

ROCK inhibition as a potential therapeutic target involved in apoptosis in hemangioma

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Abstract. Gene expression was examined in hemangiomas (HA), benign, birthmark-like tumors occurring in infancy, and confirmed in HA-derived endothelial cells (HDEC), for which cell proliferation and apoptosis were also assessed. Protein and mRNA accumulation of Rho-associated protein kinase (ROCK), vascular endothelial growth factor (VEGF), Ki-67 and proliferating cell nuclear antigen was significantly higher in proliferating phase HAs than in involuting phase HAs. In contrast, p53 and caspase-3 exhibited higher levels of accumulation in involuting than proliferating HAs. Cell apoptotic indexes were low in proliferating phase HAs and increased in involuting phase HAs. HDECs were treated with the ROCK inhibitor Y-27632. Y-27632 induced p53 expression and downregulated VEGF expression, significantly inhibited cell proliferation, and induced cell apoptosis in HA cells. The inhibitor effects were confirmed in HAs from HDEC-injected nude mice. These results indicated that ROCK is involved in p53-mediated apoptosis and VEGF expression in HA cells and suggested that such inhibition may be exploited for future HA therapies.

Introduction

Hemangiomas (HAs) are benign vascular tumors affecting approximately 10% of all infants (1,2). They generally occur within the first few months after birth, usually on the head and neck. Infantile HAs characteristically increase in size (proliferative phase) for 4-6 months followed by a rest phase, before eventually shrinking (involuting phase). The involuting phase is typically longer than the proliferative phase, and may take up

to 10 years. Severe HAs occasionally result in tissue damage. Although HAs generally disappear without physical complications, they can cause significant psychological damage in school children when considerable disfigurement is present on the face. Several treatments are available including surgery and propranolol or oral corticosteroid therapy. The causes and mechanisms of HA development are currently unknown.

Propranolol, a non-selective β -blocker used to treat high blood pressure, has been used since 2008 to treat HAs. The therapeutic action of propranolol is believed to be the inhibition of blood vessel growth, as well as the constriction of existing blood vessels (3). This drug triggers apoptosis by interacting with β -adrenergic receptors and reducing the release of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, both known to stimulate blood vessel growth (4). Corticosteroid treatment has been shown to suppress VEGF secretion from HA-derived stem cells, and VEGF-A silencing in these cells reduced vasculogenesis *in vivo*.

The pathways controlling cell proliferation and apoptosis during the proliferative and involutive phases of HA are still to be elucidated. Apoptosis may be inhibited during the proliferative phase, as is the case in other proliferative diseases. For example, the knockdown of Livin, an inhibitor of apoptosis, inhibited cell growth and invasion of gastric cancer by blocking the MAPK pathway in cancerous cells (5). Whereas, during involution, the levels of apoptosis are thought to increase in HAs (6,7). Regarding HA proliferation, the role of VEGF has recently been reported. Knockdown of VEGF in primary HA-derived endothelial cells (HDEC) inhibited cell viability and induced apoptosis (8). Knockdown of VEGF has also been reported to decrease expression of p-AKT, p-ERK, p-p38MAPK and Ki-67 and increase expression of caspase-3 (9). Similarly, knockdown of insulin-like growth factor-II, a signaling molecule involved in cell growth, migration and differentiation, reduced proliferation and increased apoptosis in involuting HA cells, possibly by blocking the cell cycle regulation of the PI3K/AKT signaling transduction pathway (10).

It is, therefore, clear that the role and regulation of proliferation and apoptosis in the proliferating and involuting phases of HAs is complex. Insight may be provided by the use of specific pathway inhibitors. p53 has been shown to be involved in apoptosis, gene transcription, and downstream

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signaling (6,11,12). Y27632 is a highly potent ATP-competitive inhibitor of Rho-associated protein kinase (ROCK), a serine-threonine kinase implicated in various cellular functions including cytoskeleton organization and cell motility (13-15) and has been shown to play a role in tumorigenesis (16) and other diseases.

