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Resveratrol Inhibits Proliferation and Promotes Apoptosis of Keloid Fibroblasts by Targeting HIF-1 α

Loubin Si[1], Qin Han[2], Xiao Long[1], Fei Long[1], Robert Chunhua Zhao[2], Jiuzhuo Huang[1], Zhifei Liu[1], Ru Zhao[1], Hailin Zhang[1] and Xiaojun Wang[3*]

[1]*Department of Plastic Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China*

[2]*Center of Excellence in Tissue Engineering, Institute of Basic Medical Sciences and School of Basic Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China*

[3]*Department of Plastic Surgery of Peking Union Medical College Hospital, Beijing, China*

[*]**Corresponding author:** Xiaojun Wang, Department of Plastic Surgery of Peking Union Medical College Hospital, Beijing, China, Tel: + 861069156114; E-mail: XiaojunWang41063@protonmail.com

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Abstract

Keloid is characterized by red, tickling, hard and irregular raised tissues, and tends to outgrow its origin. It occurs frequently in young adults and appears as refractory to prevailing therapies. Although histological studies have revealed striking similarities of keloids with cancer, such as uncontrolled cell growth, and many of the therapies in clinical practice including -fluorouracil and tamoxifen, excision, and radiation therapy were borrowed from cancer management, the etiology of keloid has remained to be explored to provide insights for developing effective therapeutic modalities. Hypoxic stress around tumors was frequently reported as conferring growth momentum to tumor cells. The hypoxia caused by fast growing of keloid fibroblasts may also be a risk factor that antagonizes therapies. Resveratrol has received intense attention in cancer researches since its anti-proliferative effect on human pulmonary artery smooth muscle cells were revealed. In this study, we first revealed that hypoxia promotes proliferation and inhibit apoptosis of keloid fibroblasts, which highlights the potential obstacle in treating keloids. Further, we demonstrated resveratrol could reverse the effect of hypoxia on keloids, which involves down-regulation of HIF-1α. Moreover, the collagen synthesis of keloid fibroblasts was also inhibited by resveratrol, which was corresponding with HIF-1α suppression. These results provide complementary evidence to the multiple molecular signalings controlled by resveratrol and suggest a pathway implicates resveratrol to regulate expression of pro-collagen expression via HIF-1α.

Keywords: Keloid; Resveratrol; HIF-1α; Collagen synthesis; Hypoxia

Introduction

Keloid is a benign derma tumor that is characterized by red, tickling, hard and irregular raised tissues, which occurs at a high frequency in young adults [1,2]. It tends to extend beyond wound boundaries and is refractory to regular treatment [3]. There have been intractable cases that suffered from the recurrence of keloid, which, if not eradicated, would lead to necrosis, suppuration and recurrent hemorrhage. Histological studies revealed that keloid contains overabundant fibroblasts undergoing mitotic division, in which excessive collagen deposition and myxoid stroma can be observed [4]. The etiology of keloid has remarkable similarity with that of tumor, which is often driven by abnormally overexpression of oncogenes, resulting in uncontrolled growth of cells [5-8]. Indeed, therapies developed to treat keloid such as interferon therapy, intralesional administration of 5-fluorouracil and tamoxifen, excision, and radiation therapy [9-11], were originated from cancer therapy. Currently, no single therapeutic modality was found to achieve best outcome.

Low oxygen level was frequently reported in solid tumors including prostate cancer, melanomas, liver cancer, breast cancer and ovarian cancer [12-16]. Hypoxic stress around tumors can be caused both by fast growing of tumor tissue, which consumes oxygen more rapidly than normal, and the perfusion defects resulting from abnormal tumor blood vessel structure and function [17]. Keloid resembles tumors in many aspects, such as uncontrollable proliferation, tendency to outgrow their origin, and resistance to therapies. Hypoxia also exists in keloid tissues, yet how much it contributes to the cancerous properties of keloids is unclear.

