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Response Gene to Complement 32 (RGC-32), a novel hypoxia-regulated angiogenic inhibitor

Xiaojin An, BS[1,2], **Yi Jin, BS**[1,2], **Hongnian Guo, PhD**[2], **Shi-Yin Foo, MD, PhD**[2], **Brittany Cully, BA**[2], **Jiaping Wu, MD**[2], **Huiyan Zeng, PhD**[3], **Anthony Rosenzweig, MD**[2], and **Jian Li, MD,PhD**[1,2]

[1]Institute of Molecular Medicine, Peking University, Beijing, China

[2]Division of Cardiovascular Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston MA 02215

[3]Division of Molecular and Vascular Medicine, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston MA 02215

Abstract

Background—Response Gene to Complement 32 (RGC-32) is induced by activation of complement and regulates cell proliferation. To determine the mechanism of RGC-32 in angiogenesis, we examined the role of RGC-32 in hypoxia-related endothelial cell function.

Methods and Results—Hypoxia/Ischemia is able to stimulate both angiogenesis and apoptosis. HIF-1α/VEGF is a key transcriptional regulatory pathway for angiogenesis during hypoxia. We demonstrated that the increased RGC-32 expression by hypoxia was via HIF-1α/VEGF induction in cultured endothelial cells. However, overexpression of RGC-32 reduced the proliferation and migration and destabilized vascular structure formation in vitro, and inhibited angiogenesis in Matrigel assays in vivo. Silencing RGC-32 had an opposing, stimulatory effect. RGC-32 also stimulated apoptosis as shown by the increased apoptotic cells and caspase-3 cleavage. Mechanistic studies revealed that RGC-32's effect on the anti-angiogenic response was via attenuating FGF2 expression and further inhibiting expression of cyclin E without impacting VEGF and FGF2 signaling in endothelial cells. In the mouse hindlimb ischemia model, RGC-32 inhibited capillary density with a significant attenuation in blood flow. Additionally, treatment with RGC-32 in the xenograft tumor model resulted in reduced growth of blood vessels that is consistent with reduced colon tumor size.

Conclusions—We provide the first direct evidence for RGC-32 as a hypoxia-inducible gene and antiangiogenic factor in endothelial cells. These data suggest that RGC-32 plays an important homeostatic role, as it contributes to differentiating the pathways for VEGF and FGF2 in angiogenesis, and provides a new target for ischemic disorder and tumor therapies.

Keywords

hypoxia; ischemia; angiogenesis; apoptosis; gene therapy

Address for correspondence: Jian Li, MD. PhD., Division of Cardiovascular Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 3 Blackfan Circle. Boston MA 02115, JLi@BIDMC.Harvard.edu.

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CLINICAL PERSPECTIVE

We and others have suggested that Response gene to complement (RGC)-32 is involved in cell cycle regulation. Our data provide the novel ability of RGC-32 that its expression increased in hypoxia/ischemia and inhibited angiogenesis in endothelial cells. Ischemia is characterized by reduced blood supply to the organs. Although angiogenesis occurs in response to ischemia, angiogenesis induced by natural compensatory processes is often inadequate. There have been many unsuccessful clinical trials testing the pro-angiogenic potential of vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), and the role of growth factor feedback molecules in attenuating angiogenic response in ischemic disease is not completely understood. RGC-32 as a downstream gene induced by hypoxia/ischemia and VEGF, possesses anti-angiogenesis capability. Inhibiting the negative feedback of VEGF is a significant potential angiogenic therapy. In addition, given that angiogenesis is an important process in tumor growth, anti-angiogenic factors can block tumor's fundamental requirements. Thereby, RGC-32 will have a clinical application for tumor retardation through its inhibition of angiogenesis. We have demonstrated that injection of RGC-32 in the xenograft tumor model resulted in reduced growth of blood vessels that is consistent with reduced colon tumor size. Therefore, it is conceivable that RGC-32 provides a new target for ischemic disorder and tumor therapies.

Introduction

Hypoxia/ischemia leading to cellular dysfunction is a complex process that involves numerous factors. In endothelial cells, it has been suggested that hypoxia induces angiogenesis via upregulation of hypoxia-inducible factor (HIF)-1 alpha protein that in turn activates the transcription of several angiogenic genes, including VEGF, VEGF receptors flt-1 and neuropilin-1, and angiopointin-2.[1] In contrast, hypoxia also directs endothelial cells toward apoptosis, which is caused by changes in p53 protein levels.[2] Although functional genomic analyses have revealed specific genes that are involved in hypoxic signaling,[3] gene regulation for maintaining endothelial cell homeostasis between angiogenesis and apoptosis under hypoxic conditions is still not fully understood.

The RGC-32 protein is localized in the cytoplasm and physically associates with cyclin-dependent kinase p34CDC2, which increases the kinase activity to induce quiescent aortic smooth muscle cells to enter S-phase[4] and plays an important role in cell proliferation by downregulating cell cycle inhibitors.[5] However, studies of RGC-32 in tumor cell growth have yielded different results. Another group found that RGC-32 showed p53-dependent transcriptional activity that suppressed tumor cell line growth via the arrest of mitotic progression.[6] The disparities between these reports may be due to different RGC-32 functions in different cell types.[7] However, nothing is known regarding the regulation of RGC-32 activity in hypoxia and angiogenesis.

We report here that RGC-32 is a novel hypoxia-inducible gene. We determined that HIF-1α and VEGF significantly increased RGC-32 expression in hypoxia and ischemia. Our results suggest that HIF-1α/VEGF-induced RGC-32 expression did not follow the canonical VEGF pathway to promote angiogenesis. Rather, overexpression of RGC-32 in endothelial cells inhibited cell proliferation and migration via downregulation of another major angiogenic protein, FGF2, to further effect cyclin E. Also, RGC-32 promoted unstable vascular structure by increasing the numbers of apoptotic cells. This work reveals a novel function for RGC-32 as a potential hypoxia-inducible inhibitor of angiogenesis and a mediator between VEGF and FGF2 pathways.

Methods

Cell Culture and Hypoxia

Human Umbilical Vein Endothelial Cells (HUVECs) (Lonza) were cultured in EBM2 containing 2% FBS with growth supplements. Hypoxia (<1%O_2) was induced using a Modular Incubator Chamber (Billumps-Rothenberg). $CoCl_2$, dimethyloxalylglycine(DMOG), and 3,4-dihydroxybenzoate(3,4-DHB) (Sigma) were used to mimic hypoxia.

Retroviral Construction

The RGC-32 (NM_014059.1) coding sequence was cloned into pBMN-GFP vector (Obigen) for retrovirus packaging. pBMN-GFP or pBMN-GFP-RGC-32 were transfected to 293T using Polyethylenimine (PEI) with pVSV-G, pJK3, and pCMVtat. The medium with retrovirus/RGC-32 was collected and filtered before being used to infect HUVECs.

RNA Analysis

Total RNA from HUVECs was extracted using TRIzol Reagent (Invitrogen) and cDNA was synthesized by reverse transcription. A 420bp RGC-32 cDNA was cloned with primers (forward: 5′-GCGCTGTGCGAGTTTGAC-3′, reverse: 5′-CCCCTCTGGCAGCAGATT-3′) for probing. Northern blots were performed using a DIG Northern Starter Kit (Roche). RNA levels were also measured by Quantitative PCR (Q-PCR) using the primers 5′-CAAAGACGTGCACTCAACCTTC -3′, and 5′- CTGTCTAAATTGCCCAGAAATGG -3′. A TaqMan probe, 5′-FAM (6-carboxyfluorescein)-ACCAGGCCACTCTCAGGCTCACCTTAA-3′TAMRA (6-carboxy-tetramethylrhodamine) was included during Q-PCR.

RGC-32 Transcription Studies

A nucleotide fragment (-1205 to +379 of human sequence at GeneBank: 28984) encompassing basal elements of the human RGC-32 promoter was cloned into a PGL-3 vector (Promega). HEK293 were transfected with the RGC-32 promoter construct co-transfected with HIF-1α cDNA using Polyethylenimine (PEI). The luciferase activity was determined using a Dual-luciferase assay system (Promega).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with ChIP-IT Express Kit (Active Motif) in accordance with the manufacturer's instructions. The RGC-32 and actin promoters were amplified with the primer pairs 5′-ATTTAATTAGCCGTCTGTGGTGAG-3′ and 5′-AGCCTGACTTTATCTAGAAGGGGT-3′, and 5′-TGCACTGTGCGGCGAAGC-3′ and 5′-TCGAGCCATAAAAGGCAA-3′, respectively. RGC-32 primers were designed to the putative hypoxia response elements (HREs) binding site for HIF-1α.

siRNA transfection

siRNA targeting human RGC-32 were synthesized by Genpharma Inc. (ZhangJiang, Shanghai). Two duplexes of siRNA of RGC-32 (siRNA1: 5′-GAUUCACUUUAUAGGAACATT-3′, 5′-UGUUCCUAUAAAGUGAAUCTG-3′; siRNA2: 5′-CAUUGCUGAUCUUGACAAATT-3′, 5′-UUUGUCAAGAUCAGCAAUGTT-3′) were confirmed to have knockdown efficiency by Northern Blotting. Another duplex of siRNA that is not targeted to any human genes was used as negative control. HUVECs were transfected with siRNA at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen).

