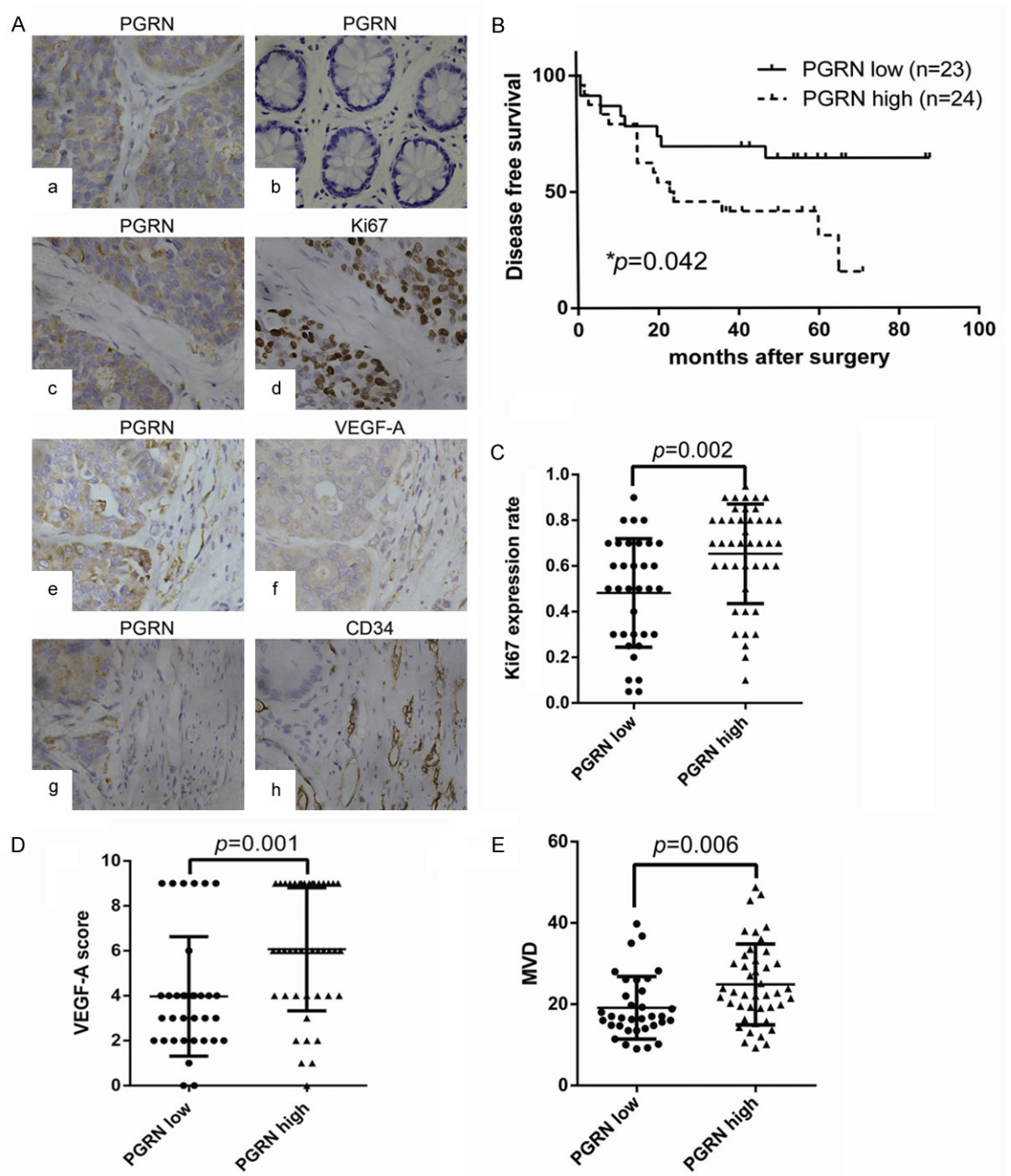

Figure 2. PGRN expression in CRC tissues. (A) Using immunohistochemical analysis, PGRN was found to be highly expressed in CRC tissues (a) compared to adjacent normal colorectal cells (b); (c and d) showed the expression of PGRN and Ki67 in tumor cells of serial sections; VEGF-A and CD34 expression was further demonstrated in (f and h), and serial sections were analyzed for PGRN in (e and g), respectively. (B) The DFS of high-PGRN expression CRC patients was lower compared with the low-PGRN cases (P = 0.042). PGRN expression was positively correlated with Ki67 expression (C), VEGF-A expression (D) and MVD (E) in immunopositivity (P = 0.002, 0.001 and 0.006, respectively). The images were all magnified by 400 folds in (A).