In this study, Y27632 was used to examine the effects on gene expression, proliferation and apoptosis in proliferating and involuting HAs. Our findings provide insight into the regulation of apoptosis in HA and potentially aid the development of therapeutic strategies.

Materials and methods

Samples. Freshly resected human HA samples were collected from the Department of General Surgery Affiliated with Xinhua Hospital and were classified according to the International Society for the Study on Vascular Anomalies criteria. Tissues and clinical information were obtained as part of an approved study at Shanghai Jiao Tong University School of Medicine. There were 49 cases of proliferating phase HAs and 47 cases of involuting phase HAs. A portion of each tissue sample was fixed with 10% formalin for immunohistochemical examination. All HA tissues were diagnosed by two independent pathologists.

The primary HDECs used in these experiments were from the Institute of Biochemistry and Cell Biology (Shanghai, China). All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study.

Cell culture and inhibitor studies. Proliferating phase HDECs were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc.), 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 g/ml streptomycin (Invitrogen). Cultures were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. For inhibitor studies, 1x10⁵ cells were treated with 2-10 μM Y-27632 (Sigma-Aldrich, St. Louis, MO, USA). Control samples were treated with phosphate-buffered saline (PBS). Recombinant VEGF-A was from R&D Systems (Minneapolis, MN, USA). Cells (1x10⁵) were stimulated with 200 ng/ml VEGF (R&D Systems). VEGF samples were treated with VEGF. A small interfering RNA (siRNA) that targeted p53 (siP53) or VEGF (siVEGF), and a negative control vector (siNC) were from Genechem (Shanghai, China). Cells (1x10⁵) were transfected with the following constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In some experiments, cells were transfected with siRNA for 24 h followed by treatment with Y-27632, a ROCK inhibitor.

Animals and tumor growth. Vascular tumors were established by subcutaneous injection of HDEC cells into the flanks of

4- to 6-week-old male nude mice (1x10⁷ cells per site, two sites per mouse, five mice per group). Mice were monitored daily, and three out of four mice developed a subcutaneous tumor. When the tumor size reached ~5 mm in length, it was surgically removed, cut into 1-2 mm³ pieces, and re-seeded individually into other mice on the right flanks. When tumor size reached ~5 mm in length, Y27632 was injected intraperitoneally daily. Negative control mice were injected with PBS. The mice were observed closely every day to monitor their general condition and to measure tumor size using a caliper. The mice were euthanized three weeks after treatment. Resulting HAs were measured and subjected to qRT-PCR and western blot analysis.

Immunohistochemical staining. Tissue sections were processed for immunohistochemical analysis of proteins as previously described (8). The following antibodies were used: anti-ROCK goat polyclonal IgG; anti-p53 goat polyclonal IgG; anti-VEGF goat polyclonal IgG; anti-Ki67 goat polyclonal IgG; anti-caspase-3 goat polyclonal IgG; and anti-PCNA goat polyclonal IgG (Santa Cruz Biotechnology, Dallas, TX, USA). Antigen unmasking was performed for VEGF and Ki-67 with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 30 min and was not necessary for the other proteins. Normal serum or PBS was used instead of antibodies in negative control samples.

Quantitative real-time PCR. Real-time PCR was performed to determine mRNA abundance. Total RNA was extracted from 1x10⁵ cells of proliferating phase HDECs or tumors using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was performed with 5 μg total RNA and an M-MLV reverse transcriptase (Promega, Madison, WI, USA) and cDNA amplification was performed using the SYBR Green Master Mix kit (Takara, Otsu, Japan), according to the manufacturer's instructions and the following primers: VEGF, 5'-ATCCAATCGAGACCCTGGTG-3' and 5'-ATCTCTCCTATGTGCTGGCC-3'; Ki-67, 5'-GGGTACCTGGTCTTAGTT-3' and 5'-ATGGTTGAGGCTGTTCC-3'; PCAN, 5'-TGATGAGGTCCTTGAGTG-3' and 5'-GAGTGGTCGTTGTCTTTC-3'; and, as a control, β-actin, 5'-AGCGAGCATCCCCCAAAGTT-3' and 5'-GGGCACGAAGGCTCATATT-3'. Real-time PCR assays were performed on an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA, USA). The expression of each gene was normalized to the β-actin gene. Relative mRNA levels were calculated by the ΔΔCt method. Three separate experiments were performed for each clone.