Growth factor and inhibitor studies

Selective growth factors and cytokines including VEGF, FGF2, TGF-β, IL-1β, TNF-α and DGF-BB (Sigma) were added to HUVECs. To specifically block secreted VEGF, the monoclonal anti-human VEGF-neutralizing antibody, murine isotype control IgG (R&D Systems), or SU4312 (Sigma), a suppressor of VEGFR2 activation that inhibits VEGFR2 phosphorylation, were pre-incubated with HUVECs at 37°C for 1 hour before hypoxia.

Western Blot Analysis

HUVECs were lysed in RIPA buffer and blot with antibodies as described.[8] Detailed information of antibodies and materials are listed in the online-only Data Supplement.

Proliferation and Migration Assays

HUVECs were seeded in 12-well plates (3×10^4 cells/well) and incubated for 5 hours counting from day 1. At the indicated time point, culture was stopped by fixing with 4% paraformaldehyde for 15 minutes and subsequently stained with 0.1% crystal violet dissolved in 10% ethanol for 20 minnutes. After washing, 10% acetic acid was added to the cells and absorbance was measured at 590 nm wavelength. HUVECs migration was measured by "wounding" assays. Cells were grown to subconfluence in 12-well plates (10^5 cells/ well) then starved for 24 hours in 0.5% serum. The cell layer was scratched with a pipette tip, producing a gap ~2-mm wide. The gap width was measured at marked locations from images taken by inverted microscope (TMS-F; Nikon) immediately after the scratching and again 6 hours later at the same marked locations as described.[9]

Apoptosis Assay

Apoptotic cells were analyzed using Vybrant Apoptosis Assay Kit 13 for FACS and Annexin V kit for Matrigel (Invitrogen). The cells were incubated with PO-PRO™-1 and 7-AAD on ice for 30 minutes. Stained HUVECs were analyzed by FACScan flow cytometry using BD FACSDiva software (BD Pharmingen). Cells that were PO-PRO™-1 positive and 7-AAD negative were counted for apoptosis to exclude necrotic cells. Caspase Inhibitor Z-VAD-FMK was from Calbiochem.

Matrigel Analysis

BD Matrigel™ Matrix Growth Factor Reduced (BD Biosciences) was coated on a pre-chilled 24-well culture plate on ice. After Matrigel solidification for 30 minutes, HUVECs were plated 10^5 cells/well with EBM2. Twenty-four to 48 hours later, the extent of network formation was observed and photographed. In vivo Matrigel angiogenesis assays were carried out as described.[10] 1×10^7 PT67 cells were infected with retroviruses expressing full-length RGC-32 or GFP cDNAs. The infected PT67 alone or mixed with SKMEL/VEGF cells (1×10^7) were suspended in 0.5ml of growth factor-reduced Matrigel (BD Biosciences) and injected subcutaneously (sc) into nu/nu mice. Tissues were photographed and fixed with 4% paraformaldehyde for immunohistochemistry. Each experiment was replicated with 4 mice.

Hindlimb ischemia model

Hindlimb ischemia in 12-week-old male FVB mice was induced by ligation of the femoral artery as described.[11] Immediately after surgery, animals received in the right hindlimb an i.m. injection consisting of 50 μl of either Retrovirus pBMN-GFP (1×10^8 pfu/ml) or pBMN-GFP-RGC-32 (1×10^8 pfu/ml) at the quadriceps. Tissue section was performed to assess the expression of retroviral-encoded GFP from injected ischemic muscle specimens 7 and 28 days after surgery. Blood flow was measured with a MoorLDI2 infrared scanner.

Tumor analysis

1×10^8 SW480 cells were implanted s.c. into BALB/c nude mice. Animals were inspected daily for tumor development. Tumors were measured using a digital caliper, and volume was calculated by length × width^2 × 0.52, which approximates the volume of an elliptical solid. After two weeks, mice harboring subcutaneous tumors (approximately 100 mm^3) were randomly divided into two groups (n=4) and were treated with an intraneoplastic inoculation of 1×10^7 PFU retrovirus of either pBMN or RGC-32 every 3 days. Mice were sacrificed on day 13 postinjection. All the animal studies were approved by the IACUC of BIDMC in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Immunostaining

Sections of in vivo Matrigel blocks, hindlimb ischemia quadriceps and colon tumor tissues were fixed in 4% Paraformaldehyde for capillary density studies. Immunohistochemistry and immunofluorescence were performed with the CD31 antibody (BD Pharmingen) as described previously.[10]

Statistics

The results are expressed as mean ± S.E.M or S.D based on triplet experiments. Statistical analysis used analysis of variance and Student's t-test (two-tailed). A p-value <0.05 was considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

RGC-32 expression is induced by hypoxia

To investigate the role of RGC-32 in angiogenesis, we initially examined RGC-32 expression under hypoxia compared with normoxia in endothelial cells. A significant increase in RGC-32 gene expression occurred from 3 hours (2.21±0.12 fold) to 36 hours (3.47±0.57 fold), and returned to baseline after 48 hours in hypoxia (Figure 1A), which was also confirmed in the Quantitative-PCR assay (Supplementary Figure 1A). Immunoblot analysis shows that RGC-32 protein response to hypoxia began at 6 hours and remained at a high level until 48 hours (Figure 1B). mRNA half-life assay indicated that RGC-32 mRNA half-life was prolonged under hypoxia (6.05 vs 1.96 hours) (Supplementary Figure 1B). These results demonstrate that the increased expression of RGC-32 in response to hypoxia is mediated, at least partially, at the posttranscriptional level, which could reasonably account for its steady elevated protein level.

HIF-1α and VEGF mediate the induction of RGC-32 expression in hypoxia

To determine whether hypoxia-induced RGC-32 mRNA was mediated by HIF-1, HUVECs were exposed to $CoCl_2$, 3,4-DHB, or DMOG, which stabilize HIF-1α expression via hydroxylase inhibition.[12] RGC-32 mRNA was significantly increased when the HIF-1 protein was protected from degradation (Figure 2A). In addition, co-transfection with the RGC-32 promoter and HIF-1α cDNA led to a significant increase in RGC-32 promoter activity (Figure 2B). ChIP assay revealed direct interaction between HIF-1α and HRE sites on the RGC-32 promoter (Figure 2C). Thus, hypoxia-induced RGC-32 expression was regulated at both the transcriptional and posttranscriptional levels via HIF-1 or HIF-1 target genes.

Compared with other reported hypoxia responsive genes, RGC-32 showed a steady, prolonged induction, suggesting that some other indirect induction may also be involved in this response.

A panel of growth factors and cytokines, VEGF, FGF-2, TGF-β, IL1-β, TNF-α and PDGF, were incubated with HUVECs for 12 hours, and RGC-32 mRNA levels were examined. As Figure 2D shows, VEGF, but not TGF-β or PDGF, significantly induced RGC-32 mRNA expression, whereas TNF-α, FGF2 and IL-1β decreased the expression of RGC-32 mRNA. VEGF was markedly induced by hypoxia in HUVECs (Figure 2E), and VEGF-induced RGC-32 expression showed a time- and dose-dependence (Figure 2F), indicating that VEGF might play an important role in hypoxia-related RGC-32 stimulation. To test this hypothesis, SU4312, a VEGF receptor 2 activation suppressor, was incubated with HUVECs for 1 hour prior to hypoxia. Hypoxia-induced RGC-32 expression was significantly blocked (Figure 2G left). In addition, treating HUVECs with anti-VEGF-neutralizing antibody prior to hypoxia decreased the hypoxia-induced RGC-32 mRNA (Figure 2G right). Thus, VEGF appears to be the predominant factor for hypoxia induction of RGC-32 in endothelial cells.

RGC-32 attenuates angiogenesis in vitro and in vivo

To test the hypothesis that RGC-32, as a downstream gene of VEGF, might enhance angiogenesis, RGC-32 cDNA carried by a retrovirus was generated in endothelial cells. RGC-32 proteins were successfully expressed in HUVECs (Figure 3A) and angiogenic activities of RGC-32 (o/e) were examined. Surprisingly, a growth curve assay showed that the RGC-32 (o/e) cells grew significantly slower compared with those infected by retrovirus vector only (Figure 3B). The migration rate of the RGC-32 (o/e) cells was dramatically decreased compared to control cells shown in Figure 3C. To further validate that RGC-32 does not act as an angiogenic enhancer within the VEGF pathways, three sequences of siRNA that targeted RGC-32 were synthesized, and two of them showed superior knockdown of RGC-32 expression (Figure 3D left). Figure 3D (middle) demonstrates that the HUVECs decreased the velocity of growth under hypoxic conditions. However, knockdown of RGC-32 by siRNA indicated that the HUVECs growth rate was accelerated in normoxia and significantly enhanced by hypoxia in comparison with the control siRNA. The migration rate was also accelerated in RGC-32 knockdown HUVECs compared with the control cells (Figure 3D right).