Resveratrol is a natural polyphenolic compound and has been reported to have various beneficial effects, such as cardio-protective, anti-cancer, anti-inflammatory and anti-oxidative functions. One of the most studied aspects was the protective function of resveratrol in hypoxic pulmonary hypertension. Csiszar et al. first demonstrated that resveratrol prevents monocrotaline-induced pulmonary hypertension in rats [18], and Chen et al. further proved that the anti-proliferative effect of resveratrol on human pulmonary artery smooth muscle cells involves decreased expression of hypoxia-induced arginase II expression and Akt-dependent signaling [19]. In this light, we assumed that resveratrol could alleviate the effect of hypoxia in keloid tissues.

In this study, we revealed that hypoxia promotes proliferation and inhibit apoptosis of keloid fibroblasts, which highlights the potential obstacle in treating keloids. Further, we demonstrated resveratrol could reverse the effect of hypoxia on keloids, which involves down-regulation of HIF-1α, a stress-responsive protein that was then found to disrupt the collagen synthesis of keloid tissues. These results provide complementary evidence to the multiple molecular signalings controlled by resveratrol and suggest a pathway implicates resveratrol to regulate expression of pro-collagen expression *via* HIF-1α.

Material and Methods

Source of keloid fibroblasts

Keloid tissues were obtained from five Chinese patients who received surgery in plastic surgery department of our hospital. Detailed information of those samples was provided in Table 1. Written informed consent of all patients was obtained and this study was approved by Ethic Committee of our hospital. None of patients had been administrated with any therapy prior to sampling nor had complicating disease that affects wound healing. All keloid tissue specimens were examined and confirmed pathologically, and the full-thickness biopsy specimens were obtained.

No.	Sex	Age	Site	Keloid Duration (yrs)
1	Female	42	Shoulder	10
2	Male	45	Back	11
3	Male	38	Chest	3
4	Female	35	Shoulder	4
5	Male	24	Ear	2

Table 1: Profile of samples for primary culture.

Cell culture

Tissues were dissected and minced into pieces to as 1 mm in diameter, which were then cultured in DMEM supplemented with 5% FBS in 5% CO_2, 37 humidified atmosphere. At day 12, the cells proliferating out of the explanted tissues were passaged, and the central of clonies in 3-6 passages were used for subsequent experiments.

Construction of hypoxic model

The air was customized containing 1% O_2, 5% CO_2 and 94% N_2, and was stored in a sterile CO_2 air tank. Before hypoxia treatment, the incubator (SANYO, Japan) was charged with the customized air overnight at stable air pressure. When the CO_2 content reached 5%, the O_2 content was 1%.

CCK-8 cell proliferation assay

The Cell Counting Kit-8 (CCK-8; Sigma, USA) was used to quantify cell proliferation. Cells were seeded in 96-well plates till the cells adhere to plate wall. Cells were divided into normal control and hypoxia challenged group, each containing 5 plates. Cell counts per 25cm^2 were quantified. Cells were seeded at a density of 1000/100 μl, 3000/100 μl and 5000/100 μl, 5 replicates each. Both experimental group and control were incubated in respective conditions for 5 days. Every 24 h, CCK-8 reagent (10 μl/well) were added to the well for 2 hours before the absorbance at 450 nm were taken within ELISA plate reader (Thermo), which was then processed with Gen5.

Annexin V-FITC apoptosis detection

FITC ANNEXIN V/DEAD CELL APOPT kit (Invitrogen, USA) was used for assess the apoptosis rate of keloid fibroblasts. 100 μL cells were suspended in 1×Annexin-binding buffer and then incubated with 5 μL fluorescein isothiocyanate (FITC) annexin V and 5 μL PI for 15 min at room temperature in darkness. After adding 400 μL to each replicate, the viability and apoptosis were analyzed by flow cytometry under 530 nm and >575 nm, respectively (BD Bioscience).

