Role of Rac1 and Cdc42 in hypoxia induced p53 and von Hippel-Lindau suppression and HIF1α activation

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · JUNE 2006

Impact Factor: 5.09 · DOI: 10.1002/ijc.21763 · Source: PubMed

10 AUTHORS, INCLUDING:

Yanglin Pan
Fourth Military Medical University
68 PUBLICATIONS **1,824** CITATIONS

SEE PROFILE

Int. J. Cancer: **118,** 2965–2972 (2006)

Role of Rac1 and Cdc42 in hypoxia induced p53 and von Hippel-Lindau suppression and HIF1α activation

Yan Xue[1], Feng Bi[1]*, Xueyong Zhang[1]*, Siyuan Zhang[1], Yanglin Pan[1], Na Liu[1], Yongquan Shi[1], Xuebiao Yao[2], Yi Zheng[3] and Daiming Fan[1]

[1]*The State Key Laboratory of Cancer Biology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi Province, People's Republic of China*
[2]*Laboratory for Cell Dynamics, School of Life Science, University of Science and Technology of China, Hefei, Anhui Province, People's Republic of China*
[3]*Division of Experimental Hematology, Children's Hospital Research Foundation, University of Cincinnati, Cincinnati, OH, USA*

Low oxygen tension can influence tumor progression by enhancing angiogenesis, a process that may involve Rho GTPases whose activities have been implicated in tumorigenesis and metastasis. In the present study, we show that hypoxia can increase the mRNA levels and intracellular activities of Rac1 and Cdc42 in a time-dependent manner. The hypoxia-stimulated activities of Rac1 and Cdc42 could be blocked by the phosphatidylinositol 3′-kinase (PI3K) inhibitor LY294002 and the protein tyrosine kinase (PTK) inhibitor genistein but were not affected by the p38MAPK inhibitor SB203580 or the MEK-1 inhibitor PD98059, suggesting that the hypoxia-mediated signals were through PI3K and PTK. Correlating with the increased activities of Rac1 and Cdc42, the expression of the pro-angiogenesis factors HIF-1α and vascular endothelial growth factor (VEGF) was upregulated by hypoxia, whereas the expression of the tumor suppressors von Hippel-Lindau and p53 was down-regulated. Dominant negative N17Rac1 and N17Cdc42 could upregulate the expression of p53 and pVHL but downregulate that of HIF-1α and VEGF under hypoxia. Furthermore, the preconditioned medium from N17Rac1 or N17Cdc42-expressing gastric cancer cells was able to inhibit the proliferation of HUVECs. Our results indicate that PI3K and PTK-mediated activations of Rac1 and Cdc42 are involved in the hypoxia-induced production of angiogenesis-promoting factors and tumor suppressors, and suggest that the Rho family GTPases Rac1 and Cdc42 may contribute to the hypoxia-mediated angiogenesis.

Key words: Rho GTPases; Rac1/Cdc42; hypoxia; angiogenesis; tumor suppressors

Rho GTPases form a subfamily of the Ras superfamily of 20–30 kDa GTP-binding proteins that have been shown to regulate a wide spectrum of cellular functions, such as cytoskeletal reorganization, membrane trafficking, transcriptional regulation and cell growth control.[1] So far, more than 20 Rho subfamily members have been identified, and these can be further subdivided according to the distinction of their sequences and functions. The subgroups include proteins that are most similar to RhoA, those that are most similar to Rac1 and Cdc42 and those that lack intrinsic GTPase activity.[2] Recent studies have provided evidence suggesting that Rho GTPases are involved in many aspects of tumor development, including tumorigenesis,[3,4] metastasis,[5,6] cell-cycle control[7,8] and apoptosis.[9,10] However, the precise mechanisms underlying the involvement of Rho GTPases in 1 important area of cancer development, tumor angiogenesis, have not yet been defined.

A number of recent studies about the Rho GTPases involvement in angiogenesis have focused the attention on the role of the small GTPases in endothelial cell regulation. For example, Rho GTPases have been shown to influence endothelial cell migration and haptotaxis/chemotaxis.[11,12] But to date, the underlying mechanisms of the regulation and the effect of Rho GTPases during tumor angiogenesis remain elusive. Some studies suggested that in tumor angiogenesis, Rac1 is the critical factor among Rho GTPases,[13] while others considered RhoA or Cdc42 as the key player of tumor angiogenesis.[14,15]

Hypoxia, a common feature of malignant tumors, can be detected in the central region of solid tumors as well as during embryonic development.[16] Hypoxia regulates many transcription factors including hypoxia-inducible factor (HIF)-1α, which in turn controls the expression of hypoxia-induced angiogenic factors such as vascular endothelial growth factor (VEGF). One important function of VEGF is to act as a survival factor in endothelial[17] and tumor cells[18] *via* VEGF receptors that are upregulated by hypoxia. Thus, the enhancement of angiogenesis by hypoxia could be considered as a prequisite for the progressive growth and metastasis of solid tumors.

Here, we have investigated the contribution of Rho GTPases to the hypoxia-regulated angiogenesis-promoting factors and tumor suppressors production. We found that two prominent members of the Rho family GTPases, Rac1 and Cdc42, are hypoxia-inducible through the action of phosphatidylinositol 3′-kinase (PI3K) and protein tyrosine kinase (PTK). The dominant-negative forms of the small GTPases have potent-effect on the hypoxia-induced HIF-1α, VEGF, von Hippel-Lindau protein (pVHL) and p53 productions. We propose that Rac1/Cdc42 may have important roles in hypoxia-induced angiogenesis through the regulation of the angiogenesis-promoting factors and tumor suppressors as well as intracellular signaling events.

Material and methods

Antibodies and reagents

Anti-Rac1 (610651, 1:1000 dilution), anti-Cdc42 (610929,1:125 dilution) antibodies were purchased from BD Corporation (BD, NY, USA). Anti-RhoA (SC-418, 1:200 dilution), anti-p53 (SC-126, 1:1000 dilution), anti-HIF-1α (SC-12542, 1:200 dilution) antibodies and enhanced chemiluminescence (ECL) reagent were from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-VHL (MS-690-P0, 1:200 dilution) antibody was from Neomarkers Corporation (Taipei, China). Anti-β-actin antibody (C2206, 1:10,000 dilution), glutathione agarose beads and genistein were

Grant sponsor: National Outstanding Youth Foundation of China; Grant number: 30325039; Grant sponsor: Ministry of Science and Technology of China; Grant number: 2002CB713701; Grant sponsor: National Natural Science Foundation of China; Grant number: 30300125.

Feng Bi's current address is: Laboratory of Signal Transduction and Molecular Targeting Therapy, West China Hospital, Sichuan University, Chengdu 610041, People's Republic of China.