Western blot assay. Protein extracts were prepared from 1x10⁵ cells of proliferating phase HDECs or 30 μg of total protein as previously described (8). Briefly, cells were harvested, extracts were prepared and separated on SDS-PAGE gels. Proteins were transferred to membranes, which, after blocking, were incubated with the diluted antibodies described above. Horseradish peroxidase-linked secondary antibodies were added to the membranes at a dilution ratio of 1:1000, and incubated. After washing, immunoreactive bands were visualized using the ECL-PLUS kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Western blot signals were quantitated using 1D

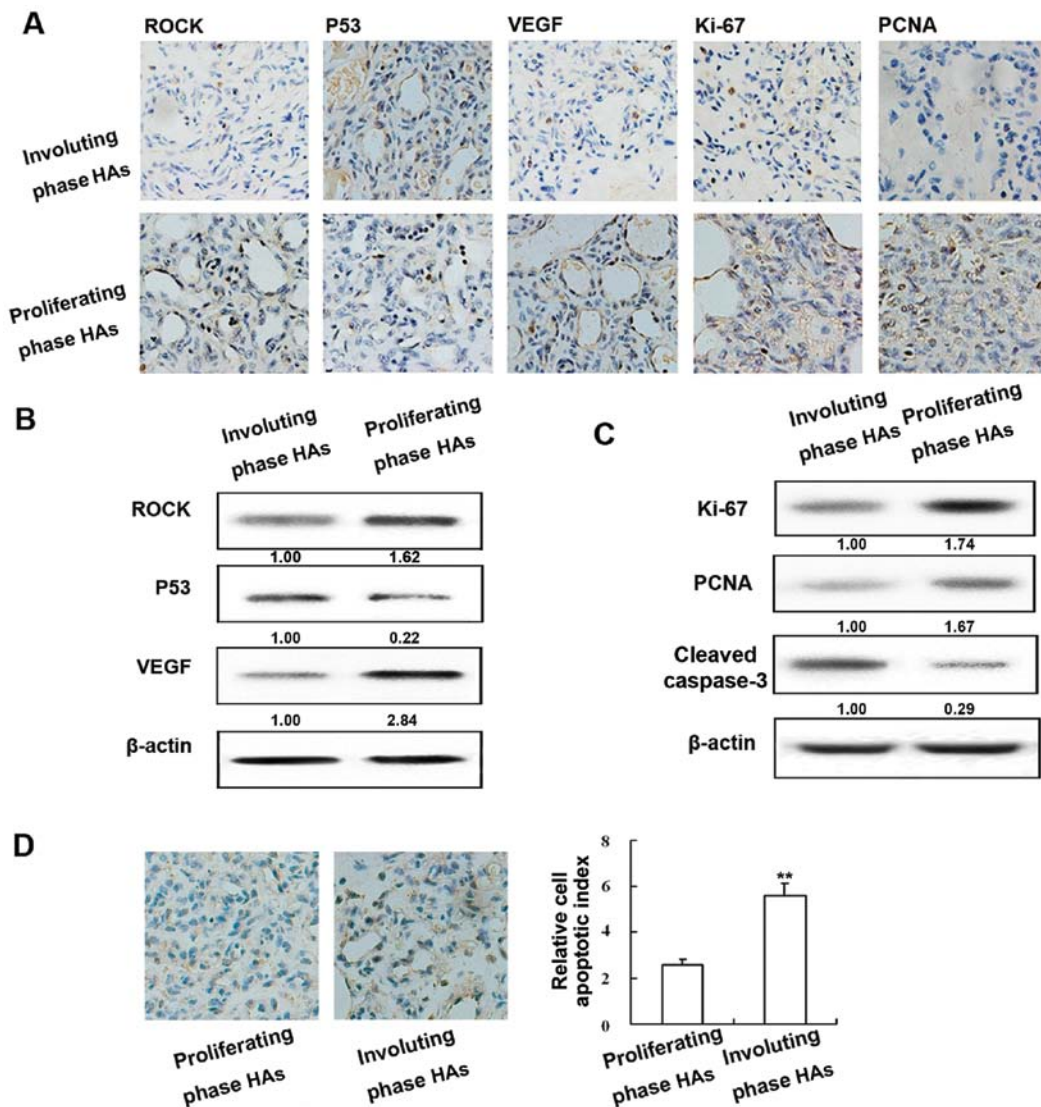


Figure 1. Expression of ROCK, p53, VEGF, Ki-67, PCNA and β -actin in HAs, according to phase. Expression was examined by (A) immunohistochemical staining (x400), ROCK, p53, VEGF, Ki-67, PCNA and cleaved caspase-3 protein levels were examined by (B and C) western blot analysis and (D) a TUNEL assay (x400) was performed to determine the apoptotic indices (** $P < 0.01$).

Image Analysis Software (Eastman Kodak, Rochester, NY, USA), and the relative protein abundance was normalized to β -actin levels. Three separate experiments were performed for each clone.

TUNEL assay. Apoptosis was detected by the TdT-mediated dUTP nick end labeling (TUNEL) method with 1×10^5 cells as previously described (8). Briefly, sections were dewaxed, incubated with blocking solution and permeabilized before detecting apoptosis using an *in situ* cell death kit (Boehringer