Caspase-3 activity assay

Caspase-3 activity assay kit (Beyotime, China) was used to determine caspase activities of keloid fibroblasts. 50 μl chilled cells lysis buffer was added to the cells which was collected by centrifugation at 600 g for 5 min. After 10 min of lysis, the lysate was centrifuged at 16000 g for 10 min. The supernatant was transferred to pre-cooled centrifuge tube, and dispensed to a 96-well plate in which 50 μl sample, 40 μl reaction buffer, and 10 μl Ac-DEVD-pNA (2 mM) substrate were mixed and incubated for 90 min at 37. Until the color change was obvious, Absorbance at 405 nm wavelength was taken with ELISA reader. In parallel, small amount of samples were used to determine the protein concentration with Bradford method, which serve to normalize the absorbance values.

Western blot

Keloid fibroblasts were lysed and total protein were extracted using RIPA. Aliquot protein extraction were then subjected to 10% sodium dodecylsulfate-polyaclylamidegel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. 5% skimmed milk was used to block the background and 0.1% Tween 20 resolved in PBS (pH 7.4), which were then incubated with primary antibodies for 60 min at 37. The following antibodies were used: anti-HIF-1α (1:250, Abcam), anti-collagen I (1:1000, Abcam), anti-collagen III (1:1000, Abcam) and β-actin (1:1000). Horseradish peroxidase (HRP) conjugated secondary antibody (diluted in 0.01M PBS) was added followed by 5 times of washes by 0.01M PBS. Enhanced chemiluminescence solution was used to visualize antigens, and Image J was employed to assess the signal intensities.

Quantitative RT-PCR

Total RNA was extracted using Trizol. cDNA synthesis was done by Revert Aid M-MulV Reverse Transcriptase, which served as template for PCR with SYBR mix (Roche Applied Science) as per the manufacturer's instructions. Each sample contained 3 replicates. The primer sequences are as follows (Invitrogen, USA): HIF1A forward: 5′-CGTTCCTTCGA TCAGTTGTC-3′, and antisense, 5′-TCAGTGGTGGCAGTG GTAGT-3′; COL1A1 forward: 5′-TACAGCGTCACTGTCGATGGC-3′, reverse: 5′-TCAATCACTGTCTTGCCCCAG-3′; COL3A1 forward: 5′-AATTTGGTGTGGACGTTGGC-3′, reverse: 5′-TTGTCGGTCACTTGCAC TGG-3′; β-catin forward: 5'-GAGACCTTCAACACCCCAGCC-3', reverse: 5'-AATGTCACGCACGATTTCCC-3'.

siRNA Transfection and construction of HIF-1

Cells were transfected with HIF-1α siRNAs and scrambled siRNAs (QIAGEN) using Lipofectamine^{TM} 2000 Transfection Reagent (Invitrogen, USA) when grew to 70-90% confluence. Two days after incubation at 37, cells were harvested for subsequent assay. For HIF-1α overexpression constructs, pAdTrack-CMV and pAdEasy-1 were transfected into HEK-293 cells to produce recombinant adenovirus, which were then used to transfect keloid fibroblasts with LipofectamineTM 2000 when the cells grown till 60% confluence.

Results

Hypoxia induces proliferation and inhibit apoptosis of keloid fibroblasts

The hypoxic microenvironment of cancer tissue promotes the proliferation and growth of cancer cells, and has been considered as a risk factor in various therapies. It was long believed that hypoxia induces tolerance to chemotherapies and substantially deteriorates the outcome [20]. Keloid resembles tumor in many aspects, such as the propensity to extend beyond wounded area, overexpression of growth factors, and resistance to chemotherapy and radiation. This enlightened us to explore the cell behavior of keloid fibroblasts under hypoxic condition. We subjected keloid fibroblasts to an atmosphere composed of 1% O_2, 5% CO_2 and 94% N_2 for 5 days. The atmosphere for control was composed of 95% air and 5% CO_2. Cells were cultured at 3 levels of seeding density: 1000 cell/100 µl, 3000 cell/100 µl, and 5000 cell/100 µl. Generally, the growth rate of hypoxia group is significantly faster than that of control (Figure 1). For 1000 cell/µl, the cell number of control outnumbered hypoxia group at day 4, and after that it dropped while hypoxia group kept growing. For 3000 cell/µl, the hypoxia group kept higher and increasing OD450 value, same as 5000 cell/µl except at day 2 both groups seemed to have equal cell numbers. These results showed that proliferation of keloid fibroblasts were enhanced under hypoxic condition.