***Correspondence to:** The State Key Laboratory of Cancer Biology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi Province 710032, People's Republic of China. Fax: +86-29-82539041. E-mail: fengbi@fmmu.edu.cn

Received 23 March 2005; Accepted after revision 8 November 2005
DOI 10.1002/ijc.21763
Published online 4 January 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – PRIMERS AND REACTION PARAMETERS OF SEVEN MAIN RHO FAMILY MEMBERS FOR PCR

Products	Sequence	Annealing temperature (°C)	No. of cycles	Size (bp)
RhoA	5′-catccggaagaaactggt-3′ 5′-tcccacaaagccaactc-3′	54	28	168
RhoB	5′-cggactcgctggagaaca-3′ 5′-gaggtagtcgtaggcttggat-3′	57	30	206
RhoC	5′-ggctgcaatccgaaag-3′ 5′-gtccacttctcaggaatgtt-3′	55	30	297
Rac1	5′-agacggagctgtaggtaaaa-3′ 5′-atgcaggactcacaaggga-3′	54	28	236
Rac2	5′-cagcctcttatgagaacgtc-3′ 5′-cgaacacggttttcaggc-3′	57	32	249
Rac3	5′-acgggaaaccagtcaact-3′ 5′-gcagccgctcaatggt-3′	57	30	249
Cdc42	5′-cgatggtgctgttggt-3′ 5′-ccaacaagcaagaaagga-3′	55	28	312
β_2-microglobulin	5′-ctatccagcgtactccaa-3′ 5′-aagtcaacttcaatgtcgg-3′	54	26	118

from Sigma (Sigma, USA). Peroxidase-conjugated goat anti-mouse/rabbit IgG was from Zhong Shan Corporation (Amersham Pharmacia Biotech, Beijing, China). TRIZOL Reagent was from GIBCO (Invitrogen, Carlsbad, USA), RT-PCR kit was from MBI Corporation (MBI, CA, USA), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], LY294002, SB203580 and PD98059 were from Promega Corporation (Promega, USA).

Cell culture

Human gastric cancer cell lines AGS, KATOIII (ATCC, USA), MKN-45, MKN-28 (Cell Bank, Japan), SGC-7901 (Science Academe of China) and human hepatocellular carcinoma cell lines HepG2 (ATCC), SMMC-7721, HCC (Science Academe of China) were all preserved in our institute. Cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin and were passaged at 80–90% confluence with Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. Human umbilical vein endothelial cells (HUVECs, ATCC) were cultured in medium 199 with Earl's salts supplemented with 20% FCS, acidic FGF (20 ng/ml), gentamicin (5 μg/ml), and heparin (17.6 U/ml). For the hypoxic condition, tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA), which was flushed with 1% O_2, 5% CO_2 and 94% N_2, sealed and placed at 37°C.[19]

Cell transfection

AGS cells were plated in a 6-well plate and grown overnight to 70–80% confluence without antibiotics. Cells were transfected using Lipofectamine Plus (Invitrogen) and 1.6 μg of DNA per well in serum-free RPMI-1640 (Invitrogen), according to the manufacturer's instructions: 20 μl Plus Reagent were dissolved in 100 μl media and 12 μl Lipofectamine were dissolved in 100 μl media for each well. Nine wells were transfected respectively with dominant-negative mutant of RhoA, Rac1 or Cdc42 (pCEFL-GST-N19RhoA, pCEFL-GST-N17Rac1 and pCEFL-GST-N17Cdc42, 3 wells for each) expressing vectors, and 3 wells were transfected with pCEFL-GST-neo control plasmid. Five hours later, the transfection media was replaced with RPMI-1640 supplemented with 10% FBS. Selection for stable transfected cells with 800 μg/ml geneticin (Invitrogen) was started 2 days after transfection. After 2 weeks, geneticin was reduced to the maintenance dose of 400 μg/ml. Four weeks after transfection, these polyclonal, stable-transfected cells were trypsinized and transferred to T-75 flasks. Monoclones were picked in a 6-well plate and expanded for an additional 2 months.

Semi-quantitative RT-PCR

The total RNA of cells was extracted using Trizol reagent according to the manufacturer's protocol. The PCR primers and reaction parameters that were used for Rho family genes amplification are listed in Table I. The reaction conditions of PCR (*e.g.* RhoA) were as follows; Initial denaturation at 95°C for 4 min, 28 cycles of denaturation at 94°C for 40 sec, annealing at 54°C for 40 sec and extension at 72°C for 1 min in a Touchgene Gradient thermal cycler (Techne, Cambridge, UK). Appropriate cycles were chosen to assure the termination of PCR amplification before reaching stable stage in each reaction. Gene expression was presented by the relative yield of the PCR product from target sequences to that from the β_2-microglobilin gene. Mean values from 3 independent experiments were taken as results.

Western blotting

For whole-cell extracts, cells were washed with ice-cold PBS and collected by scraping. Cell pellets were homogenized in extraction buffer (50 mM Tris–HCl, 0.1% SDS, 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% NP-40 and 0.5% sodium orthovanadate), and incubated at 4°C for 20 min, centrifuged 20 min at 12,000 g/min. Protein levels in the extracts were quantified using the BioRad DC protein assay. For western blotting, whole cell extracts (50–100 μg/lane) were resolved in 8–12% SDS polycrylamide gels and transferred onto nitrocellulose membrane (0.45 μm, Millipore, USA) in 25 mM Tris-base, 190 mM glycine and 20% methanol using a semidry blotter. Membranes were blocked with 8% fat-free milk and 0.1% Tween-20 in TBS. Primary antibodies were used at the concentration recommended by the suppliers. Detection of monoclonal and polyclonal antibodies was performed using horseradish peroxidase-conjugated goat antimouse/antirabbit immunoglobulins, respectively, and ECL.

Pull-down assay

Active RhoA, Rac1 and Cdc42 in cell lysates (500 μg) were respectively precipitated with 15 μg GST-RBD (containing amino acids 8–89 of Rhotekin) or GST-PBD (containing amino acids 51–135 of PAK1), which was expressed in *E. coli* and bound to agarose beads. The precipitates were washed 3 times in washing buffer (50 mM Tris pH 7.2, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin), and the bound proteins were eluted by boiling for 5 min and resolved in 12% polyacrylamide gels, transferred to nitrocellulose membranes, and respectively immunoblotted with anti-RhoA, anti-Rac1 and anti-Cdc42 antibodies as described earlier. The experiments were carried out in triplicates.