To further verify RGC-32's mechanism of action, we used the Matrigel assay to introduce genes of interest into the vascular endothelium in vivo.[13] On day 3 after implantation of Matrigel plugs containing various cell mixtures, angiogenic responses were evaluated macroscopically (Figure 3E, top panels) and by histology and immunohistochemistry for the endothelial cell marker CD31 (Figure 3E, bottom panels). Plugs containing only PT67 cells did not significantly induce angiogenesis (Figure 3E-a/e). However, strong angiogenesis was induced in plugs containing SK-MEL/VEGF cells (Figure 3E-c/g). When PT67/RGC-32 cells were included in the Matrigel, the angiogenic response induced by SKMEL/VEGF cells was strikingly inhibited (Figure 3E-d/h). Taken together, these results suggest that RGC-32 does not have pro-angiogenesis capability, instead it can inhibit endothelial cell proliferation and migration and VEGF-induced angiogenesis.[14]

RGC-32 induces apoptosis

When the effect of RGC-32 in Matrigel capillary structure formation in vitro was examined, it did not show significant differences at an early stage (10-24 hours) between RGC-32 (o/e) and control cells. However, at a late stage (40 hours), the vascular structures formed by RGC-32 (o/e) cells were less stable, but no significant difference was seen when the cells were incubated with the caspase inhibitor Z-VAD-FMK compared with control cells. This indicated that endothelial cell apoptosis took place in RGC-32 (o/e) cells, which was confirmed by the increase of Annexin V positive cells (Figure 4A). PO-PRO™-1/7-aminoactinomycin D staining measured the number of apoptotic cells (Figure 4B). The obvious changes were observed after cells had undergone hypoxia for 48 hours. The number of apoptotic cells

increased from 7.7% to 14.2% for controls, but increased from 8.5% to 22.1% for RGC-32 (o/e) HUVECs. In agreement with the increased apoptotic cell number, there was also a significant increase of cleaved caspase 3 in RGC-32 (o/e) HUVECs after 48 hour hypoxia (Figure 4C). These results suggest that RGC-32 accelerates hypoxia-induced endothelial apoptosis.

RGC-32 attenuates FGF2-related cyclin E expression

One possible mechanism that might explain the non-canonical VEGF angiogenic pathway is that RGC-32 could be a VEGF negative feedback regulator.[15] However, there were no significant differences found between RGC-32 (o/e) cells and control cells in terms of phosphorylation of eNOS, Akt and MAP kinases (ERK and p38) by VEGF stimulation (Supplementary Figure 2A). Examination of three major angiogenic pathways[16] in RGC-32 (o/e) cells showed marked decreases in the expressions of the FGF2 isoforms 23kd and 17kd (Figure 5A), and RGC-32 down-regulated FGF2 expression enhanced by hypoxia (Figure 5B). However, no effect on the FGF2-stimulated signaling pathway was observed (Supplementary Figure 2B). Meanwhile, we examined the protein levels of anti-angiogenic genes, including angiostatin and endostatin, but no significant differences were found between RGC-32 (o/e) and control cells (data not shown). These results suggest that RGC-32 had no effect on the VEGF downstream pathway, but did influence expression of FGF2.

In light of RGC-32 perturbations of the cell cycle described for malignant transformation,[6, 17] we postulated that RGC-32 might act in conjunction with cyclin in response to the down-regulation of FGF2. Figure 5C indicate that RGC-32 inhibited FGF2-stimulated cyclin E, but not cyclin B, D and E2F. VEGF-induced cycline E was also attenuated in RGC-32 o/e cells in comparison to controls. Collectively, these data suggested that RGC-32 impacts FGF2's pro-proliferating pathway via inhibiting FGF2's up-regulation of cyclin E in endothelial cells, which explains RGC-32's ability to reduce proliferation.

RGC-32 impaired perfusion recovery after hindlimb ischemia

In the mouse hindlimb ischemia, the expression of RGC-32 was markedly induced in all ischemic muscle beds (Figure 6A). To assess whether RGC-32 can impact the normal vascular response to ischemia, RGC-32 and control retroviruses were delivered to quadriceps immediately after the procedure. The expression of the delivered gene was confirmed by GFP (Supplementary Figure 3). Blood perfusion in ischemic hindlimbs was measured by Doppler analysis on days 0, 7, 14, 21 and 28. The blood flow in the pBMN vector-only treated quadriceps area was improved 88% of the non-ischemic control at day 14. However, treatment with RGC-32 decreased the blood flow recovery, showing about 64% of the non-ischemic control (Figure 6 B&C). The angiographic analysis at day 7 and 28 after ligation of the femoral artery revealed a decreased number of vessels in the RGC-32–treated group (Figure 6D).

RGC-32 reduced tumor angiogenesis

To determine whether RGC-32 has the ability to regulate tumor angiogenesis, an intraneoplastic inoculation of either pBMN or RGC-32 retrovirus started when tumors were around 100mm^3 Growth of RGC-32 expressed tumors was greatly inhibited. Compared to the control, the average tumor size in the RGC-32 group recessed by 35% on day 9 and 45% on day 13 (Figure 7A). Additionally, we found that suppression of tumor growth by RGC-32 (Figure 7B) was associated with reduced angiogenesis, particularly with a reduction in number of vessels, which was assessed by CD31 staining (Figure 7 C & D).

0RGC-32 in regulation of FGF2 and cyclin E was also found in RGC-32 treated tumor tissues. Figure 7E indicates that RGC-32-treated tumor tissue significantly reduced expression of FGF2, cyclin E, and endothelial cell marker CD31.

Discussion

The principal finding of this study is that RGC-32 is a hypoxia-induced anti-angiogenesis factor in endothelial cells. We demonstrate that the mechanism of HIF-1α/VEGF-induced RGC-32 expression inhibits angiogenesis via RGC-32-dependent attenuation of the FGF2 pathway for cyclin E expression. Several lines of evidence support our finding. First, RGC-32 expression was induced by hypoxia in both mRNA and protein levels. Second, inhibitory studies indicated that hypoxia-induced RGC-32 expression is dependent on HIF-1α stabilization, and HIF-1α is able to stimulate RGC-32 promoter activity. Third, RGC-32 was induced by VEGF but did not effect VEGF signaling. Instead, RGC-32 significantly downregulated FGF2 and attenuated FGF2-dependent cyclin E expression. Finally, the ability of RGC-32 to inhibit endothelial cell proliferation and migration could be blocked by inhibition of RGC-32. Injection of RGC-32 in the mouse hindlimb ischemia and the tumor xenograph models can reduce the number of blood vessels in association with downregulation of FGF2 and cyclin E.

Our data provide evidence that hypoxia induced RGC-32 expression via the HIF1α/VEGF pathway. The results for RGC-32 as an inducible gene were consistent with previous reports indicating that RGC-32 mRNA expression is induced by C5b-9 complement activation,[4] steroid hormones[18] and TGF-β.[19] However, we did not observe TGF-β induced RGC-32 expression in endothelial cells, although this might be due to TGF-β activating RGC-32 through specific Smad and RhoA signaling to initiate cell differentiation in smooth muscle cells.[19]

Unlike other VEGF-induced genes, such as COX-2,[8] RGC-32 did not follow the canonical VEGF-induced angiogenic pathway. One possible mechanism that might explain the role of RGC32 during proliferation and vascular growth is that RGC-32 could be a VEGF negative feedback regulator. Similarly, Delta-like ligand 4 (Dll4) was dynamically induced by VEGF, but Dll4 blockade enhanced angiogenic sprouting while suppressing ectopic pathological neo-vascularization in the retinal vasculature.[15] In the present study, RGC-32 did not influence VEGF-mediated signaling pathways indicated by VEGF-stimulated phosphorylation of eNOS, Akt and MAPK (ERK, P38) that showed no significant differences in RGC-32 overexpressed endothelial cells compared with control cells. We demonstrated in this report that RGC-32 significantly attenuated expression of FGF2, but it did not interrupt the expressions of the FGF receptor 1 and angiopointin-1/Tie2 pathways. The anti-angiogenic proteins angiostatin and endostatin were also not regulated by RGC-32.

Although FGF2 is involved in angiogenesis, its expression regulation during hypoxia is poorly documented. In HUVECs we have observed that FGF2 protein was downregulated in 23 kd and 17 kd isoforms under hypoxic conditions. This result contradicted another report showing that FGF2 was induced at a protein level concomitant with a decrease in FGF2 mRNA caused by internal ribosome entry site (IRES) during hypoxia.[20] However, our data is in agreement with previous reports suggesting that hypoxia induced expression of VEGF but not FGF2.[21, 22] Interestingly, despite the fact that expression of FGF2 was reduced by RGC-32, it had no effect on the FGF2-stimulated signaling pathways.

In contrast to these active constitutive forms, we found in RGC-32 overexpressing cells that cyclin E expression was attenuated in response to FGF2. Cyclin E is one of the major cyclins that is involved in the G1 to S phase transition. This result is consistent with a previous report showing that RGC-32 suppressed the growth of glioma cells via p53 regulation.[6] However, RGC-32 did not directly alter activation of cyclin E in endothelial cells. Our results suggest that RGC-32 inhibits endothelial cell proliferation not by directly interrupting the cell cycle, but via influencing FGF2 pathways, in which cyclin E and cyclin-dependent kinase (cdks) 2 and 4 were expressed after FGF2 infusion[23]. Furthermore, FGF2 decreased levels of the cdk inhibitor p27 (Kip1) to enhance association of cyclin E–cdk2.[24]

RGC-32 had no impact on vascular structure formation, but it did decrease its stability. This suggests that RGC-32 not only inhibits angiogenesis, but also stimulates apoptosis. RGC-32 has been shown to be a direct transcriptional target of p53 in human cells of various tissue origins.[6] Cell cycle regulation and apoptosis are the most important features of p53-dependent tumor suppression. The regulator of FGF2 transcription (RFT) is a transcriptional repressor and induces glioma cell death by its overexpression, suggesting that RFT regulates the G1–S transition and apoptosis via the p53/p21^{Waf1} pathway.[25] Thus, down-regulation of FGF2 expression by RGC-32 may trigger suppression of cell growth, especially mitotic progression through the p53 pathway in endothelial cells. Moreover, the FGF2–treated group in the hindlimb ischemia increased angiogenesis and number of collateral vessels after ligation of the femoral artery,[26] therefore, downregulation of FGF2 expression by RGC-32 could impact the return of blood flow to the ischemic limb and inhibit progressive neovascularization.