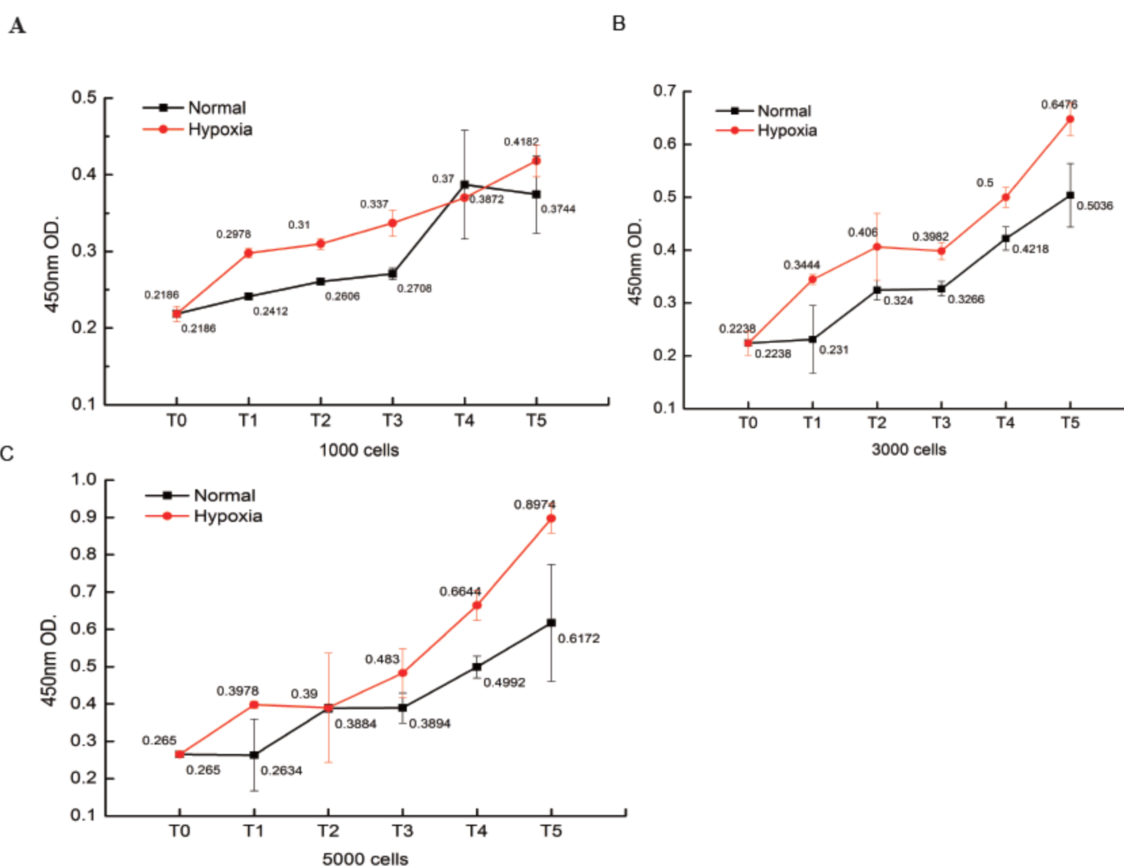
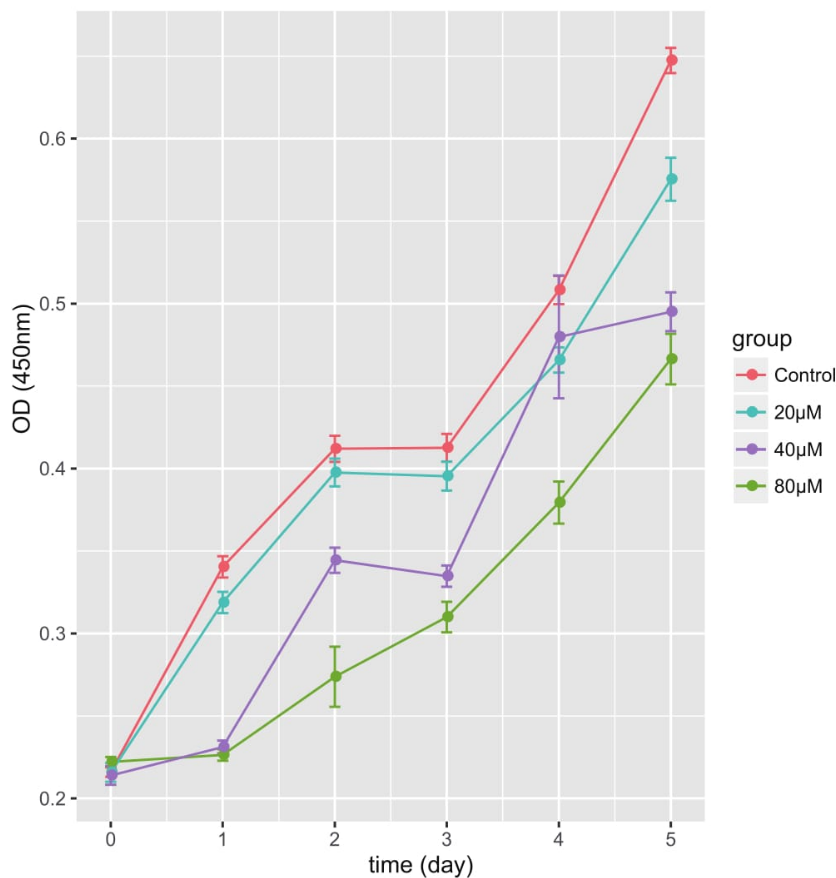