Indirect angiogenic effects

Indirect angiogenic effects of Rho GTPases were estimated by effects on the production of angiogenic growth factor (VEGF) by tumor cell lines. Cells were plated onto 6-well plates at a density

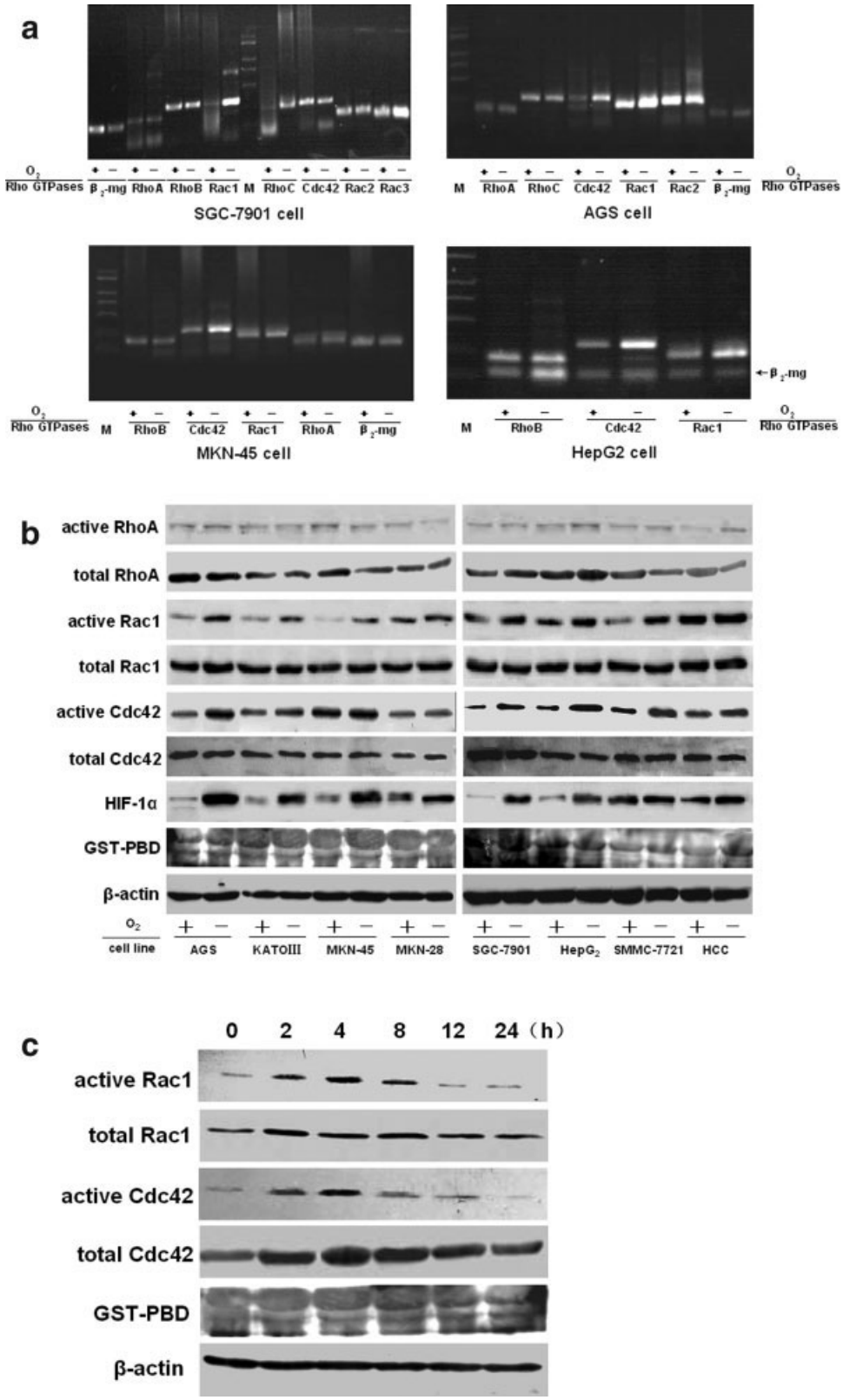

FIGURE 1 – Activity and expression of Rho GTPases are regulated by oxygen tension. (*a*) Transcription level of Rho GTPases was determined in AGS, MKN-45, SGC-7901 and HepG2 cells after exposure to hypoxia. Cells were incubated in conditions of normoxia (20% O_2, marked as '+') and hypoxia (1%O_2, marked as '−') for 4 hr, total RNA was extracted and semiquantitive RT-PCR was performed, β_2-microglobulin was taken as internal controls. Data suggested that mRNA expressions of Rac1 and Cdc42 were upregulated by 4 hr of hypoxia, other members of Rho GTPases did not show obvious sensitivity to hypoxia. (M: Marker) (*b*) Modulation of Rac1 and Cdc42 activities under hypoxia in different cancer cell lines. The activities of Rac1 and Cdc42 under hypoxia were investigated by GST-PBD pull-down assay in the following cancer cell lines (human gastric cancer cell lines AGS, KATOIII, MKN-45, MKN-28 and SGC-7901, human hepatocarcinoma cell lines HepG2, SMMC-7721 and HCC). Cells were incubated in conditions of normoxia (20%O_2, marked as '+') and hypoxia (1%O_2, marked as '−') for 4 hr, the activity of Rac1 was increased in AGS, KATOIII, MKN-45, SGC-7901 and SMMC-7721 cells and the activity of Cdc42 was increased in AGS, SGC-7901, HepG2 and SMMC-7721 cells in conditions of hypoxia. β-actin and GST-PBD were taken as internal controls. (*c*) Time kinetics of Rac1 and Cdc42 activity under hypoxia. AGS cells were incubated in conditions of hypoxia (1% O_2) for 0–24 hr, the activities of Rac1 and Cdc42 were analyzed by GST-PBD pull-down assay. The hypoxia-induced Rac1 and Cdc42 activities responded in a time-dependent manner with a maximum at 4 hours. β-actin and GST-PBD were taken as internal controls.

of 1×10^5 cells. After attachment overnight, media was aspirated off and serum-free RPMI-1640 were added to the wells. The cells were incubated for a total of 48 hr under normoxic or hypoxic conditions. After 0–36 hr, the supernatant was centrifuged at 2,000 rpm for 5 min to remove debris, aliquoted out and stored at −80°C until further use. The ELISA for VEGF was conducted according to R&D systems duo-set protocols.[20] Plates were read on a plate reader at 405 nm. VEGF production per cell was calculated from the standard curves obtained. Data were expressed in pg VEGF/10^5 cells/ml.

HUVEC isolation

HUVEC were isolated from umbilical cords, obtained from the Department of Obstetrics, Xijing Hospital, Xi'an, by the collagenase perfusion technique.[21] Briefly, the umbilical vein was cannulated and 5 ml of prewarmed type 1 Collagenase (Sigma, USA) was infused through it. The free end of the cord was clamped and then incubated at 37°C in a 5% CO_2 environment. The vein was flushed with 20 ml of HUVEC medium. The isolated endothelial cells were centrifuged at 1,500 rpm for 5 min and then the pellet was resuspended in 5 ml of HUVEC medium and plated on a pregelatinized tissue culture flask. Cells isolated from at least 2 umbilical cords were pooled for further culture. Cells for experiments were used between passage 2 and 6.