Mannheim, Mannheim, Germany). Positive cells were visualized by fluorescence microscopy. As a control, the reaction mixture was incubated without enzyme to detect nonspecific staining. The apoptotic index was calculated from the ratio of the number of positively stained tumor cells to the total number of tumor cells counted per section.

Cell viability assay. Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, proliferating phase HDECs

treated with $10 \mu\text{M}$ Y-27632 or PBS as control were incubated in 96-well-plates to a density of 1×10^5 cells per well in DMEM medium supplemented with 10% FBS. At 0, 24, 48 and 72 h, $20 \mu\text{l}$ MTT was added, and cells were subsequently incubated with $150 \mu\text{l}$ DMSO for 5 min. The color reaction was measured at 570 nm on an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA). The proliferation activity was calculated for each clone.

Cell apoptosis analysis. To detect cell apoptosis, proliferating phase HDECs (1×10^5 cells) were trypsinized, washed with cold PBS and resuspended in the binding buffer of the Cell Apoptosis Propidium Iodide kit (KeyGen Biotech, Nanjing, China). According to the manufacturer's instructions, fixed cells were incubated in darkness with Annexin V conjugated to fluorescein isothiocyanate and propidium iodide for 20 min at room temperature. Annexin V binding buffer was added to the mixture before the fluorescence was measured on a FACsort flow cytometer (BD, Quebec, Canada). Cell apoptosis was analyzed using the Cell Quest software (Becton Dickinson,