Figure 1: Growth curves of keloid fibroblasts under hypoxia and normoxia. At three different cell seeding densities A at 1000 cells, B at 3000 cells and C at 5000 cells, the difference between growth rate of keloid fibroblasts under hypoxia and normoxia were compared. Y-axis suggests OD value of the culture, and x-axis represent duration under set conditions. Error bars indicate standard deviation.

Resveratrol attenuates effect of hypoxia on keloid fibroblasts

Resveratrol has been reported as an anti-cancer adjuvant. Similar effect of resveratrol on keloid fibroblasts were also observed under hypoxic condition. Supplemented with different concentration of resveratrol and incubated under hypoxia for 5 days, the proliferation of keloid fibroblasts was reduced (Figure 2A), which seemed to display a dose dependent pattern (3000 cells/100 µl). After 2 days of treatment, the cell viability displayed remarkable divergence. At 80 µM, the OD450 value indicating cell numbers showed significant difference. Under microscope, we observed excessive number of resveratrol treated cells were shrunk and deformed, suggestive of apoptosis. Annexin-V flow cytometry was performed to assess cell apoptosis of keloid fibroblasts treated with 80 µM resveratrol under hypoxia. Results showed that the apoptosis rates of resveratrol treated cells were significantly higher than control after 48 h (Figure 2B). No significant difference in necrosis was observed between keloid and normal fibroblasts (Figure 2B). To further examine the difference in apoptosis, we compared the caspase-3 activity of keloid fibroblasts treated with resveratrol and control under hypoxia. These results suggest that resveratrol could alleviate the effect of hypoxia on keloid fibroblasts.