Proliferation assay

HUVEC cells were cultured in a standard medium with 90–100% confluence, then trypsinized, seeded on 96-well plates and allowed to attach for 24 hr, as described earlier. Medium was then removed and replaced by the medium previously pre-conditioned by different cells as described earlier. After 24 hr, MTT assay was performed to assess the proliferation of HUVECs.

Statistical analysis

Statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by LSD multiple comparisons tests through using statistical SPSS software package (SPSS, Chicago, USA), and the difference was considered significant when $p < 0.05$.

Results

Modulation of Rho GTPases activities by hypoxia

To examine the potential effect of hypoxia on Rho GTPase, the mRNA expression levels of a few prominent Rho GTPases in 4 cancer cell lines, *i.e.*, human gastric cancer cell line AGS, MKN-45, SGC-7901 and human hepatocellular carcinoma cell line HepG2 were measured by semi-quantitive RT-PCR under normoxic or hypoxic conditions. Comparing to normoxia, hypoxia could significantly increase the mRNA expression of *Rac1* and *Cdc42*, but not other Rho GTPases examined, including *RhoA*, *RhoB*, *RhoC*, *Rac2* and *Rac3* (Fig. 1*a*). The effect of hypoxia on the expressions and activities of Rac1 and Cdc42 were further examined in the following cancer cell lines: human gastric cancer cell line AGS, KATOIII, MKN-45, MKN-28, SGC-7901 and human hepatocellular carcinoma cell line HepG2, HCC and SMMC-7721. As shown in Figure 1*b*, the activity of Rac1 was increased in AGS, KATOIII, MKN-45, SGC-7901 and SMMC-7721 cells and the activity of Cdc42 was increased in AGS, SGC-7901, HepG2 and SMMC-7721 cells in conditions of hypoxia. Among them, the Rac1 and Cdc42 activities of gastric cancer cell line AGS showed the most sensitivity to hypoxia. Moreover, accumulations of HIF-1α protein are observed in most cell lines (AGS, KATOIII, MKN-45, MKN-28, SGC-7901 and HepG2 cells) by 4 hr of hypoxia, similarly, the expression of HIF-1α in AGS cells also showed the most sensitivity to hypoxia (Fig. 1*b*). When the changes of Rac1 and Cdc42 activities in AGS cells were evaluated over time, the hypoxia-induced activities increased at 2 hr timepoint after hypoxia induction, reaching a maximum at 4 hr and coming back to the basal level after 8 hr (Fig. 1*c*). These results indicate that hypoxia could result in the activation of the Rho GTPases Rac1 and Cdc42 at both the mRNA and protein activity levels, and Rac1 and Cdc42 might be involved in oxygen-regulated gene expression and hypoxia-induced angiogenesis.

Effect of hypoxia on the expression of angiogenesis-promoting factors and tumor suppressors

Angiogenesis is a complex process that occurs in a series of interrelated steps and involves the release of pro-angiogenic factors. One of the most important angiogenic factors is VEGF.

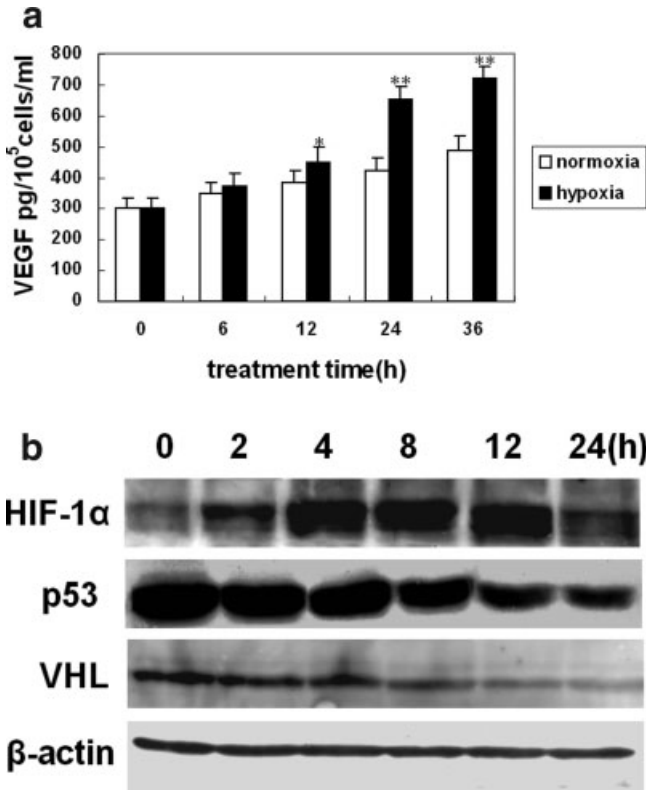

Figure 2 – Effect of hypoxia on the protein expression of VEGF, HIF-1α, p53 and VHL. (*a*) Effect of hypoxia on the production of VEGF in pre-conditioned medium. AGS cells were incubated for the indicated time (0–36 hr) in normoxic (21% O_2) or hypoxic (1% O_2) conditions. After the incubation, the medium was recovered for VEGF assay by ELISA and the cell number was counted. Results were expressed in pg VEGF/10^5cells/ml and presented as mean ± SD ($N = 6$). * indicates $p < 0.05$, ** indicates $p<0.01$ *vs* corresponding normoxia. (*b*) Effect of hypoxia on the protein expression of HIF-1α, p53 and VHL. AGS cells were incubated in 1% O_2, 5% CO_2 and 94% N_2 for 0–24 hr, cell proteins were analyzed by Western blotting. The expression of HIF-1α increased in a time-dependent manner and diminished after 12 hr. Oppositely, p53 and VHL were significantly reduced by hypoxia. The decreases of p53 and VHL were evident at 8 hr.

VEGF regulates both vascular endothelial cell migration, proliferation and permeability, and functions as an anti-apoptotic factor for newly formed blood vessels.[22] To study the effect of hypoxia on the regulation of VEGF, we measured VEGF concentration in the cell culture medium of AGS cells over time (0–36 hr), in conditions of normoxia or hypoxia. As shown in Figure 2*a*, VEGF production/secretion to the cell culture medium was increased in the cells under hypoxia in a time-dependent manner, with the significant difference after 12 hr treatment comparing to normoxia. To investigate the underlying mechanism, the expression of transcription factor HIF-1α, known to be responsible for VEGF regulation, was studied by Western blotting. Figure 2*b* shows that the expression of HIF-1α was very weak under normoxia and increased as early as 2 hr after exposure to 1% O_2, reached a peak at 4 hr and diminished after 12 hr, suggesting that hypoxia affects HIF-1α generation that precedes the VEGF induction.