As a cell cycle regulator, RGC-32 has shown two opposite results for tumor growth. In addition to targeting the G/M phase transition and suppressing tumor growth,[6] RGC-32 has also reportedly activated cdc2 kinases to induces cell cycle activation.[17] These contradictory results have been explained by the possibility that RGC-32 may play dual roles by enhancing cell proliferation and acting as a tumor suppressor gene in certain types of cancers.[7] The model in the present study demonstrated that RGC-32 reduced colon cancer tumor size with reduced numbers of vessels. We also observed a significant decrease of VEGF-induced blood vessel growth by RGC-32 in Matrigels. Many stimuli including hypoxia can increase a major angiogenic factor (e.g. VEGF) expression in tumor cells, which is correlated with increased microvessel counts and poor prognosis[27] in many human cancers. However, hypoxia/ischemia -induced gene regulation in tumor growth is not fully understood. We document here that RGC-32 has the ability to promote both anti-angiogenesis and pro-apoptosis in endothelial cells, which results in decreased blood vessel growth during colon cancer proliferation and, in turn, reduced tumor size. Overexpression of FGF2 mRNA was associated with significantly increased risk for tumor recurrence[28] Targeting FGF receptors and/or FGF signaling can affect both the tumor cells directly and tumor angiogenesis.[29] Our study indicated that decreased FGF2 expression in the RGC-32 treated xenograph model may not be limited to vessels due to the FGF signaling involved in both the cancer cells and surrounding vasculature to enhance proliferation and resistance to cell death, thereby, enhancing tumor progression.[29] Our results add an additional mechanism, anti-angiogensis, for RGC-32 as a tumor suppressor.

RGC-32 inhibited endothelial cell proliferation and migration. However, it is not clear how this may correlate to other recently reported functions, such as cell differentiation[19] or inflammation.[30] Further studies will be required to determine if RGC-32 as a hypoxia-inducible gene effects hypoxia/ischemia-related cell dysfunction in other pathways. In summary, we demonstrated for the first time that RGC-32 is a hypoxia inducible gene dependent on HIF-1α and VEGF. Induced RGC-32 performed anti-angiogenic activity through downregulating endothelial cyclin E via the FGF2 pathway. This study reveals the important role that RGC-32 plays in homeostasis of hypoxic endothelial cells. This may contribute to understanding the crosstalk between different angiogenic gene pathways and also highlights the potential for tumor treatment by targeting RGC-32.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Simons M. Angiogenesis: where do we stand now? Circulation 2005;111:1556–66. [PubMed: 15795364]
2. Stempien-Otero A, Karsan A, Cornejo CJ, Xiang H, Eunson T, Morrison RS, Kay M, Winn R, Harlan J. Mechanisms of hypoxia-induced endothelial cell death. Role of p53 in apoptosis. J Biol Chem 1999;274:8039–45. [PubMed: 10075703]
3. Li, J.; Sellke, FW. Cambridge University Press. Endothelial Biomedicine. Vol. Chapter 65. 2007. Cardiomycytes-Endothelial Cell Interaction (Review); p. 602-608.
4. Badea T, Niculescu F, Soane L, Fosbrink M, Sorana H, Rus V, Shin ML, Rus H. RGC-32 increases p34CDC2 kinase activity and entry of aortic smooth muscle cells into S-phase. J Biol Chem 2002;277:502–8. [PubMed: 11687586]
5. Oram SW, Liu XX, Lee TL, Chan WY, Lau YF. TSPY potentiates cell proliferation and tumorigenesis by promoting cell cycle progression in HeLa and NIH3T3 cells. BMC Cancer 2006;6:154. [PubMed: 16762081]
6. Saigusa K, Imoto I, Tanikawa C, Aoyagi M, Ohno K, Nakamura Y, Inazawa J. RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest. Oncogene 2007;26:1110–21. [PubMed: 17146433]
7. Vlaicu SI, Cudrici C, Ito T, Fosbrink M, Tegla CA, Rus V, Mircea PA, Rus H. Role of response gene to complement 32 in diseases. Arch Immunol Ther Exp (Warsz) 2008;56:115–22. [PubMed: 18373239]
8. Wu G, Mannam AP, Wu J, Kirbis S, Shie JL, Chen C, Laham RJ, Sellke FW, Li J. Hypoxia induces myocyte-dependent COX-2 regulation in endothelial cells: role of VEGF. Am J Physiol Heart Circ Physiol 2003;285:H2420–9. [PubMed: 12881220]
9. Horowitz A, Murakami M, Gao Y, Simons M. Phosphatidylinositol-4,5-bisphosphate mediates the interaction of syndecan-4 with protein kinase C. Biochemistry 1999;38:15871–7. [PubMed: 10625452]
10. Zeng H, Qin L, Zhao D, Tan X, Manseau EJ, Van Hoang M, Senger DR, Brown LF, Nagy JA, Dvorak HF. Orphan nuclear receptor TR3/Nur77 regulates VEGF-A-induced angiogenesis through its transcriptional activity. J Exp Med 2006;203:719–29. [PubMed: 16520388]
11. Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. Mouse model of angiogenesis. Am J Pathol 1998;152:1667–79. [PubMed: 9626071]
12. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54. [PubMed: 11595184]
13. Hoang MV, Senger DR. In vivo and in vitro models of Mammalian angiogenesis. Methods Mol Biol 2005;294:269–85. [PubMed: 15576918]
14. Folkman J. Antiangiogenesis in cancer therapy--endostatin and its mechanisms of action. Exp Cell Res 2006;312:594–607. [PubMed: 16376330]
15. Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci U S A 2007;104:3219–24. [PubMed: 17296940]
16. Oettgen P. Transcriptional regulation of vascular development. Circ Res 2001;89:380–8. [PubMed: 11532898]
17. Fosbrink M, Cudrici C, Niculescu F, Badea TC, David S, Shamsuddin A, Shin ML, Rus H. Overexpression of RGC-32 in colon cancer and other tumors. Exp Mol Pathol 2005;78:116–22. [PubMed: 15713436]

18. Chen QM, Alexander D, Sun H, Xie L, Lin Y, Terrand J, Morrissy S, Purdom S. Corticosteroids inhibit cell death induced by doxorubicin in cardiomyocytes: induction of antiapoptosis, antioxidant, and detoxification genes. Mol Pharmacol 2005;67:1861–73. [PubMed: 15755911]
19. Li F, Luo Z, Huang W, Lu Q, Wilcox CS, Jose PA, Chen S. Response gene to complement 32, a novel regulator for transforming growth factor-beta-induced smooth muscle differentiation of neural crest cells. J Biol Chem 2007;282:10133–7. [PubMed: 17327222]
20. Conte C, Riant E, Toutain C, Pujol F, Arnal JF, Lenfant F, Prats AC. FGF2 translationally induced by hypoxia is involved in negative and positive feedback loops with HIF-1alpha. PLoS ONE 2008;3:e3078. [PubMed: 18728783]
21. Aparicio S, Sawant S, Lara N, Barnstable CJ, Tombran-Tink J. Expression of angiogenesis factors in human umbilical vein endothelial cells and their regulation by PEDF. Biochem Biophys Res Commun 2005;326:387–94. [PubMed: 15582590]
22. Brogi E, Wu T, Namiki A, Isner JM. Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. Circulation 1994;90:649–52. [PubMed: 8044933]
23. Olson NE, Kozlowski J, Reidy MA. Proliferation of intimal smooth muscle cells. Attenuation of basic fibroblast growth factor 2-stimulated proliferation is associated with increased expression of cell cycle inhibitors. J Biol Chem 2000;275:11270–7. [PubMed: 10753937]
24. Frederick TJ, Wood TL. IGF-I and FGF-2 coordinately enhance cyclin D1 and cyclin E-cdk2 association and activity to promote G1 progression in oligodendrocyte progenitor cells. Mol Cell Neurosci 2004;25:480–92. [PubMed: 15033176]
25. Kano H, Arakawa Y, Takahashi JA, Nozaki K, Kawabata Y, Takatsuka K, Kageyama R, Ueba T, Hashimoto N. Overexpression of RFT induces G1-S arrest and apoptosis via p53/p21(Waf1) pathway in glioma cell. Biochem Biophys Res Commun 2004;317:902–8. [PubMed: 15081425]
26. Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 2003;9:604–13. [PubMed: 12669032]
27. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–201. [PubMed: 11689883]
28. Barclay C, Li AW, Geldenhuys L, Baguma-Nibasheka M, Porter GA, Veugelers PJ, Murphy PR, Casson AG. Basic fibroblast growth factor (FGF-2) overexpression is a risk factor for esophageal cancer recurrence and reduced survival, which is ameliorated by coexpression of the FGF-2 antisense gene. Clin Cancer Res 2005;11:7683–91. [PubMed: 16278388]
29. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. Endocr Relat Cancer 2004;11:709–24. [PubMed: 15613447]
30. Tanaka T, Takada H, Nomura A, Ohga S, Shibata R, Hara T. Distinct gene expression patterns of peripheral blood cells in hyper-IgE syndrome. Clin Exp Immunol 2005;140:524–31. [PubMed: 15932515]