PGRN stimulated proliferation and regulated Ki67 expression in CRCs

To study the effect of PGRN expression on proliferation in CRC cells, proliferation marker Ki67 was detected in CRC tissues. PGRN was directly correlated with Ki67 expression in immunopositivity (P = 0.002, **Figure 2A** and **2C**), revealing its proliferative potential. In CRC cell line, when PGRN expression was inhibited by

Table 3. Relationship between PGRN and clinical parameters in CRCs

Clinical parameters	Cases (n)	PGRN		X^2	P
		low	high		
Age (year)					
≤65	40	17	23	2.7	0.136
>65	37	17	20		
Gender					
Male	49	20	29	0.609	0.435
Female	28	14	14		
Differentiation					
Mediate-low/low	25	11	14	0.000	0.985
High/mediate	52	23	29		
Tumor size					
≤5 cm	39	18	21	0.128	0.820
>5 cm	38	16	22		
Clinical stage					
I/II	43	24	19	5.367	0.021*
III/IV	34	10	24		
Lymph node metastasis					
-	47	25	22	3.994	0.046*
+	30	9	21		

*P < 0.05 was significant.

shRNA in SW1116, the expression of Ki67 decreased both at mRNA and protein levels (**Figure 3A** and **3C**). In contrast, PGRN up-regulation was associated with elevated Ki67 expression (**Figure 3B** and **3D**). Using the MTT assay, the cell growth rate decreased significantly when PGRN was down-regulated in SW1116; whereas increased in SW1116 overexpressed PGRN (**Figure 3E** and **3F**). Besides, the cell proliferation was also inhibited when PGRN was downregulated in HT29 cells which highly expressed PGRN (**Figure 3G**).

To determine whether apoptosis contributed to the growth inhibition, we analyzed Bcl-2 expression of SW1116 with PGRN-shRNA or PGRN-vector. Compared to the control group, Bcl-2 expression did not change significantly regardless of up or down regulation of PGRN (**Figure 3A-D**). Consistent with these results, flow cytometric analysis revealed that the apoptosis rates of SW1116 were not markedly altered as well (data not shown).

PGRN promoted angiogenesis and regulated VEGF-A expression in CRCs

In CRC tissues, the MVD was positively correlated with PGRN expression (P = 0.006) (**Figure 2A** and **2E**). Besides, VEGF-A, one of the most important VEGF family members, was found to be positively associated with PGRN expression (P = 0.001) (**Figure 2A** and **2D**). These data strongly indicated that PGRN may be involved in the angiogenic process of CRCs.

We then investigated the effect of PGRN on angiogenic factor expression in CRC cell line. Depletion of PGRN expression could remarkably down-regulate VEGF-A as well as other angiogenic factors, such as VEGF-C and FGF-1 (**Figure 4A**). Meanwhile, the level of secreted VEGF-A was also decreased in the media of SW1116 cell line transfected with PGRN-shRNA (**Figure 4C**). Conversely, overexpression of PGRN resulted in the up-regulation of VEGF-A (**Figure 4B** and **4C**).

TNFR2/Akt and ERK signaling pathways are involved in PGRN-induced Ki67 and VEGF-A expression in CRCs

To identify the potential molecular mechanisms of PGRN overexpression in CRC proliferation and angiogenesis, we analyzed the expression levels of several signaling proteins by Western blotting assays and showed that PGRN overexpression markedly stimulated the phosphorylation of ERK and Akt, whereas the levels of total ERK and Akt did not alter (**Figure 5A**). In addition, treatment of SW1116 cells with recombinant PGRN (rPGRN) increased the phosphorylation of ERK and Akt as well, leading to more expression of Ki67 and VEGF-A in a dose-dependent manner (**Figure 5E**).