In contrary to VEGF and HIF-1α, the tumor suppressor gene products p53 and VHL have inhibitory effects on angiogenesis.[23–28] Mutation or loss of function of either of them is associated with highly vascularized malignant tumors. p53 inhibits HIF-1 activity by targeting the HIF-1α subunit for Mdm2-mediated ubiquitination and proteasomal degradation.[23] p53 can also inhibit hypoxia-inducible factor stimulated transcription.[24] Conversely, the loss of

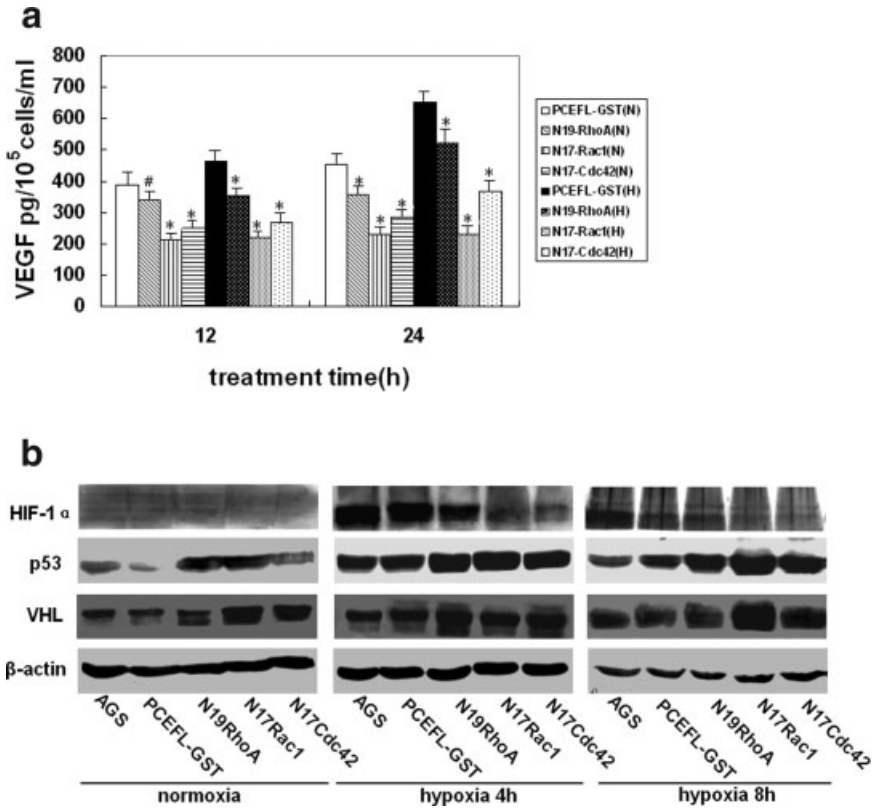

FIGURE 3 – Regulation of hypoxia-responsive factors by Rho GTPases. (*a*) AGS cells were incubated for the indicated time in normoxic (21% O_2) or hypoxic (1% O_2) conditions. After the incubation, the medium was recovered for VEGF assay by ELISA and the cell number was counted. Results were expressed in pg VEGF/10^5cells/ml and presented as mean ± SD ($N = 6$). N: normoxia, H: hypoxia. # indicates $p < 0.05$, ** indicates $p < 0.01$ *vs* corresponding AGS/PCEFL-GST. (*b*) Regulation of hypoxia-responsive angiogenesis-related moleculars by Rho GTPases. Cells were incubated in 1% O_2, 5% CO_2 and 94% N_2 for 0–24 hr, cell lysis were analyzed by Western blotting. In contrast to the suppressed levels of HIF-1α, expression of p53 and VHL was significantly upregulated by N19RhoA, N17Rac1 and N17Cdc42. Compared to N17Rac1 and N17Cdc42, however, N19RhoA did not induce significant change in the expression of HIF-1α under hypoxia.

p53 enhances hypoxia-induced HIF-1α levels and augments HIF-1-dependent expression of VEGF in tumor cells. VHL disease is a hereditary cancer syndrome characterized by the development of highly vascular tumors that overproduce hypoxia-inducible mRNAs such as *VEGF*.[25] The product of the *VHL* tumor suppressor gene, pVHL, is a component of a multiprotein complex that bears structural and functional similarity to SCF (Skp1/ Cdc53 or Cullin/F-box) ubiquitin ligases. In the presence of oxygen, pVHL, in association with elongin B and elongin C, binds directly to HIF-1α subunits and targets them for polyubiquitination and destruction. Cells lacking functional pVHL can not degrade HIF and thus overproduce mRNAs encoded by HIF target genes.[26,27] When the AGS cells were grown under hypoxia, in contrast to VEGF and HIF-1α, the expression of p53 and VHL were reduced in a time-dependent manner. The decrease of p53 or VHL was most evident at 8 hr after hypoxia induction (Fig. 2*b*). Together, these data suggest that hypoxia can promote the induction of HIF-1α and VEGF and a concurrent suppression of p53 and VHL.

Regulation of hypoxia-responsive genes by Rho GTPases

To examine if the observed elevation of Rho GTPase activities by hypoxia might be involved in the hypoxia-induced gene induction, we transfected AGS cells with vectors expressing dominant-negative mutant form of RhoA, Rac1 or Cdc42 (N19RhoA, N17Rac1 and N17Cdc42, respectively). Expression of N17Rac1 or N17Cdc42 resulted in the significant decrease of HIF-1α and VEGF and an upregulation of p53 and VHL production under hypoxia (Figs. 3*a* and 3*b*). Compared with N17Rac1 or N17Cdc42- expressing cells, however, N19RhoA did not induce significant changes in the expression of HIF-1α under hypoxia while displaying an ability to inhibit VEGF production (Fig. 3*a*) and stimulate p53 and VHL generation (Fig. 3*b*). Among the 3 Rho GTPases, Rac1 appeared to be the most effective in regulating the hypoxia-responsive factors, HIF-1α, VEGF, p53 and VHL production.

Involvement of PI3 kinase and protein tyrosine kinase in hypoxia-induced activities

To investigate the potential signal transduction pathways regulating Rho GTPases in the hypoxia-induced activities, we utilized 50 μm LY294002, a PI3K inhibitor, 50 μm genistein, a tyrosine kinase inhibitor, 20 μm PD98059, a MEK1 inhibitor and 10 μm SB203580, a p38 MAPK inhibitor to treat the cells and followed by the examination of the activity of Rho GTPases and the expression of HIF-1α. As shown in Figure 4, LY294002 and genistein suppressed hypoxia-induced HIF-1α expression, while PD98059 and SB203580 could only partially inhibit HIF-1α expression. Moreover, LY294002 and genestein also inhibited Rac1 and Cdc42 activities in response to hypoxia. In contrast, neither PD98059 and SB203580 attenuated their activities in response to hypoxia. These results are in accordance with the finding of Hirota and Semenza[29] and suggest that both protein tyrosine kinase and PI3-kinase are required for the hypoxia-induced activation of the Rac1/Cdc42 and HIF-1α expression.