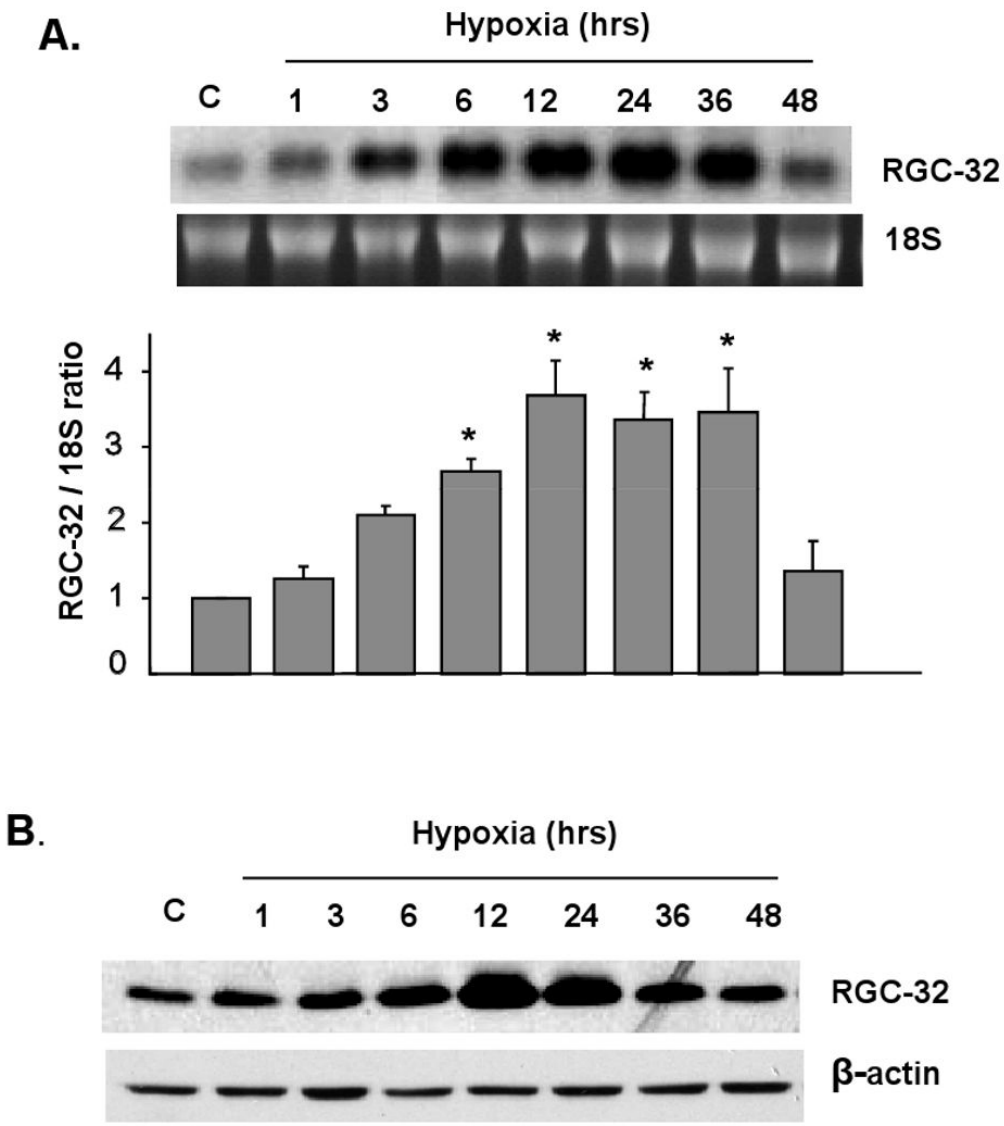
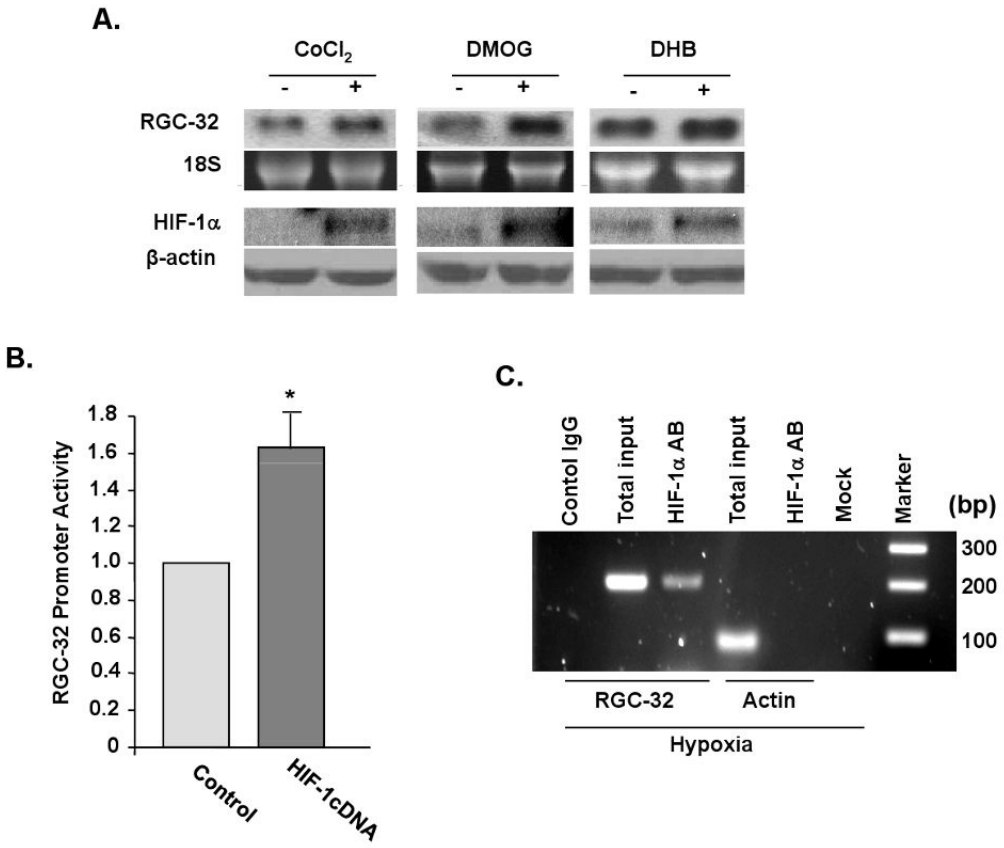


Figure 1. Effect of hypoxia on RGC-32 expression

RGC-32 expression in HUVECs was assessed after varying durations of hypoxia, from 1 h to 48 h using Northern (A) and Western (B) blotting. Both mRNA and protein were upregulated by hypoxia. Results are means ± SEM, based on 3 experiments quantified by Image J; *$P <$ 0.05.