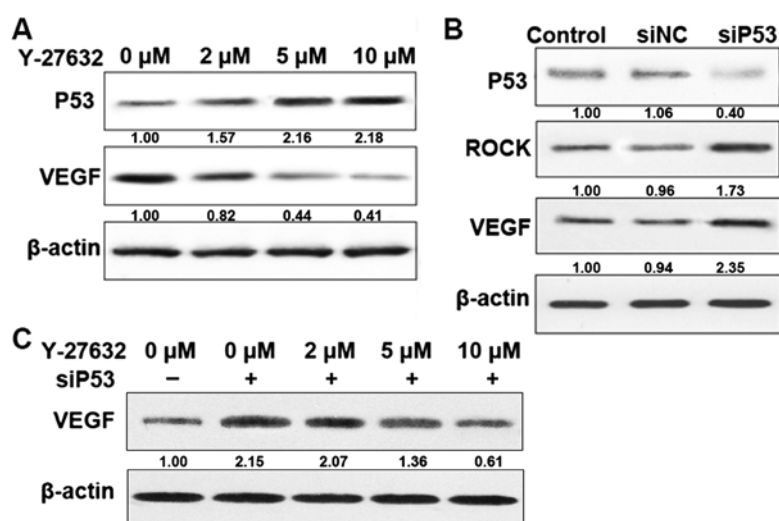


Figure 2. Effect of Y-27632 and siP53 on the expression of VEGF. (A) Treatment with increasing concentrations of Y-27632 (0, 2, 5, 10 μ M) resulted in a dose-dependent decrease in the expression of VEGF and a dose-dependent increase in p53. (B) Effect of siP53 on the expression of ROCK, VEGF and β -actin in primary HDECs. (C) Effect of increasing concentrations of Y-27632 and siP53 on the expression of VEGF.

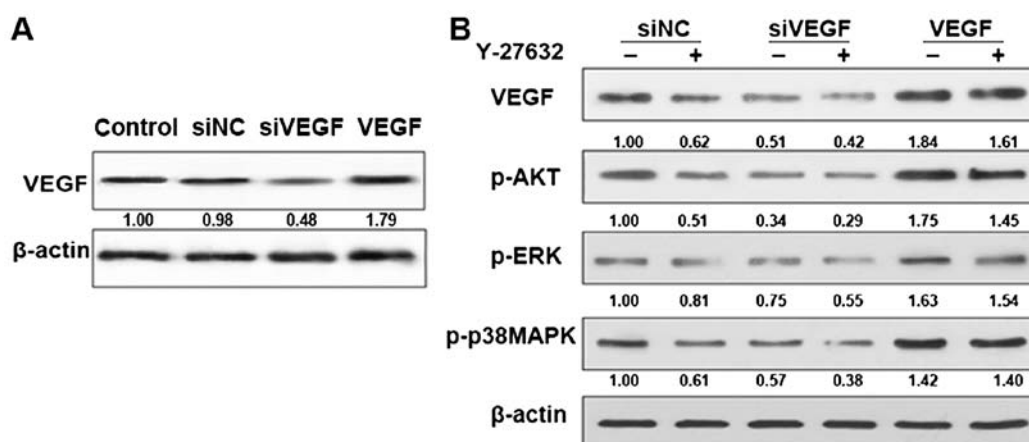


Figure 3. Effect of Y-27632 on the expression of AKT and ERK signaling pathways. (A) VEGF expression was examined by western blot analysis. (B) VEGF and Y-27632 on the expression of pAKT, pERK, p-p38MAPK and β -actin in primary HDECs.

Franklin Lakes, NJ, USA). Three separate experiments were performed for each clone.

Statistical analysis. Statistical analysis was performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the differences between groups. The Fisher's Least Significant Difference method of multiple comparisons was used when the probability for ANOVA was statistically significant. Statistical significance was $P < 0.05$.

Results

Expression of ROCK, p53, VEGF, Ki-67, PCNA and caspase-3 in human HAs and apoptotic index of human HAs. The expression of ROCK, p53, VEGF, Ki-67, PCNA and caspase-3 was examined in proliferating and involuting HAs by immunohistochemical staining (Fig. 1A) and western blot analysis. ROCK and VEGF exhibited similar patterns of protein; expression was highest in proliferating HA tissue and expression in

involuting HAs was higher than normal tissue. In contrast, p53 protein was most abundant in involuting HAs and its levels were higher in normal tissue than in proliferating HAs (Fig. 1B).

As shown in Fig. 1C the expression of Ki-67, PCNA and cleaved caspase-3 was examined in proliferating and involuting HAs, as well as in normal tissue by immunohistochemical staining and western blot analysis (Fig. 1C). Positive staining of apoptotic cells in different phase HAs was examined using the TUNEL assay (Fig. 1D). The amount of apoptotic cells was markedly higher in involuting HAs compared to proliferating HAs.