Effect of Rho GTPases on HUVEC cells proliferation

Previous studies suggest that Rho family GTPases can regulate the intracellular pathways as well as the extracellular matrix compositions critical for endothelial cell migration, proliferation and tube formation.[12] Next, we used the conditioned medium (CM) derived from N19RhoA, N17Rac1 or N17Cdc42-expressing cells to examine the effect of the Rho protein-mediated extracellular components on the proliferation rate of the vascular endothelial cells, HUVECs. To exclude the influence caused by the cell numbers in different group, the growth rates of the 3 dominant-negative mutants stable-transfected cell lines (N19RhoA, N17Rac1 and N17Cdc42) were studied. Results showed that the growth rates of 3 dominant-negative mutants had no difference in 48 hr compared with parental cell line (AGS) and empty vector transfectant (AGS-PCEFL-GST), although they had difference after 3 days.[30,31] HUVEC cells grown in serum-free medium condi-

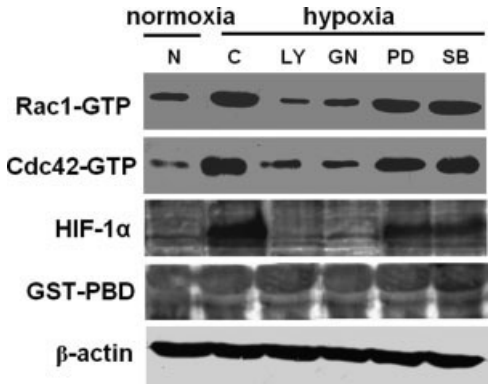

FIGURE 4 – Effect of inhibitors on hypoxia-induced Rho GTPases activation and HIF-1α expression. AGS cells were pretreated with 50 μm LY294002 (LY, a PI3K inhibitor), 50 μm genistein (GN, a PTK inhibitor), 20 μm PD98059 (PD, a MEK-1 inhibitor), 10 μm SB203580 (SB, a P38MAPK inhibitor) and then exposed to 20 or 1% O_2 for 4 hr prior to whole cell lysate preparation. Hundred micrograms of aliquots were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting using anti-HIF-α antibody. For detection the activity of Rho GTPases, AGS cells were exposed to 1% O_2 for 4 hr and harvested. Lysates were subjected to pull-down assay (N, normoxia; C, control).

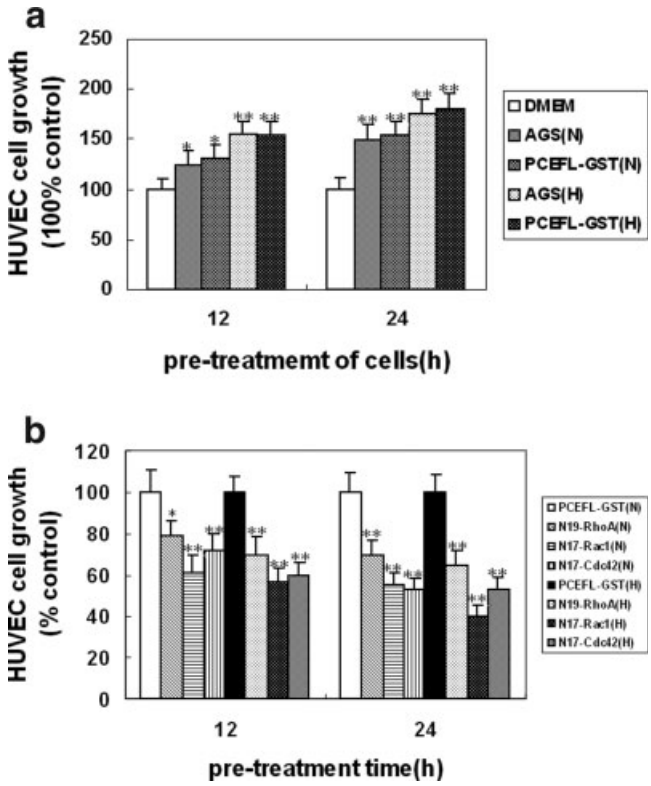

FIGURE 5 – Effect of pre-conditioned media (obtained from N19RhoA, N17Rac1, N17Cdc42 transfected AGS cells) on HUVEC cells proliferation. (*a*) HUVECs were grown to 70–80% confluence and were then exposed to preconditioned media (derived from AGS and AGS/PCEFL-GST cells pre-treated in conditions of normoxia and hypoxia for 12 to 24 hr) for 24 hr and serum-free DMEM was taken as control. N: normoxia, H: hypoxia. Mean ± SD, $N = 6$, * indicates $p < 0.05$; ** indicates $p < 0.01$ *vs* corresponding control. (*b*) HUVECs were grown to 70–80% confluence and were then exposed to pre-conditioned media (derived from PCEFL-GST or N19-RhoA, N17-Rac1 and N17-Cdc42 transfected AGS cells pre-treated in conditions of normoxia and hypoxia for 12 or 24 hr) for 24 hr. N: normoxia, H: hypoxia. Mean ± SD, $N = 6$, * indicates $p < 0.05$; ** indicates $p < 0.01$ *vs* corresponding AGS/PCEFL-GST.

tioned by gastric cancer cells AGS and PCEFL-GST transfected AGS cells showed a significant increase in the proliferation rate, directly proportional to the length of conditioning the medium with cells (Fig. 5*a*). In contrast to this, the HUVEC cells grown in serum-free medium conditioned by N19RhoA, N17Rac1, or N17Cdc42 cells showed a significant decrease in the proliferation rate, correlating to the time of conditioning the medium (Fig. 5*b*). These results show that the Rho GTPases may be involved in the generation of extracellular components of the cancer cells important for endothelial cell growth.

Discussion

Angiogenesis is a complex process involving endothelial cell (EC) migration, proliferation and tube formation. In this process, the cytoskeleton dynamics regulated by Rho GTPases may control EC movement and organization of cell-to-matrix and cell-to-cell contacts. Previous studies have shown that on the one hand VEGF signaling required Rac1 activation during chemotaxis and Rac1 and Cdc42 were activated during haptotaxis on type I collagen,[13] on the other hand, Rac1 activation induced an increase in endothelial cell-stress fiber and focal adhesion and was required for the activation of endothelial cell haptotaxis. Other studies have also suggested that VEGF-induced actin cytoskeletal changes in ECs require RhoA and Rho kinase, and the activation of RhoA-Rho kinase signaling is involved in the VEGF-induced EC migration and angiogenesis *in vitro*.[14] Moreover, it has been known that many stimuli such as EphrinA1, Sphingosine 1-phosphate, tissue factor, NSAIDs, cerivastatin and reactive oxygen species[32–34] could regulate angiogenesis through modulation of the Rho GTPases Rac1, Cdc42 and RhoA, further implicating the Rho GTPases as important regulators of tumor angiogenesis.