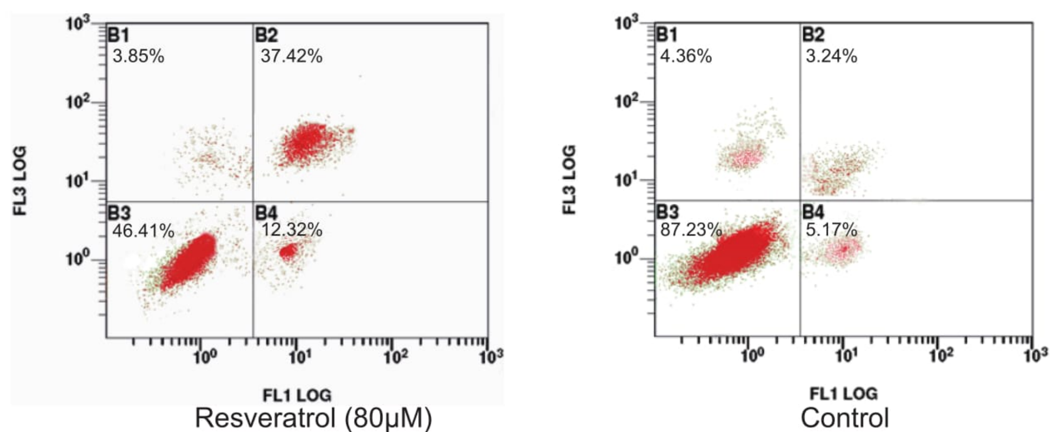


Figure 2: Resveratrol attenuates effect of hypoxia on keloid fibroblasts. A). The growth curves of keloid fibroblasts cultured with different concentration of resveratrol under hypoxia for 5 days, and the OD values were taken every 24 h. Error bars indicate standard deviation. B). Annexin V apoptosis flow cytometry analysis of keloid fibroblasts treated with 80 μM resveratrol for 2 days. Upper left quadrant represent necrosis, upper right represents late apoptosis, and lower right represents early apoptosis.

Suppression of HIF-1α accounts for attenuation on proliferation and induction of apoptosis in hypoxia-challenged keloid fibroblasts

HIF-1α was identified as an effector in increase of cell number, inhibition of proliferation in various cell types given inadequate supply of oxygen [21-24]. Since we observed the hypoxia-induced inhibition of apoptosis and enhancement of proliferation was attenuated by resveratrol, we suspected this reversal may be related to HIF-1α, an established marker of cellular response to hypoxia. Western blotting showed that HIF-1α expression of hypoxia challenged cells was higher than control, which was mitigated by resveratrol treatment (Figure 3a). Moreover, the HIF-1α deficient keloid fibroblasts showed stagnant growth and increase proportion of cells undergoing apoptosis (Figures 3b and 3c), suggesting that HIF-1α might be involved in the mechanism underlying pharmaceutical effect of resveratrol on keloid fibroblasts.

Overexpression of HIF-1α reverses the inhibition of proliferation and induction of apoptosis by resveratrol

Given the role of HIF-1α in resveratrol induced anti-proliferation and apoptosis was confirmed, we then sought to testify whether HIF-1α is critical for the effect exerted by resveratrol. Compared with untreated keloid fibroblasts, resveratrol treatment of cells overexpressing HIF-1α did not cause significant changes in cell number after 48 h (Figures 4A and 4B). The active caspase-3 of resveratrol treated cells was also rescued by overexpression of HIF-1α, and both were significantly increased in untreated HIF-1α overexpressing cells (Figure 4C). These results suggest that HIF-1α might be a target of resveratrol.

HIF-1α promotes collagen synthesis and resveratrol inhibits it

A pronounced characteristic of keloid fibroblasts is the excessive accumulation of collagen fiber, and collagen I and collagen III are critical proteins implicated in this pathogenesis. To address if resveratrol affects this pathological process, we performed western blot analysis. The protein level of collagen I and collagen III were substantially reduced in resveratrol treated cells, and HIF-1α overexpression seemed to alleviate this effect (Figure 5). These highlight a potential mechanism underlying the pharmacological effect of resveratrol.