ROCK regulates p53 and VEGF expression. To confirm the effects of various ROCK inhibitors on p53 and VEGF expression in HDECs, protein levels were determined in untreated cells and cells treated with Y-27632 or transfected with siRNA. Treatment with increasing concentrations of Y-27632 (0, 2, 5, 10 μ M), a potent inhibitor of ROCK, resulted in a dose-dependent decrease in the expression of VEGF and a

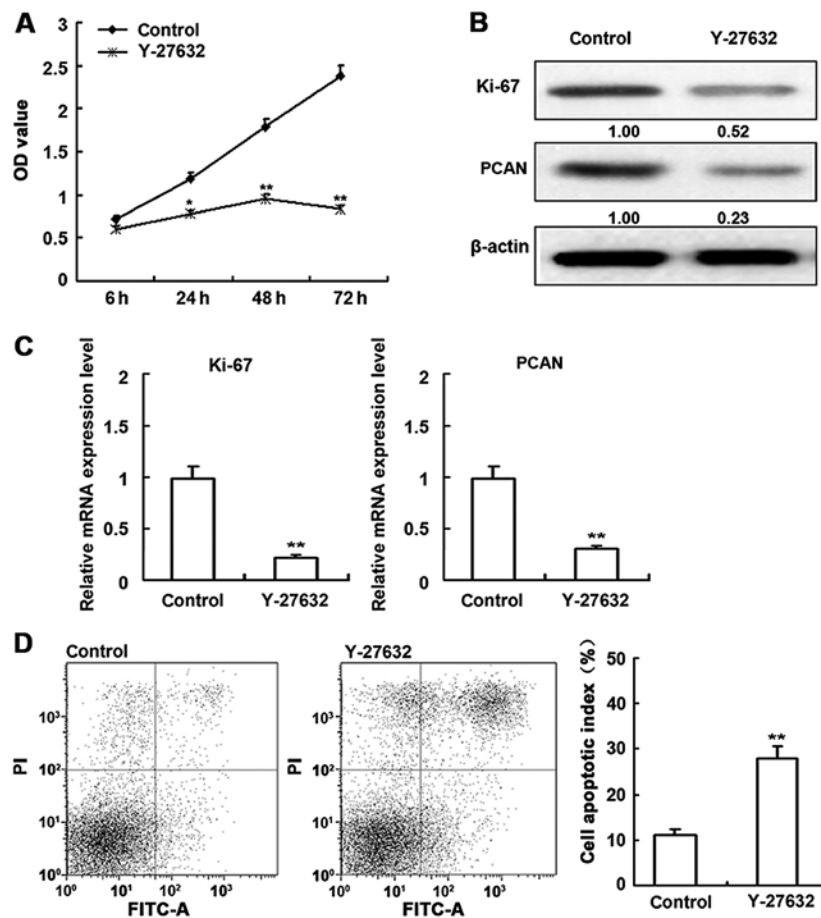


Figure 4. Effect of Y-27632 on proliferation, gene expression and apoptosis. (A) MTT assay. Ki-67 and PCAN mRNA levels detected by (B) western blot and by (C) real-time PCR analysis. (D) Flow cytometric analysis. (* $P < 0.05$, ** $P < 0.01$).

dose-dependent increase in p53 (Fig. 2A). Cell transfected with siP53 resulted in an increase in ROCK and VEGF expression. However, cell transfected with siP53 for 24 h followed by treatment with increasing concentrations of Y-27632, decreased VEGF expression in a dose-dependent manner (Fig. 2C). This result indicated that crosstalk between ROCK and p53 regulates VEGF expression, and the effect of ROCK on VEGF is mediated at least in part by p53.

Effect of ROCK inhibitors on AKT and ERK pathways. The fact that Y-27632 reduced VEGF levels in cells supports the idea that ROCK is involved in AKT and ERK signaling which has a close relationship with VEGF signaling. To this end, we treated control and VEGF knockdown cells with or without Y-27632 and monitored the levels of pAKT, pERK and p-p38MAPK. As shown in Fig. 3, pAKT, pERK and p-p38MAPK levels were decreased in control cells treated with Y-27632 indicating that the pathway is inhibited. However, in the VEGF knockdown cell levels of pAKT, pERK and p-p38MAPK were induced to a much lower level. In the VEGF cells, levels of pAKT, pERK and p-p38MAPK was the opposite. This finding supports the idea that ROCK contributes to AKT and ERK pathway activation in HDECs.