VEGF is one of the most potent angiogenesis stimulating factors. Enhanced expression of VEGF has been observed in many human cancers including colorectal, breast, non-small cell lung and ovarian cancers.[35] The *VEGF* gene and several other related genes regulated by hypoxia are under the control of the transcription factor HIF-1.[36] HIF-1 is composed of 2 subunits of the basic helix-loop-helix PAS domain protein family, HIF-1α and HIF-1β/ARNT.[37] The mRNA levels of HIF-1α are similar in normoxia and hypoxia, but undergo an O_2-dependent degradation under non-hypoxic conditions *via* the ubiquitin−proteasome pathway.[38] Under hypoxic conditions, however, HIF-1α protein translocates and accumulates to the nucleus, where it forms an active complex with HIF-1β and activates transcription of target genes.

Our current studies provide evidence that hypoxia can significantly increase the secretion of VEGF in the cell culture medium and 1 possible mechanism of this might be through the upregulation of HIF-1α and downregulation of p53 and VHL (Fig. 2). HIF-1α can stimulate the transcription of VEGF gene while p53 and VHL can inhibit the expression of VEGF and promote the degradation of HIF-1α.

Our results also suggest that hypoxia can significantly increase the mRNA expression and the activities of Rac1 and Cdc42 (Fig. 1). However, little change was shown in the total Rac1 or Cdc42 protein levels. The 2 possible explanations are: (i) post-transcriptional or post-translational modification and degradation; (ii) the Western blotting analysis may not be sensitive enough to detect the differences. Moreover, dominant-negative N17Rac1 and N17Cdc42 significantly down-regulated the expression of VEGF and HIF-1α while up-regulated the expression of p53 and VHL (Fig. 3), indicating that Rac1 and Cdc42 acted as the key mediators of the hypoxia-regulated production of the pro-angiogenesis factors VEGF and HIF-1α and the tumor suppressors p53 and VHL. The fact that the conditioned medium from N17Rac1 and N17Cdc42 expressing gastric cancer AGS cells could inhibit

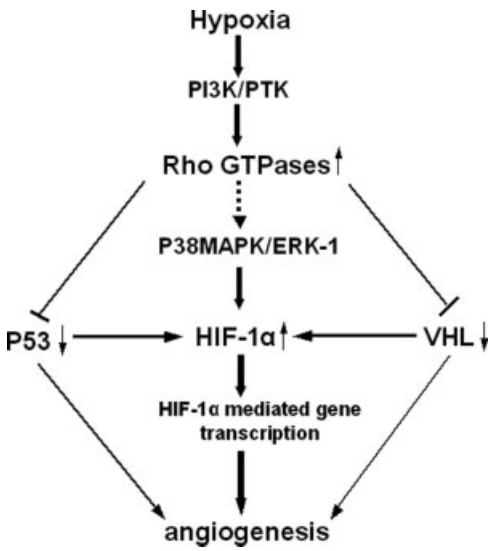

FIGURE 6 – Signal transduction pathways leading to hypoxia-induced tumor angiogenesis.

the proliferation of vascular endothelial cells (Fig. 5) further suggesting that Rho GTPases may be involved in the generation of extracellular components of the cancer cells which are important for endothelial cell growth. Together, our findings may shed new light on the role of Rac1 and Cdc42 in the hypoxia-induced angiogenesis.

Loss of wild type *p53* and *VHL* contributes to the development of angiogenic phenotype by stimulating the production of VEGF, the inducer of angiogenesis.[39,40] Recent studies showed that p53 and VHL could inhibit VEGF expression by regulating the transcriptional activity of Sp1 or by down-regulating the Src kinase activity, under both normoxic and hypoxic conditions.[41,42] Furthermore, VHL can promote the degradation of HIF-1α by ubiquitin-mediated proteolysis[26] and p53 can suppress HIF-1α-stimulated transcription,[24] loss of p53 function could amplify HIF-1α−dependent responses to hypoxia.[43] Our experiments using the dominant-negative forms of Rho GTPases in hypoxia indicate that Rho GTPases could influence HIF-1α and VEGF expression, possibly by the regulation of p53 and VHL (Fig. 3). Another possibility is that the Rho GTPases may be able to directly control the transcriptional regulation of HIF-1α and VEGF independent of p53 or VHL regulation. Further studies might be needed to explore the mechanism in detail.

In addition to changes in cellular redox,[44,45] hypoxia signal transduction may also require kinase/phosphatase activity.[46] In certain cell types, PI3K activity may also be needed for hypoxia-induced HIF-1α expression.[47,48] Reporter assays involving expression of constitutively activative or dominant-negative PI3K or Akt demonstrated that the PI3K/Akt pathway modulates hypoxia-induced HIF-1 activation and induces HIF-1 activity in non-hypoxic cells. Thus, the signaling pathway from the putative O_2 sensor to HIF-1 may contain several intermediate molecules. The inhibitory effects of LY294002 and genistein on the activation of Rac1/Cdc42 (Fig. 4) indicate that Rac1/Cdc42 are downstream components of these hypoxia-induced signal transduction network and that activation of Rac1/Cdc42 may represent an intermediate step in the process. In contrast, the p38MAPK and MEK-1 could not affect the activity of Rac1/Cdc42, only partially affected HIF-1α expression. They might be either acting downstream of Rac1/Cdc42 or independent of this process (Fig. 4). Further delineation of both the upstream signals responsible for Rac1/Cdc42 activation as well as the downstream signals leading to angiogenesis in response to hypoxia will be our future goal (Fig. 6). It is possible that specific Rho GTPase targeting may have important therapeutic values for cancer therapy, and that individual Rho GTPases might be useful targets in developing angiogenic inhibitors.