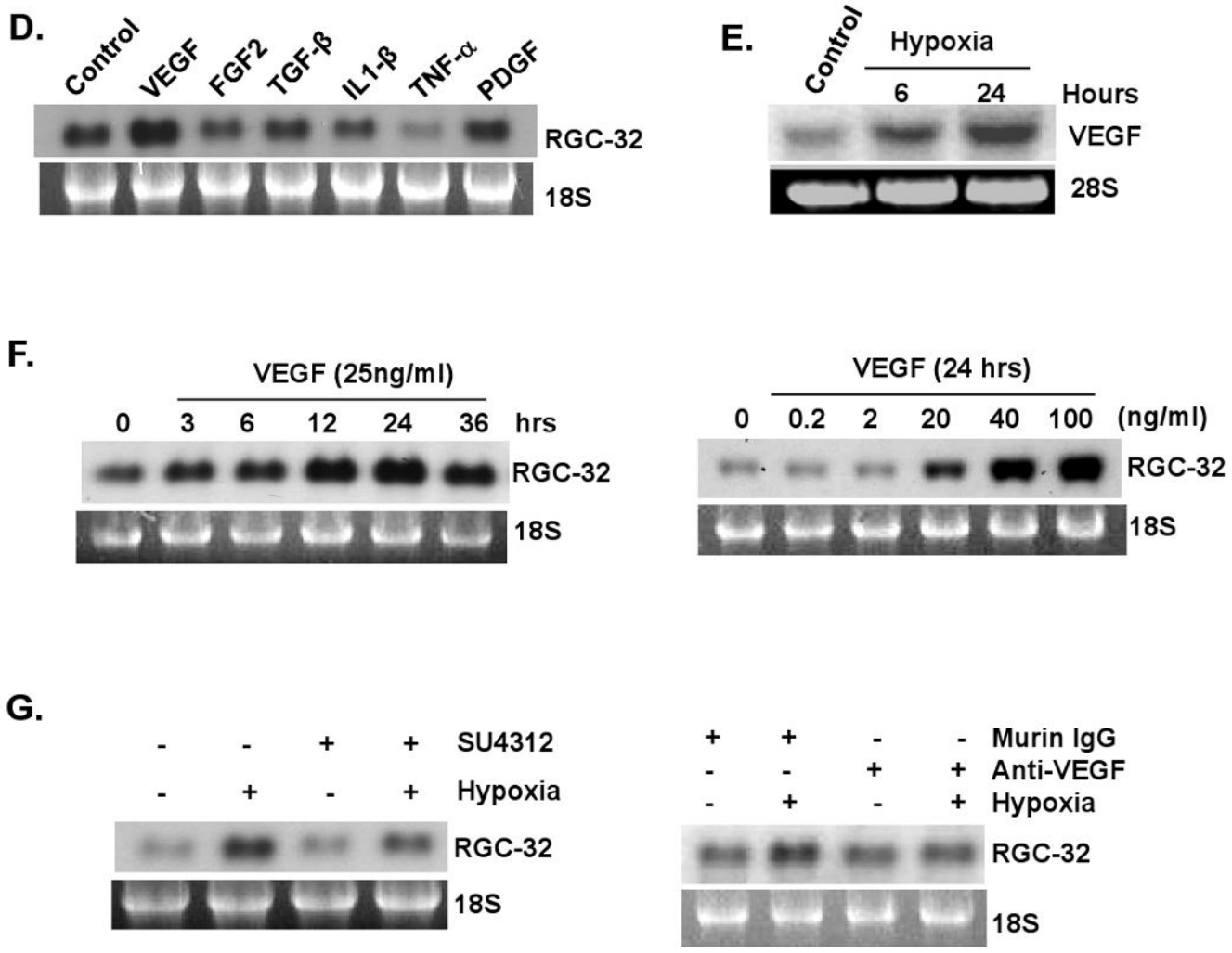
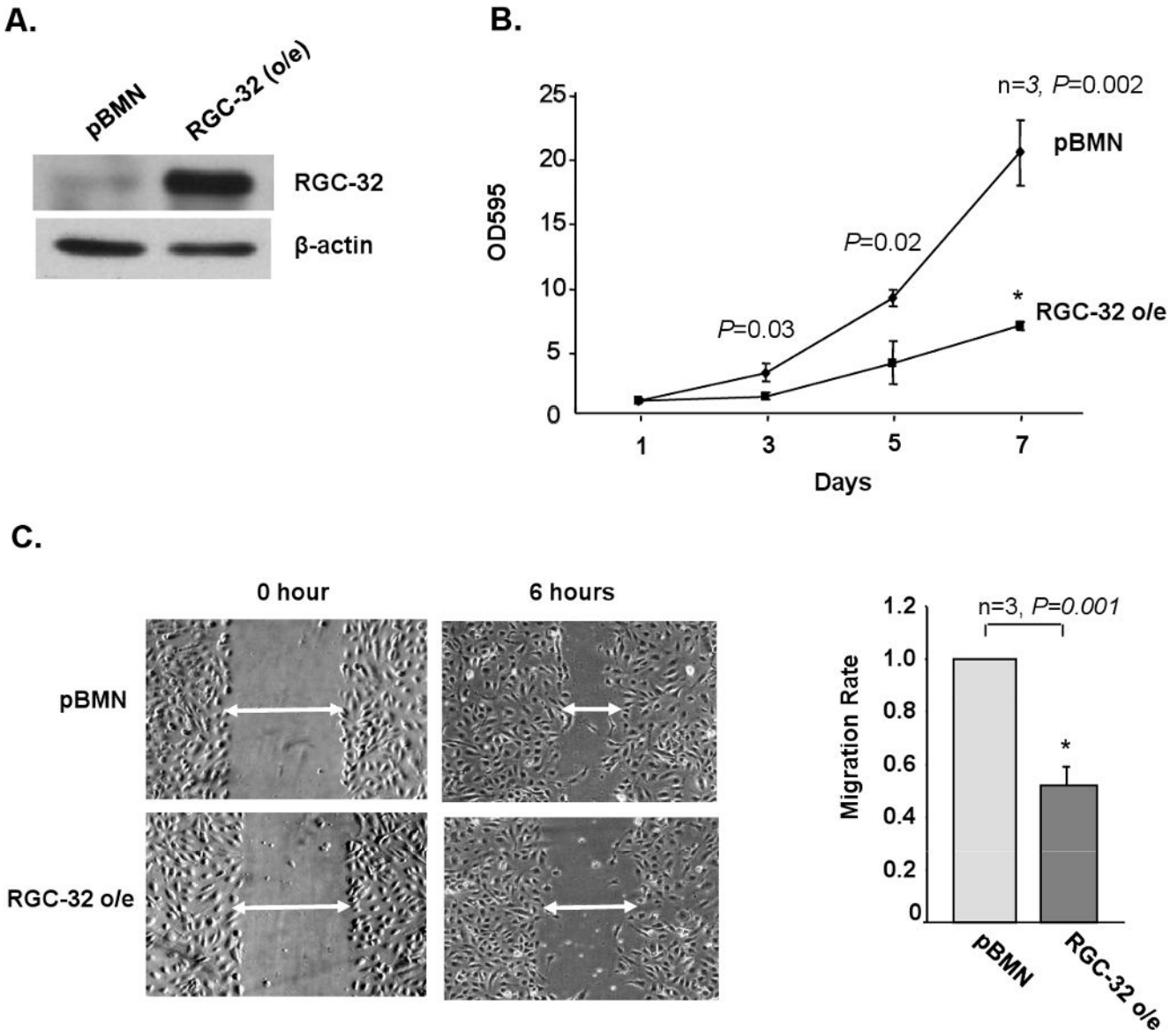


Figure 2. Effect of HIF-1α and VEGF on RGC-32 expression

(A) RGC-32 mRNA was significantly induced by $CoCl_2$, DMOG, and 3,4-DHB in HUVECs under normoxic conditions with HIF-1α accumulated. (B) RGC-32 promoter activity was increased 1.62±0.22 fold in luciferase assay in response to HIF-1α. Results are means ± SD. *P<0.05. (C) ChIP assay showing the RGC-32 promoter co-immunoprecipitating with HIF1α antibody in hypoxic conditions. (D) HUVECs were stimulated by VEGF (25ng/ml), FGF2 (25ng/ml), TGF-β(10ng/ml), IL-1β(10ng/ml), TNF-α (10ng/ml) or PDGF (20ng/ml) for 12 hours, and RGC-32 expression was analyzed by Northern blot. (E) VEGF was upregulated by hypoxia in HUVECs. (F) RGC-32 was upregulated by VEGF in a time (left) and dose dependent manner (right). (G) RGC-32 induced by VEGF was partially inhibited by SU4312 under hypoxia (left) and by anti-VEGF-neutralizing antibody (right).



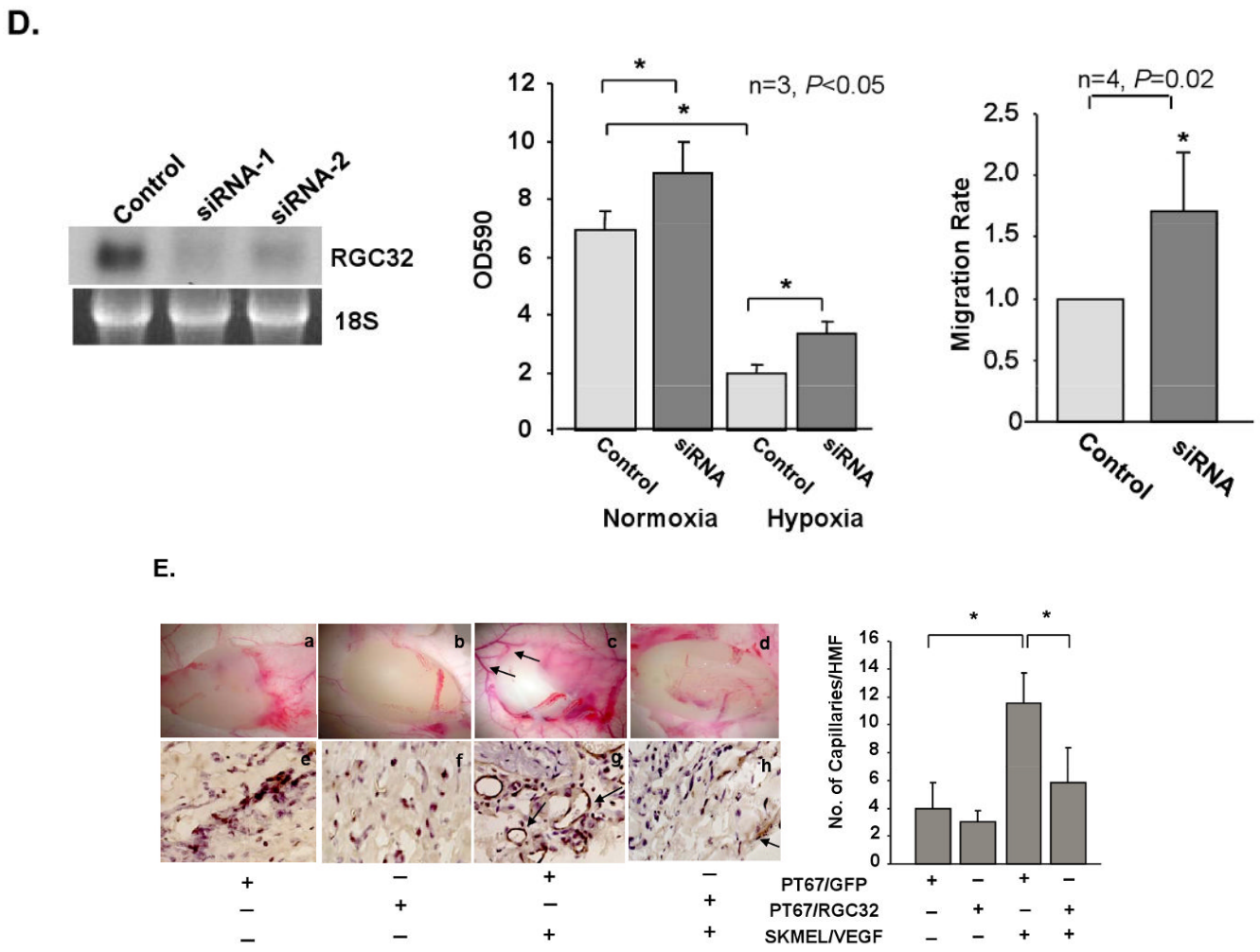


Figure 3. Effect of RGC-32 on angiogenesis

(A) RGC-32 overexpression in HUVECs was determined by Western blot. (B) Growth curve analysis indicates that RGC-32 (o/e) attenuated 51% of endothelial cell proliferation. (C) Migration assay for RGC-32 (o/e) shows about 45% lower migrating rates compared to controls. (D) Two siRNA sequences of RGC-32 showed knockdown efficiency (left). RGC-32 knockdown increased HUVECs proliferation (middle) and migration (right) compared to negative siRNA controls. (E) Macroscopic (top) and CD-31 stained microscopic (bottom) images of the angiogenic response induced 3 days after implantation of Matrigels with VEGF-A165–secreting SKMEL/VEGF cells and PT67 cells packaging pBMN-GFP or pBMN-RGC32. Note that VEGF stimulated blood vessels (c/g arrows) can be blocked by RGC-32 treatment (d/h).