Effect of ROCK inhibitors on cell viability. To examine the effect of the inhibitors on proliferation, HDECs were subjected to an MTT assay 6, 24, 48 and 72 h after treatment

(Fig. 4A). Y-27632 significantly diminished proliferation in a time-dependent manner. The effects of Y-27632 on Ki-67 and PCAN protein and mRNA levels were also determined (Fig. 4B and C). Compared to untreated cells, Y-27632 decreased Ki-67 and PCAN expression. Assays performed on HDECs (Fig. 4D) indicated that cell proliferation was decreased and apoptosis was increased by Y-27632 treatment. Taken together, these data indicate that ROCK inhibition induces apoptosis. The corresponding effects on Ki-67 and PCAN supports this conclusion.

Effect of ROCK inhibitors in vivo. To confirm the *in vivo* effects of ROCK inhibitor treatment, nude mice injected with HDECs (to induce HAs) were treated with Y-27632 for 18 days before HAs were resected and subjected to RT-PCR and western blot analysis (Fig. 5). Mice treated with Y-27632 displayed considerably smaller tumors and higher levels of cleaved caspase-3 as well as lower levels of VEGF, Ki-67 and PCAN expression than in control mice (Fig. 5B and C). In addition, treatment with Y-27632 also inhibited the AKT and ERK signaling (Fig. 5D). These results are in agreement with the results obtained with HDECs.

Discussion

Understanding the switch from the proliferative phase to the involutive phase is essential for the management and treatment

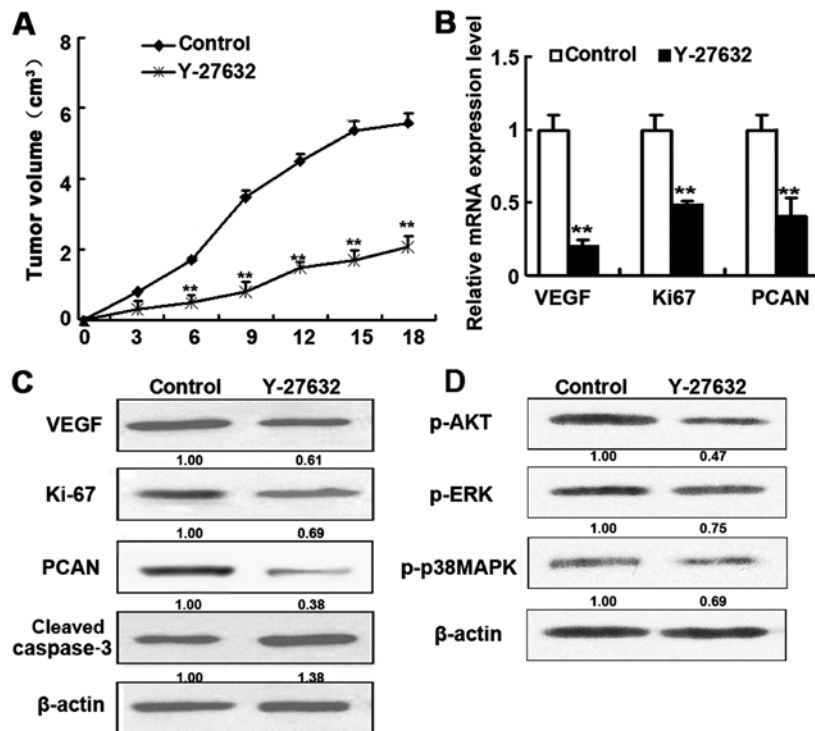


Figure 5. Effect of Y27632 treatment on tumor growth in nude mice 18 days after injection with proliferating HDECs. (A) Tumor size was measured. (B) Expression of VEGF, Ki-67, PCAN and β -actin in tumor tissue was examined by real-time PCR. (C) Ki-67, PCAN and cleaved caspase-3 protein levels was examined by western blot analysis. (D) Expression of pAKT, pERK, p-p38MAPK and β -actin was examined by western blot analysis.

of HAs. As apoptosis plays a major role in HA involution, we examined the expression of several genes involved in apoptosis and cell proliferation in proliferating and involuting HAs. Expression in HAs was confirmed in HDEC lines, for which proliferation and apoptosis could be conveniently measured, and the differential responses to inhibitors were determined.