References

1. Nobes C, Hall A. Regulation and function of the Rho subfamily of small GTPases. Curr Opin Genet Dev 1994;4:77–81.
2. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, et al. Initial sequencing and analysis of the human genome. Nature 2001; 409:860–921.
3. Qiu RG, Chen J, McCormick F, Symons M. A role for Rho in Ras transformation. Proc Natl Acad Sci USA 1995;92:11781–5.
4. Roux P, Gauthier-Rouviere C, Doucet-Brutin S, Fort P. The small GTPases Cdc42Hs, Rac1 and RhoG delineate Raf-independent pathways that cooperate to transform NIH3T3 cells. Curr Biol 1997;7: 629–37.
5. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000;406:532–5.
6. Ridley AJ. Rho GTPases and cell migration. J Cell Sci 2001;114: 2713–22.
7. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–12.
8. Robles AI, Rodriguez-Puebla ML, Glick AB, Trempus C, Hansen L, Sicinski P, Tennant RW, Weinberg RA, Yuspa SH, Conti CJ. Reduced skin tumor development in cyclin D1-deficient mice highlights the oncogenic ras pathway in vivo. Genes Dev 1998;12:2469–74.
9. Coleman ML, Olson MF. Rho GTPase signaling pathways in the morphological changes associated with apoptosis. Cell Death Differ 2002; 9:493–504.
10. Pervaiz S, Cao J, Chao OS, Chin YY, Clement MV. Activation of the Rac GTPase inhibits apoptosis in human tumor cells. Oncogene 2001; 20:6263−8.
11. Soga N, Namba N, McAllister S, Cornelius L, Teitelbaum SL, Dowdy SF, Kawamura J, Hruska KA. Rho family GTPases regulate VEGF-stimulated endothelial cell motility. Exp Cell Res 2001;269:73–87.
12. Cascone I, Giraudo E, Caccavari F, Napione L, Bertotti E, Collard JG, Serini G, Bussolino F. Temporal and spatial modulation of Rho GTPases during in vitro formation of capillary vascular network. Adherens junctions and myosin light chain as targets of Rac1 and RhoA. J Biol Chem 2003;278:50702–13.
13. Soga N, Connolly JO, Chellaiah M, Kawamura J, Hruska KA. Rac regulates vascular endothelial growth factor stimulated motility. Cell Commun Adhes 2001;8:1–13.
14. van Nieuw Amerongen GP, Koolwijk P, Versteilen A, van Hinsbergh VW. Involvement of RhoA/Rho kinase signaling in VEGF-induced endothelial cell migration and angiogenesis in vitro. Arterioscler Thromb Vasc Biol 2003;23:211–7.
15. Dormond O, Foletti A, Paroz C, Ruegg C. NSAIDs inhibit αVβ3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis. Nat Med 2001;7:1041–7.
16. Ryan HE, Lo J, Johnson RS. HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005–15.
17. Nor JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 1999;154:375–84.
18. Baek JH, Jang JE, Kang CM, Chung HY, Kim ND, Kim KW. Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis. Oncogene 2000;19: 4621–31.
19. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93:266–76.
20. Ishibashi H, Shiratuchi T, Nakagawa K, Onimaru M, Sugiura T, Sueishi K, Shirasuna K. Hypoxia-induced angiogenesis of cultured human salivary gland carcinoma cells enhances vascular endothelial growth factor production and basic fibroblast growth factor release. Oral Oncol 2001;37:77–83.
21. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745–56.
22. Martiny-Baron G, Marme D. VEGF-mediated tumour angiogenesis: a new target for cancer therapy. Curr Opin Biotechnol 1995;6:675–80.
23. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. Genes Dev 2000;14:34–44.

24. Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, Neckers L. p53 inhibits hypoxia-inducible factor-stimulated transcription. J Biol Chem 1998;273:11995–8.
25. Stratmann R, Krieg M, Haas R, Plate KH. Putative control of angiogenesis in hemangioblastomas by the von Hippel-Lindau tumor suppressor gene. J Neuropathol Exp Neurol 1997;56:1242–52.
26. Pal S, Claffey KP, Dvorak HF, Mukhopadhyay D. The von Hippel-Lindau gene product inhibits vascular permeability factor/vascular endothelial growth factor expression in renal cell carcinoma by blocking protein kinase C pathways. J Biol Chem 1997;272:27509–12.
27. Krieg M, Haas R, Brauch H, Acker T, Flamme I, Plate KH. Up-regulation of hypoxia-inducible factors HIF-1α and HIF-2α under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene 2000;19:5435–43.
28. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res 1999;59:5830–5.
29. Hirota K, Semenza GL. Rac1 activity is required for the activation of hypoxia-inducible factor 1. J Biol Chem 2001;276:21166–72.
30. Liu N, Bi F, Pan Y, Sun L, Xue Y, Shi Y, Yao X, Zheng Y, Fan D. Reversal of the malignant phenotype of gastric cancer cells by inhibition of RhoA expression and activity. Clin Cancer Res 2004; 10(18 Part 1):6239–47.
31. Xue Y, Bi F, Zhang XY, Liu N, Pan YL, Shi Y. The effect of Rho GTPases on tumor proliferation. Chin J Biochem Mol Biol 2004;20: 664–9.
32. Deroanne C, Vouret-Craviari V, Wang B, Pouyssegur J. EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. J Cell Sci 2003;116:1367–76.
33. Jung ID, Lee J, Yun SY, Park CG, Choi WS, Lee HW, Choi OH, Han JW, Lee HY. Cdc42 and Rac1 are necessary for autotaxin-induced tumor cell motility in A2058 melanoma cells. FEBS Lett 2002;532: 351–6.
34. Okamoto H, Yatomi Y, Ohmori T, Satoh K, Matsumoto Y, Ozaki Y. Sphingosine 1-phosphate stimulates G(i)- and Rho-mediated vascular endothelial cell spreading and migration. Thromb Res 2000;99:259–65.
35. Gasparini G, Harris AL. Clinical implications of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. J Clin Oncol 1995;13:765−82.
36. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1α. Nature 1998;392:405–8.
37. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 1995;92:5510–4.
38. Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.
39. Zietz C, Rossle M, Haas C, Sendelhofert A, Hirschmann A, Sturzl M, Lohrs U. MDM-2 oncoprotein overexpression, p53 gene mutation, and VEGF up-regulation in angiosarcomas. Am J Pathol 1998;153: 1425–33.
40. Wizigmann-Voos S, Breier G, Risau W, Plate KH. Up-regulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res 1995;55:1358–64.
41. Mukhopadhyay D, Knebelmann B, Cohen HT, Ananth S, Sukhatme VP. The von Hippel-Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. Mol Cell Biol 1997;17:5629–39.
42. Pal S, Datta K, Mukhopadhyay D. Central role of p53 on regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) expression in mammary carcinoma. Cancer Res 2001;61: 6952–7.
43. Schmid T, Zhou J, Kohl R, Brune B. p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). Biochem J 2004;380:289–95.
44. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci USA 1998;95:11715–20.
45. Agani FH, Pichiule P, Chavez JC, LaManna JC. The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. J Biol Chem 2000;275:35863–7.
46. Wang GL, Jiang BH, Semenza GL. Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1. Biochem Biophys Res Commun 1995;216:669–75.
47. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 1997;90:3322–31.
48. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, Semenza GL. Modulation of hypoxia-inducible factor 1α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60:1541–5.