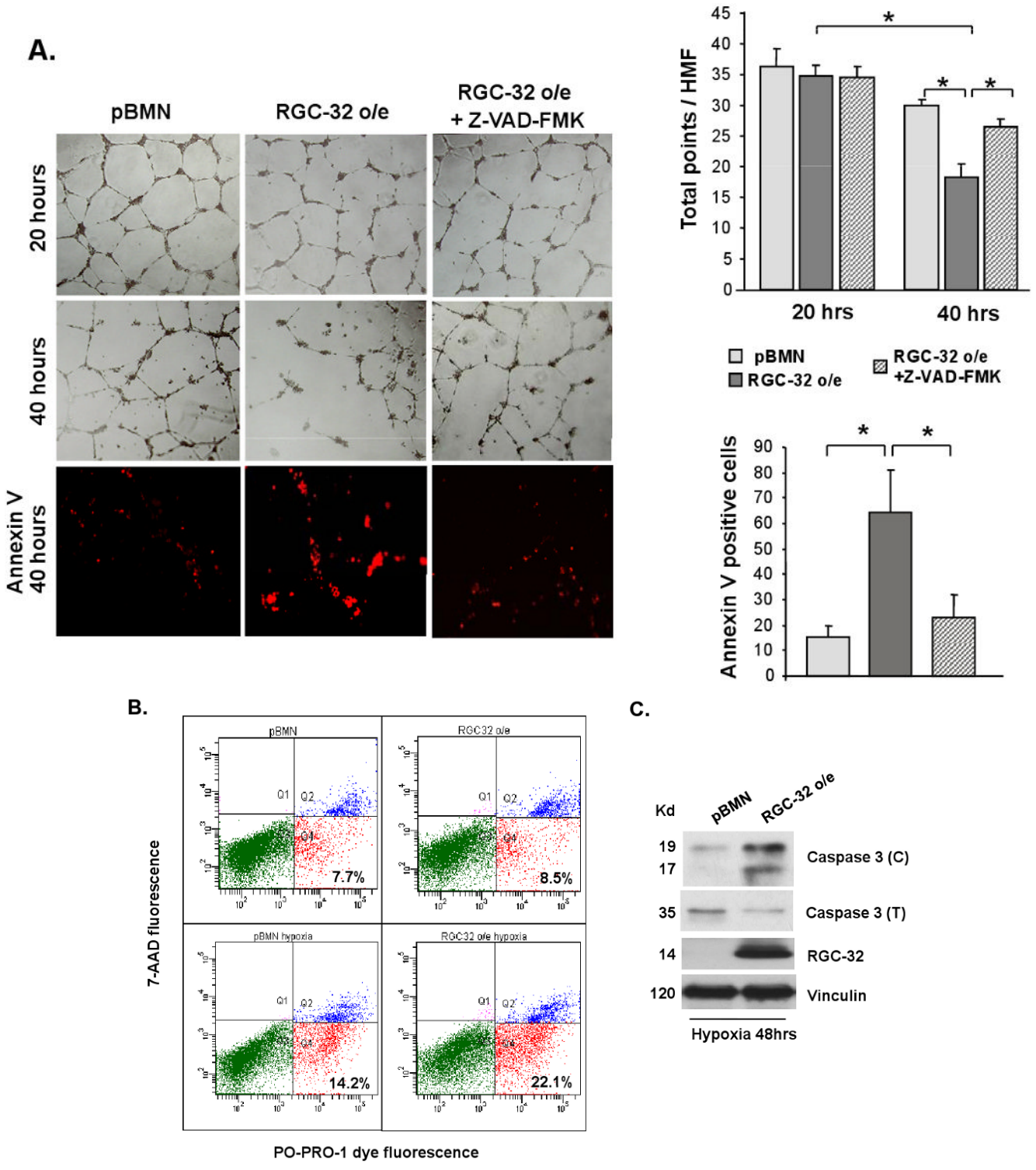


Figure 4. RGC-32 enhances endothelial cell apoptosis

(A) Vascular structure formation in Matrigel showed no significant change at 20 hours between RGC-32 (o/e) and control HUVECs. At 40 hours, the vascular structure of RGC-32 (o/e) cells was unstable from enhanced apoptosis shown by Annexin V staining (lower penal). (B) Flow

cytometry analysis indicated that the number of apoptotic cells increased in RGC-32 (o/e) HUVECs in hypoxia. (C) Western Blot shows increase of cleaved caspase 3 in RGC-32 (o/e) HUVECs.

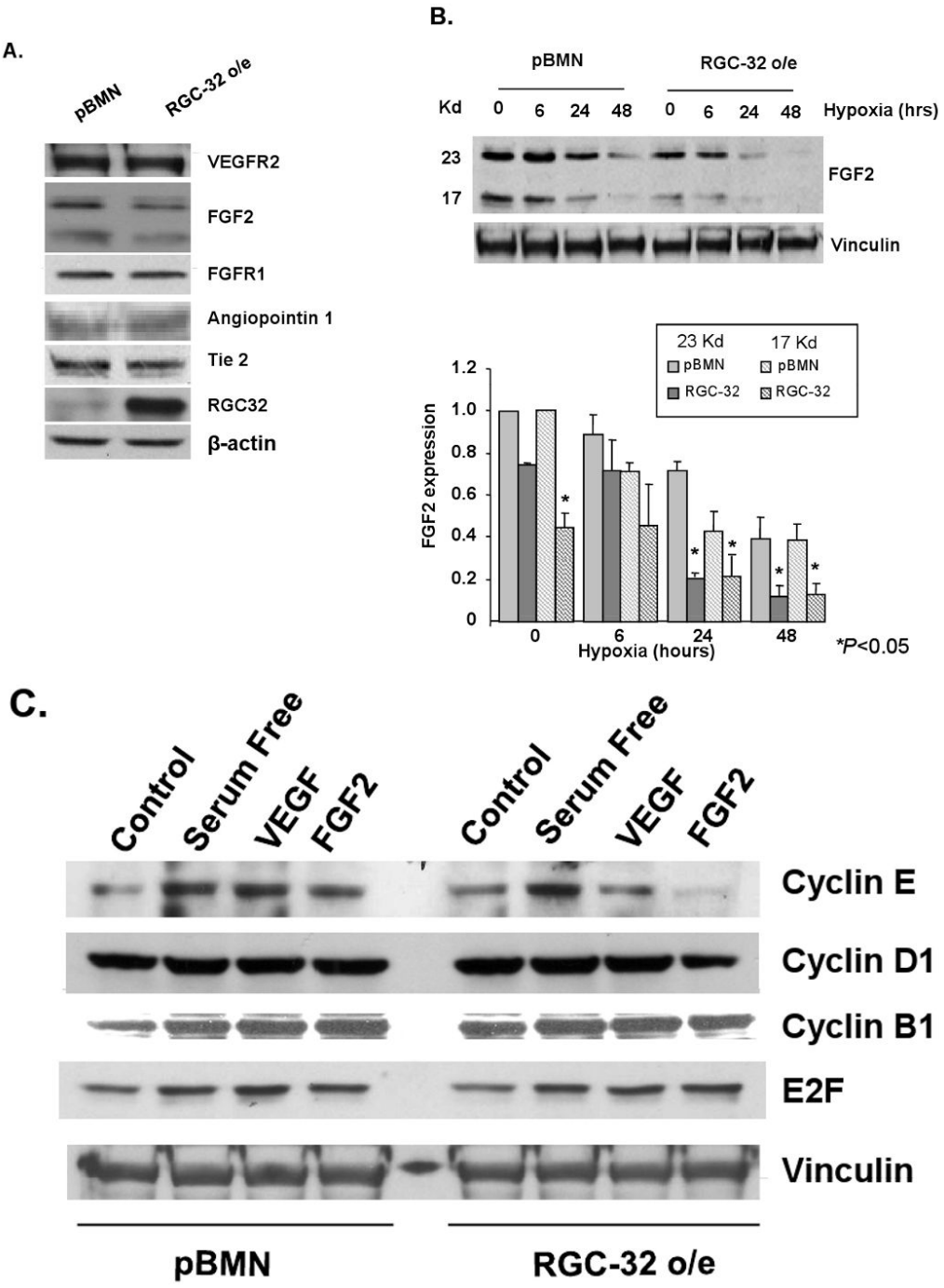


Figure 5. RGC-32 attenuates FGF2 and FGF2-stimulated cyclin E

(A) Three major angiogenesis pathway proteins including VEGFR2, FGF/FGFR1 and Angiopointin 1/Tie 2 were examined. Note the decreased expression of FGF2 in RGC-32 (o/e) HUVECs and the lack of significant changes for other tested genes. (B) Decreased FGF2 expressions in RGC-32 (o/e) HUVECs show in normoxia and hypoxia at 6, 24 and 48 hours. (C) Western Blotting analysis indicated that FGF2-stimulated cyclin E, but not cyclin B and D, was attenuated in RGC-32 o/e cells.