Pretreatment of the cells with ERK inhibitor (U0126) or Akt inhibitor (LY294002) alone markedly abrogated the impact of PGRN on the expression of Ki67 and VEGF-A in CRC cells (**Figure 5B** and **5C**).

It has been reported that PGRN could bind to TNFR, especially TNFR2, in kinds of autoimmune disease and lung inflammation [10, 29]. We therefore examined whether TNFR2 was required for PGRN to promote the expression of ki67 and VEGF-A in CRCs. As shown in **Figure 5D** and **5F**, the expression of ki67 and VEGF-A in SW1116 transfected with PGRN-vector or incubated with rPGRN was increased and this effect was partly blocked by antibody against TNFR2. Consistently, the phosphorylation of

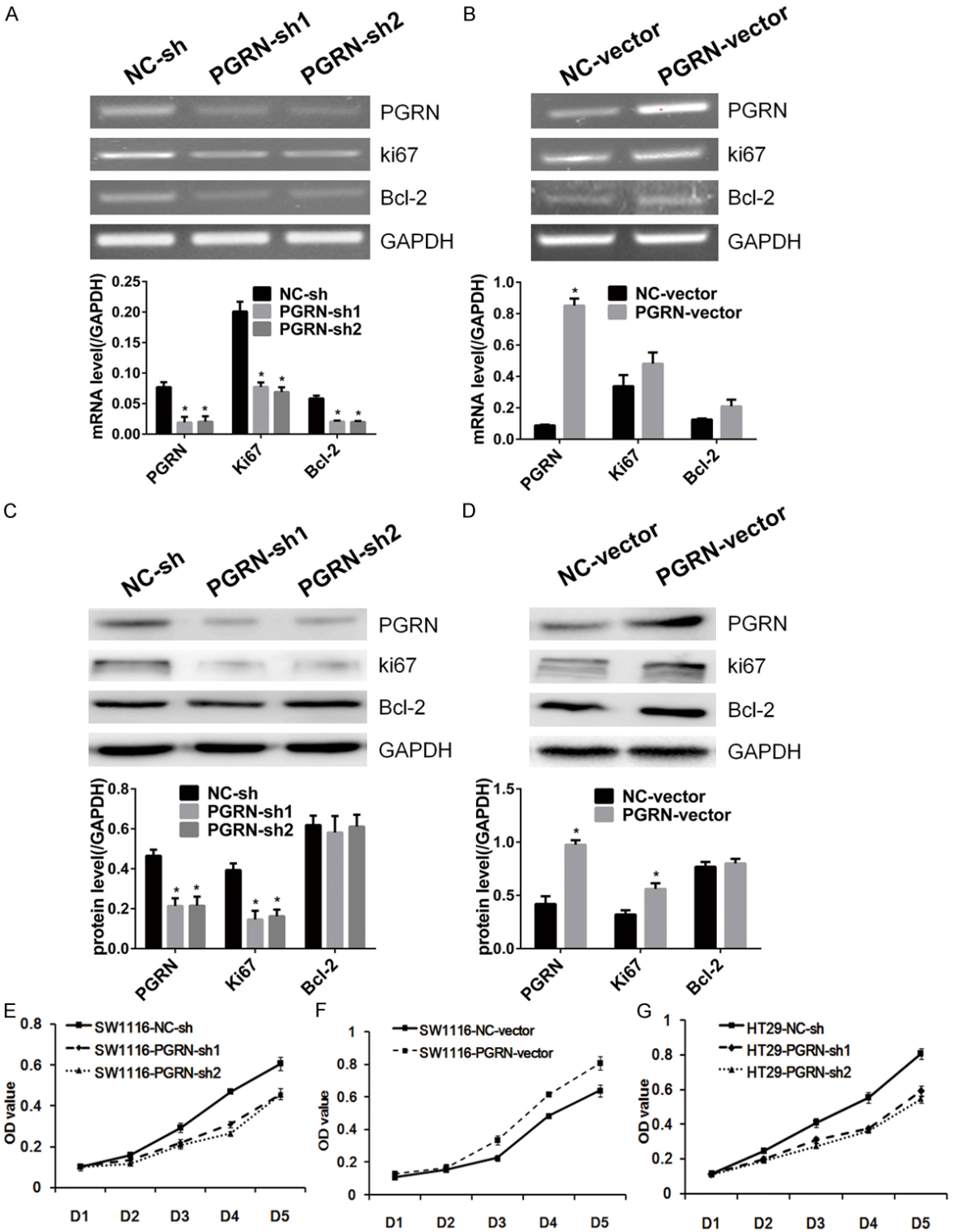

Figure 3. PGRN stimulated proliferation and regulated Ki67 expression in CRCs. When PGRN expression was inhibited by sh1RNA or sh2RNA in SW1116, the expression of Ki67 and Bcl-2 was assayed both at mRNA (A) and protein levels (C). While up-regulate PGRN, Ki67 and Bcl-2 expression was estimated as shown in (B) mRNA level and (D) protein level. Using the MTT assay, the growth rate was analyzed in SW1116 transfected with PGRN-shRNA or PGRN-vector compared to the control group (E and F). (G) In HT29 cells, the cell proliferation was also assayed when PGRN was downregulated using sh1RNA or sh2RNA. $^{*}P < 0.05$.