Our results indicated that the examined genes exhibited two distinct patterns of protein and mRNA accumulation. Consistent with their roles in cancer and cell proliferation, ROCK, VEGF, Ki-67 and PCNA exhibited the highest levels in proliferating HA tissue. ROCK expression is associated with cancer progression and is elevated in several types of cancer (17); Ki-67 and PCNA are well established markers for cell proliferation [see, for example (18)]; and VEGF has been shown to control endothelial cell proliferation and migration (19). The inverse expression pattern (i.e. higher levels in the involutive phase than in the proliferative phase) was observed for p53 and caspase-3. p53 is a known cancer suppressor, and mutations in this gene contribute to malignant progression of cancer (20). Caspases are a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death, and caspase-3 plays a specific role in apoptosis (21). Thus, the high levels of expression of these two genes in involuting phase HAs was also expected.

There is accumulating data for the essential role of ROCK in VEGF function; VEGF mediates angiogenesis (2), venular permeability (22) and neovascularization (23) and is therefore upregulated in expression in the proliferative phase in HAs. p53 is also a regulator of VEGF function, typically by modifying VEGF transcription, and disruption of this interaction plays a role in cancer progression (24-27). p53 was found to be

downregulated in expression in the involutive phase in HAs. p53 knockdown by siRNA increased expression of VEGF, indicating that p53 contributes to VEGF inhibition in cells. It may, therefore, be a fine balance and interactions between ROCK, p53 and VEGF that modulate the switch between the proliferative and involutive phases in HAs. In addition, the fact that ROCK inhibitor stimulated apoptosis in proliferating HA suggests that the corresponding pathways act upstream of p53-mediated apoptosis.

VEGF signaling is thought to promote tumor angiogenesis via regulation of the PI3K/AKT and ERK pathways (28). There is growing evidence to suggest the significant role of the activated PI3K/AKT and ERK pathways in cancer, and one study reported that the AKT and ERK signaling pathways were involved in the pathogenesis of canine HAs (29), while another confirmed the involvement of the PI3K/AKT pathway in the development of human HAs (30). Upregulation of Ki67 and PCNA in our study is indicative of activation of the PI3K/AKT pathway (31). Similar genetic regulatory pathways has been reported previously [see for example Ma *et al* (32), Takeba *et al* (25)]. In the current study, ROCK inhibition decreased the VEGF expression *in vitro* and *in vivo*. We found this coincided with decreased pAKT, pERK and p-p38MAPK expression, suggesting ROCK downregulation may inhibit the AKT and ERK pathways in part by decreasing VEGF expression. Clarifying the interactions that orchestrate the switch from the proliferating to the involuting phase may provide future therapeutic targets.

The fact that similar ROCK inhibitor effects were observed in both HDECs and HAs from HDEC-injected nude mice confirms that HDEC lines are useful for modeling HA

development. In addition, the inhibitory effect of Y-27632 on HA development was confirmed *in situ* with live animals. In a recent study, low concentrations of rapamycin, a known inhibitor of mTOR, were shown to inhibit HA growth in nude mice (33). Although the toxicity of rapamycin prevents it from being used in an unmodified form for HA therapy, these results, together with our findings, suggest that inhibitors, such as Y-27632, could potentially be developed as effective therapies for the treatment of HAs. As these results were obtained in immunodeficient mice, further work is necessary to determine the role of the nude mutation in the observed mediation of HA development.

Acknowledgements

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