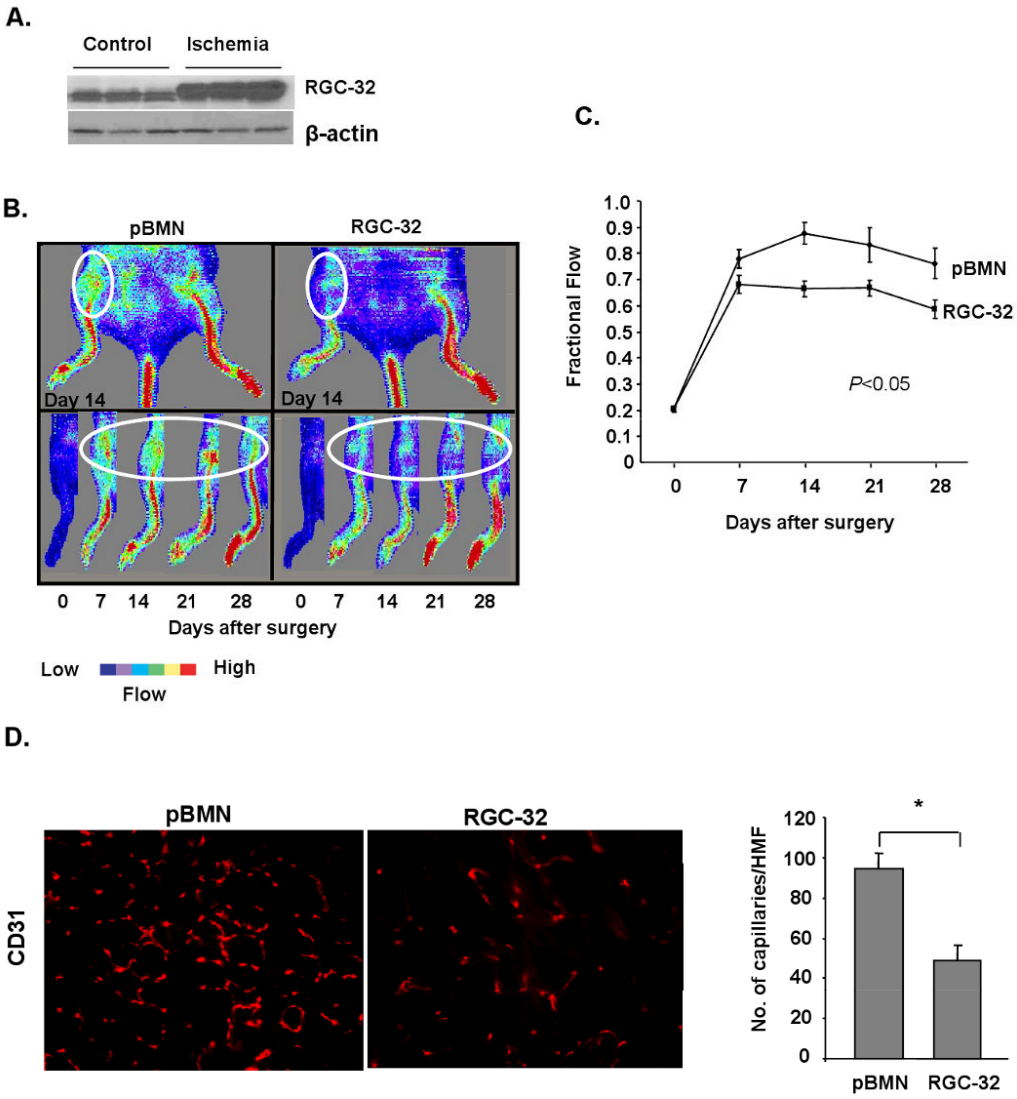


Figure 6. RGC-32 inhibited angiogenesis and blood flow recovery after ischemia in vivo

(A) RGC-32 protein expression increased after femoral artery ligation. (B) Infrared imaging from mice subjected to femoral artery ligation and retroviral injection of either pBMN or pBMN-RGC-32 on quadriceps. (C) Quantification of blood-flow recovery after treating either control or RGC-32 retrovirus. Data are means ± SEM n=8. (D) Sections of quadriceps from pBMN or RGC-32 o/e mice stained for CD31 (left) and quantification of capillaries (right).

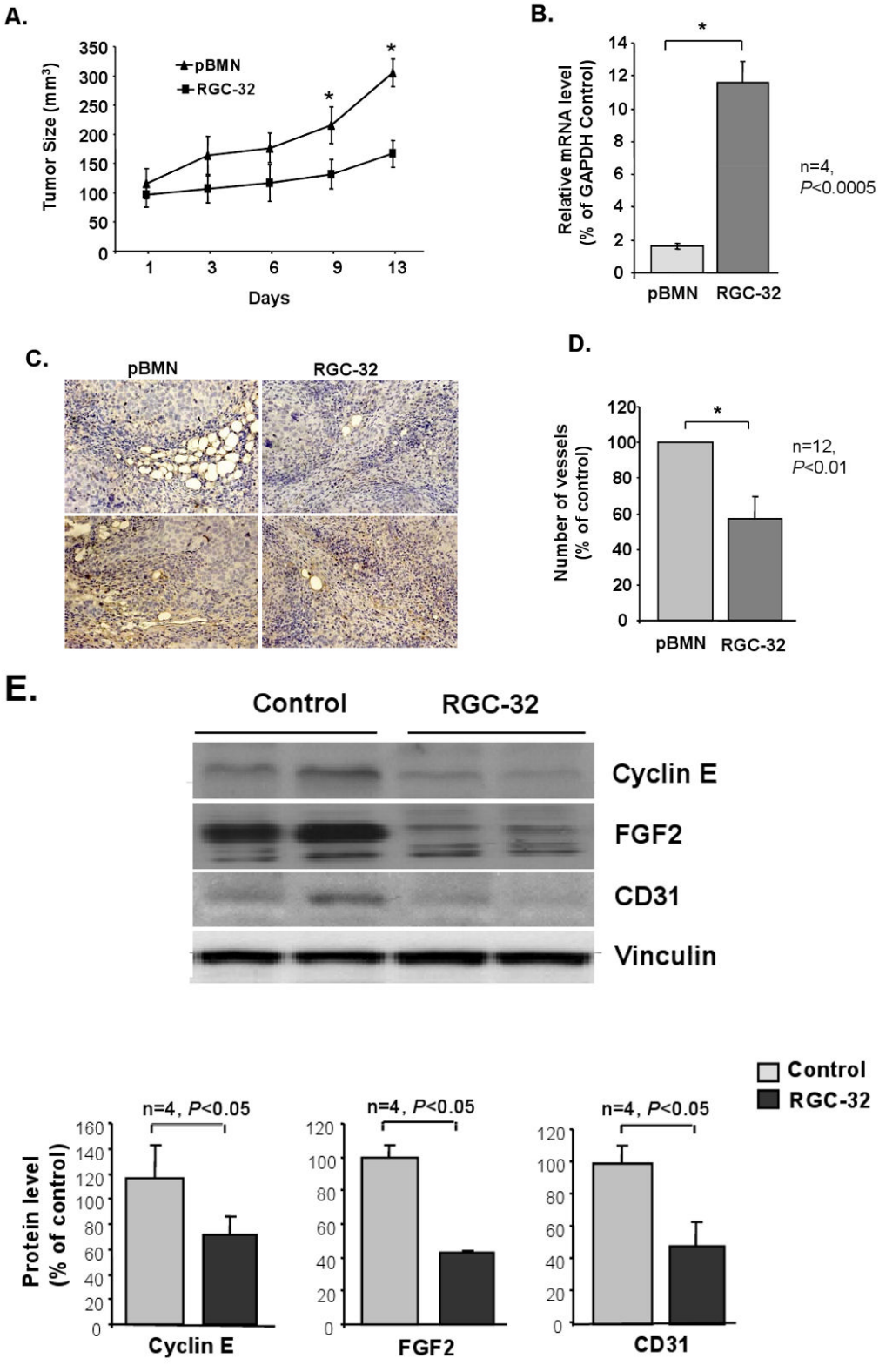


Figure 7. RGC-32 inhibited angiogenesis in SW480 melanoma tumor

(A) Nude mice bearing established SW480 xenograft were treated with control or RGC-32 retrovirus. Note: tumor size was significantly reduced as shown on day 13 after treatment. Data are means ± SEM, * $P<0.05$ n=4. (B) Q-PCR analysis shows increased RGC-32 mRNA expression in the RGC-32 virus treated tumor compared to the control (* n=4, $P<0.0005$). Representative view of tumor section with CD31 staining (C) and statistic analysis of vessel number with 12 HMF counts n=12; $P<0.01$ (D) on day 13 after treatment. (E) Western blotting indicated that FGF2, cyclin E and CD31 were downregulated in RGC-32 treated mice.