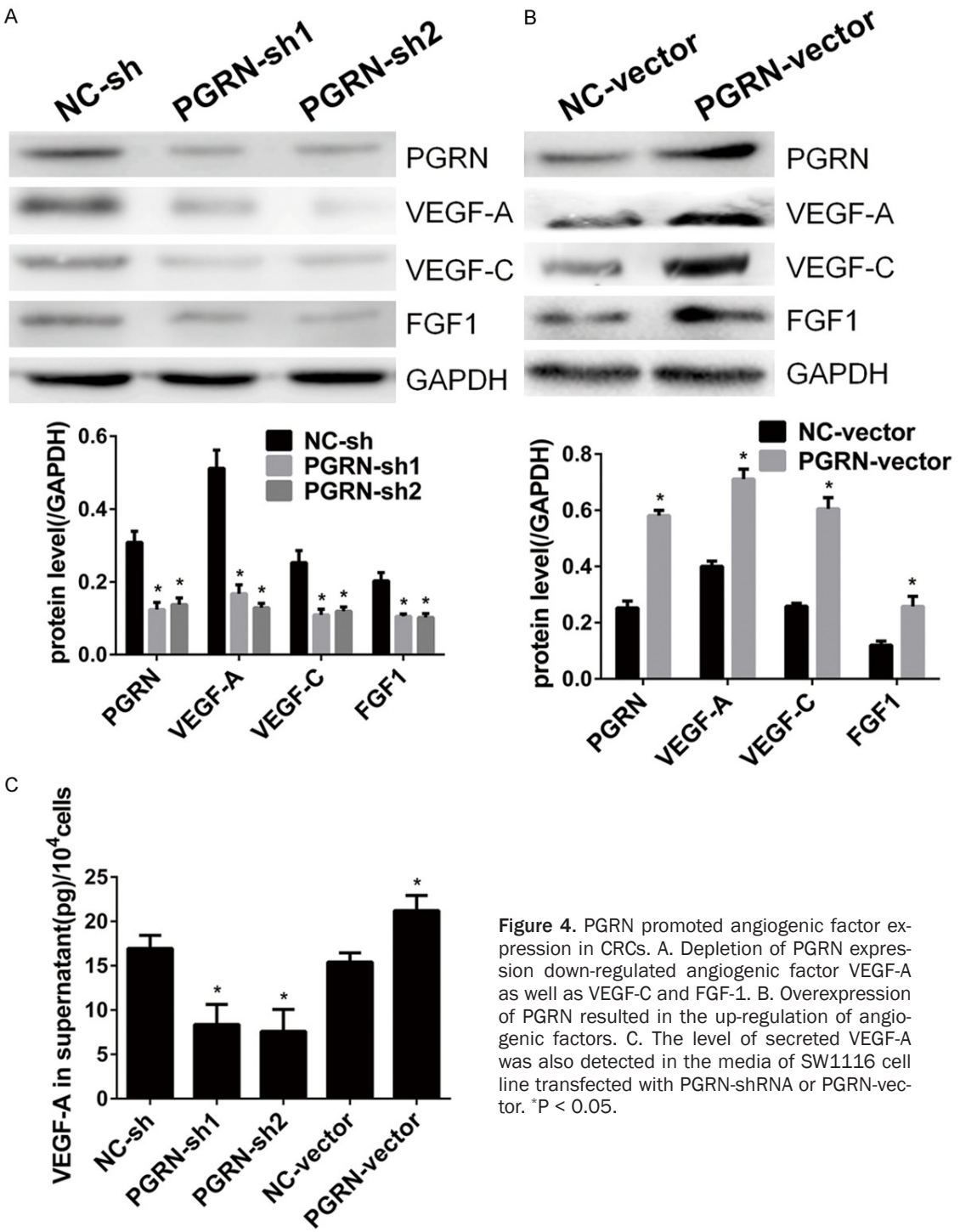

Figure 4. PGRN promoted angiogenic factor expression in CRCs. A. Depletion of PGRN expression down-regulated angiogenic factor VEGF-A as well as VEGF-C and FGF-1. B. Overexpression of PGRN resulted in the up-regulation of angiogenic factors. C. The level of secreted VEGF-A was also detected in the media of SW1116 cell line transfected with PGRN-shRNA or PGRN-vector. $^{*}P < 0.05$.

Akt was also blocked modestly by pretreatment with anti-TNFR2 antibody. Nevertheless, the anti-TNFR2 antibody treatment did not significantly reduce the phosphorylation of ERK in either PGRN-vector group or rPGRN addition group, suggesting that TNFR2 may be only required for Akt but not for ERK pathway in CRC cells.

CRCs-derived PGRN activated angiogenic properties of HUVECs and regulated VEGF-A expression in HUVECs

The former study demonstrated the potential role of PGRN in CRC angiogenesis. We then further assessed the function of PGRN derived from CRC cells on activating the angiogenic property of HUVECs directly.

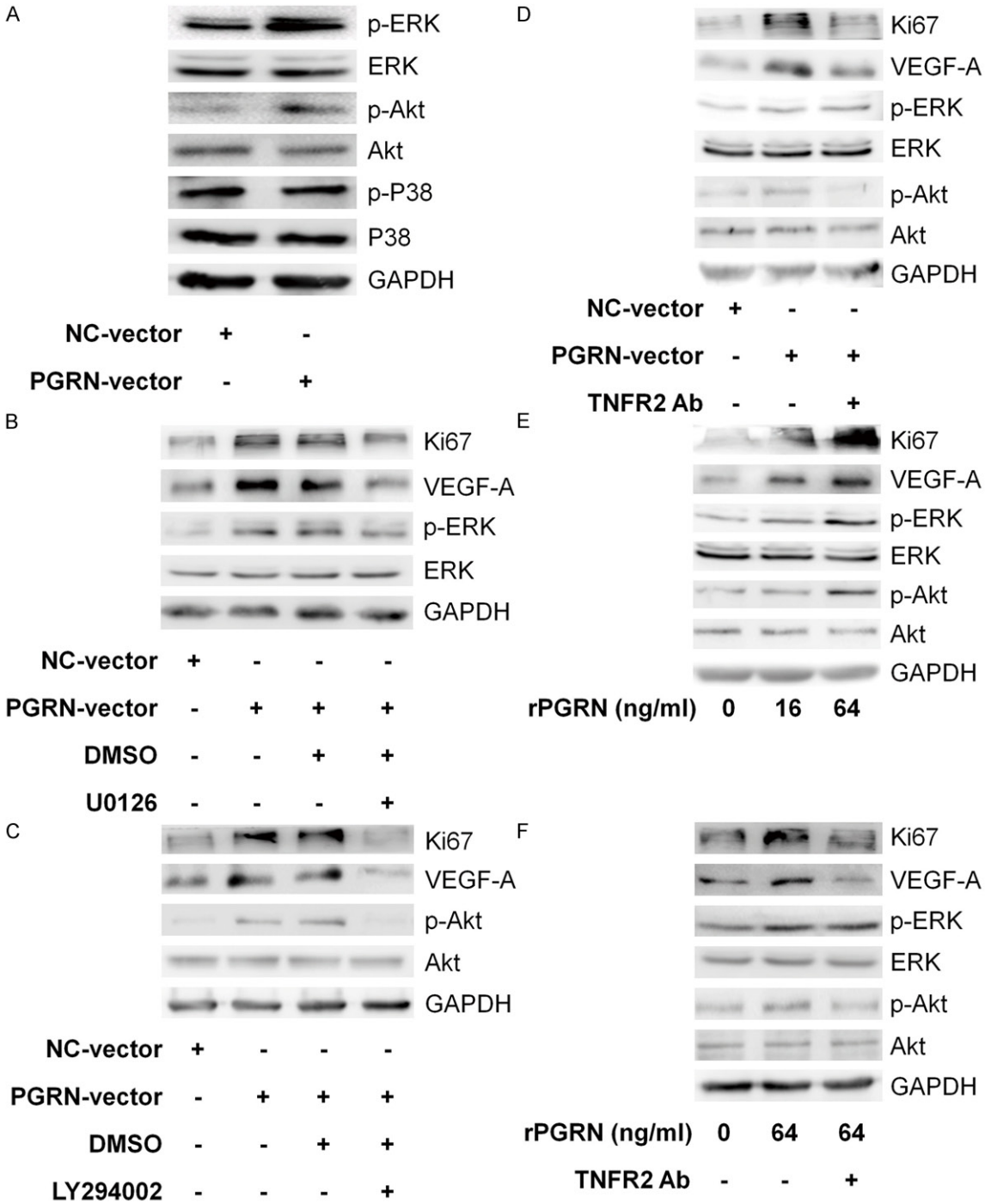

Figure 5. TNFR2/Akt and ERK signaling pathway is involved in PGRN-induced Ki67 and VEGF-A expression in CRCs. A. PGRN overexpression stimulated the phosphorylation of ERK and Akt. B and C. ERK or Akt selective inhibitor (U0126 or LY294002) abrogated the expression of Ki67 and VEGF-A in PGRN overexpressed CRC cells. D. The phosphorylation of Akt was blocked modestly by pretreatment with anti-TNFR2 antibody. E. Treatment of SW1116 cells with rPGRN increased the phosphorylation of ERK and Akt, leading to more expression of Ki67 and VEGF-A in a dose-dependent manner. F. The anti-TNFR2 antibody treatment reduced the phosphorylation of Akt in rPGRN addition group.

Firstly, HUVECs were cultured with conditioned media from SW1116 transfected with PGRN-vector or with exogenous rPGRN. The expression of Ki67 and VEGF-A in HUVECs was found

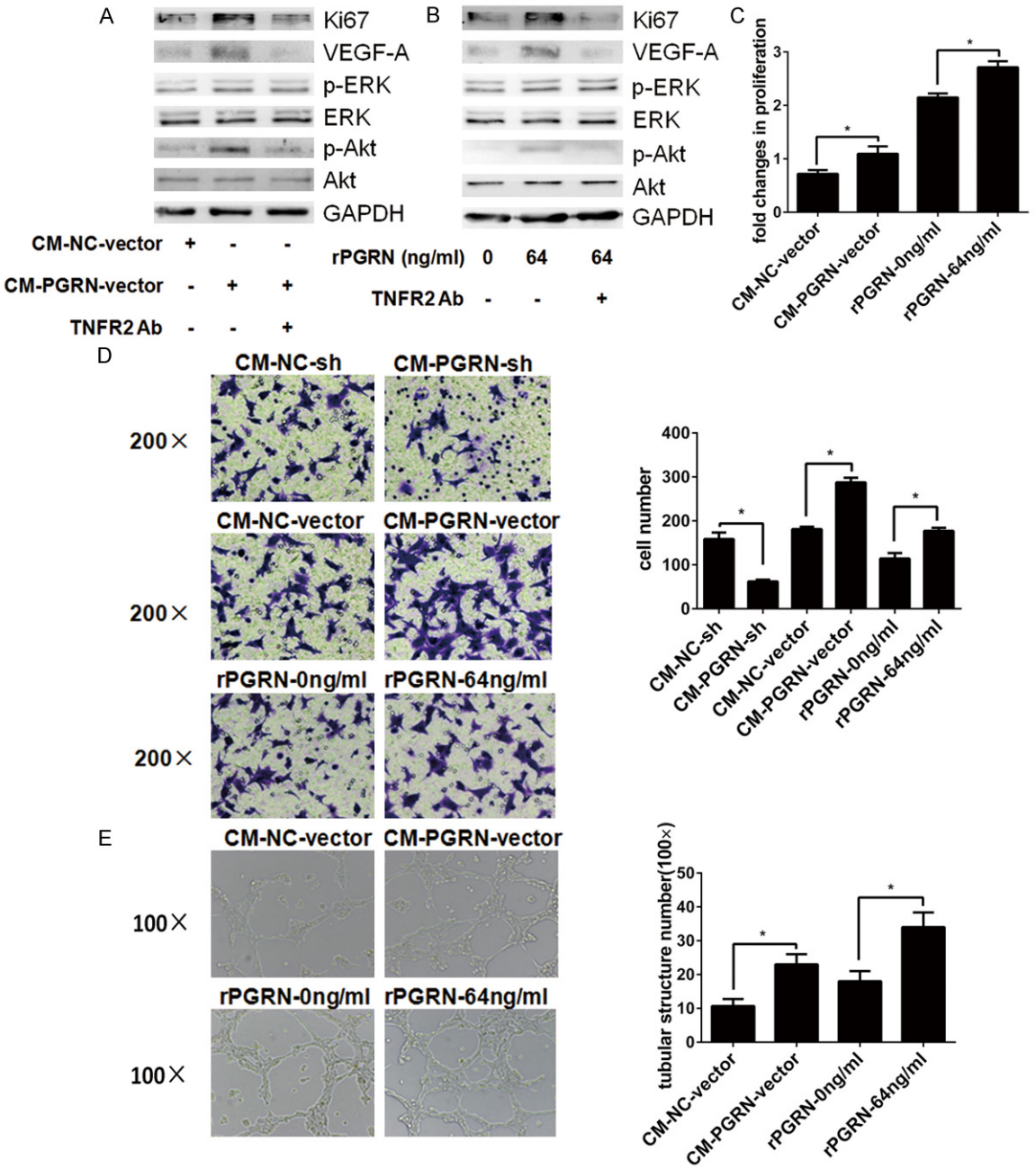

Figure 6. CRCs-derived PGRN activated angiogenic property of HUVECs and regulated VEGF-A expression. (A) The expression of Ki67/VEGF-A and phosphorylation of Akt and ERK in HUVECs were increased when cultured with CM from SW1116 transfected with PGRN-vector; anti-TNFR2 antibody effected Akt phosphorylation. (B) With exogenous rPGRN, Ki67/VEGF-A expression and phosphorylation of Akt or ERK were elevated; while Akt phosphorylation was abrogated in the presence of anti-TNFR2 antibody. The ability of proliferation (C), migration (D), and tubule formation (E) in HUVECs was assayed when cultured with CM from SW1116 transfected with PGRN-shRNA/PRGN-vector or with rPGRN.

to be markedly induced by CM from PGRN-vector group (**Figure 6A**) or exogenous rPGRN (**Figure 6B**).

We next tested the effect of CRCs-derived PGRN on activating HUVECs by analyzing their proliferation rate, migration, and tubule formation ability. The MTT assay showed that the growth rate of HUVECs was significantly increased when cultured in PGRN-vector CM or high concentration of rPGRN (64 ng/ml) (**Figure 6C**). Besides, a significant increase in migration

of HUVECs was observed when PGRN-vector CM of SW1116 or the rPGRN was added (**Figure 6D**). In contrast, CM from SW1116 with PGRN-shRNA significantly decreased the migratory ability of HUVECs. Moreover, exposure of HUVECs to CM from SW1116 with PGRN-vector or rPGRN significantly increased the ability of forming tubules as compared with their control group (**Figure 6E**).

CRCs-derived PGRN regulated Ki67 and VEGF-A expression through TNFR2/Akt and ERK signaling pathways in HUVECs

Since TNFR2/Akt and ERK are critical downstream pathways of PGRN in CRC cells, we next examined whether CRCs-derived PGRN could also have effects on Akt and ERK phosphorylation in HUVECs.

As shown in **Figure 6A** and **6B**, Akt and ERK phosphorylation were significantly increased in HUVECs when cultured with CM from SW1116 overexpressed PGRN or with rPGRN. After pretreatment with anti-TNFR2 antibody, CM from PGRN overexpressing SW1116 cells or rPGRN could stimulate the phosphorylation of ERK but not phosphorylation of Akt (**Figure 6A** and **6B**). These findings indicate that the upregulation of Ki67 and VEGF-A expression by CRCs-derived PGRN was mediated by TNFR2/Akt and ERK signaling pathways in HUVECs.

Discussion

PGRN plays crucial roles in wound healing, cartilage development and inflammatory arthritis [7-10]; and functions as a neurotrophic factor since loss of PGRN could cause frontotemporal dementia [32]. There are emerging evidences suggesting that abnormal expression of PGRN is correlated with tumorigenesis [10-21]. However, the expression level of PGRN in human CRCs and its potential role in CRC tumorigenesis are still unclear.

Our study showed for the first time that PGRN was highly expressed in CRCs and associated with higher tumor TNM staging, more lymph node metastasis, and shorter DFS in CRC patients; which may suggest that PGRN could be a useful biomarker to predict clinical response and survival in CRCs.

We also demonstrated that PGRN overexpression significantly promoted CRC cell growth rate, Ki67 expression and VEGF-A expression. Thus, PGRN may promote proliferation and angiogenesis in CRCs. Besides the important role of PGRN in tumor growth, it could also actively confer malignancy for cancer cells. For example, PGRN overexpression in SW13 adenocarcinoma cells could promote tumor-forming ability in mice [33]. PGRN combined with Tert (telomerase) and SV40 (blocking the Rb and P53 tumor suppressors) could transform primary human ovarian epithelial cells [34] and uterine smooth muscle cells [35] to malignant cells. Our observations together with these previous studies suggest that PGRN could play an oncogenic role in tumorigenesis.

In the present study, CRCs-derived PGRN not only stimulated VEGF-A expression in CRC cells, but could act as an stimulating factor for HUVECs, suggesting its direct and indirect roles in tumor angiogenesis. Besides, PGRN was reported to play a protective role in endothelial cells, since PGRN treatment inhibited ICAM-1 expression in human brain microvascular endothelial cells [36] and suppressed the expression of ICAM-1 and VCAM-1 in HUVECs [37]. Whether endothelial cell protection was also involved in the angiogenic effect of CRCs-derived PGRN may be warranted in future studies.

Moreover, our results demonstrated that PGRN overexpression regulated Ki67 and VEGF-A expression through phosphorylation of Akt and ERK signaling pathways in both CRC cells and HUVECs, while the anti-TNFR2 antibody could block the phosphorylation of Akt as well as the expression of Ki67 and VEGF-A partially. TNFR, especially TNFR2, was found to have high binding-affinity to PGRN in bone marrow-derived macrophages and chondrocytes [10]. However, there were some controversies for PGRN-TNFR interaction. For example, PGRN was found not to bind to TNFR, and cannot antagonize TNF-KB, Akt, ERK pathway in BV2 microglia and bone marrow-derived macrophages [38]. Here, we reported for the first time that whether or not PGRN could bind to TNFR, TNFR was required for the stimulation of Akt pathway in CRC cells and HUVECs. Therefore, our findings suggested the important roles of PGRN and TNFR in CRCs tumorigenesis and angiogenesis, which may be promising for the development of targeted therapy for CRC patients.

In summary, we demonstrated that PGRN overexpression was associated with poor prognosis of CRC patients and induced tumor prolifera-

tion and angiogenesis in CRC cells. CRCs-derived PGRN could also activate HUVECs through directly stimulating cell proliferation, migration, tubule formation as well as VEGF-A expression. In addition, the effect of PGRN on CRC cells and HUVECs was at least partially dependent on TNFR2/Akt and ERK signaling pathways. Thus, our findings provide new insight into the mechanism of the function of PGRN on tumor proliferation and angiogenesis of CRCs.

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Disclosure of conflict of interest

None.

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