Discussion

Tumors proliferate rapidly, consuming excess of oxygen and producing hypoxic microenvironment. The hypoxic microenvironment in turn confers proliferative advantage to tumor cells. Keloid, generally considered as benign hyperplastic dermal tumors, thrives in hypoxic microenvironment and exhibits similar characteristics as tumor, including uncontrolled proliferation, migration and invasion, and escape from apoptosis. During wound repairing, the injury area is in hypoxic state, which induces fibroblast proliferation and angiogenesis. Therefore, we suspected that hypoxia contributes to the tumor-like characteristics of keloids. In cell viability assay, the hypoxia challenged cells showed growth more rapidly than cells under normal condition. These observations were in accordance with multiple lines of evidence showing effects of low oxygen environment on cell growth in various cell types [25]. Furthermore, the decreased apoptosis rate was also observed in hypoxia treated keloid fibroblasts. Taken together, we deemed that the hypoxia exists in wound area may confer keloid fibroblasts with growth advantage and resistance to apoptosis, and contributes to the resistance of keloids to treatment, though the underlying molecular mechanism remained to be determined.

Resveratrol is a natural polyphenol compound found abundant in grapes and berries [26]. For the past decade, resveratrol has gained much attention in anti-cancer therapy [27,28], and was integrated into food supplements. Apart from being recognized for its anti-proliferative and pro-apoptotic properties in carcinogenesis, resveratrol is also renowned for its ability to reduce energy expenditure *in vivo* [29]. Recently, the inhibition of resveratrol on cellular glucose metabolism was highlighted in several studies [30]. Anthony et al. reported that resveratrol inhibited phosphatidylinositol 3-kinase (PI-3K) signaling and glucose metabolism, accompanied by cell-cycle arrest, in germinal center-like LY1 and LY8 human diffuse large B-cell lymphomas (DLBCLs) [31]. Consistently, Mara et al. revealed that resveratrol treatment decreased the ability of HepG2 cells to utilize glucose and amino acids as well as slowed down the cell cycle in S phase but without inducing apoptosis [32]. Furthermore, they also demonstrated that Sirt1, an energy status sensitive protein that harness NAD+ as co-enzyme for its acetylase activity, was increased at the presence of resveratrol. Sirt1 was a deacetylase that is sensitive to NAD +/NADH. Decrease level of NAD+ during hypoxia downregulated *SIRT1*, which inhibits deacetylation and leads to activation of HIF-1α [33]. Conversely, when the glycolysis was blocked, SIRT1 can be upregulated, resulting in excess of deacetylated HIF-1α, impairing its activity. This evidence, points to an inhibitory effect of resveratrol on hypoxia-induced biological processes. Indeed, in the present study, we observed that the hypoxia-induced proliferation and inhibition of apoptosis in keloid fibroblasts was attenuated by resveratrol treatment, which indicate therapeutic value of using resveratrol in treating keloid fibroblasts.

HIF-1α is a subunit of HIF-1, a transcriptional regulator of cellular and physiological processes in response to hypoxia stress. The expression of HIF-1α is tightly regulated by hypoxia stress, while HIF-1β is constitutively expressed. Accumulation of HIF-1α promotes its binding with HIF-1β to form active dimers, which were identified as a transcriptional factor of over 100 genes implicated in cellular proliferation, apoptosis, and angiogenesis [34,35]. Overexpression of HIF-1α has been shown to be associated with many human cancers and promote their metastasis. Moreover, the expression level of HIF-1α showed correlation with aggressive tumor phenotype, such as more advanced tumor grade. Therefore, HIF-1α is a potential marker for evaluating prognosis, mortality risk or therapeutic efficacy [36]. In keloid, the abnormal expression of HIF-1α was also frequently reported. Zhang et al found that HIF-1α accumulation was augmented in a co-culture of keloid fibroblasts and human mast cells, which involved activation of ERK1/2 and increase of VEGF [37]. Ma et al. demonstrated that HIF-1α was highly expressed in hypoxia-exposed keratinocytes and epithelial layer of keloid tissue [38]. They further found that vimentin and fibronectin, markers of epithelial-to-mesenchymal transition, were upregulated during hypoxia, concurrent with reduced expression of E-cadherin and zonula occludens-1 (ZO-1), which can be reversed by silencing of HIF-1α.

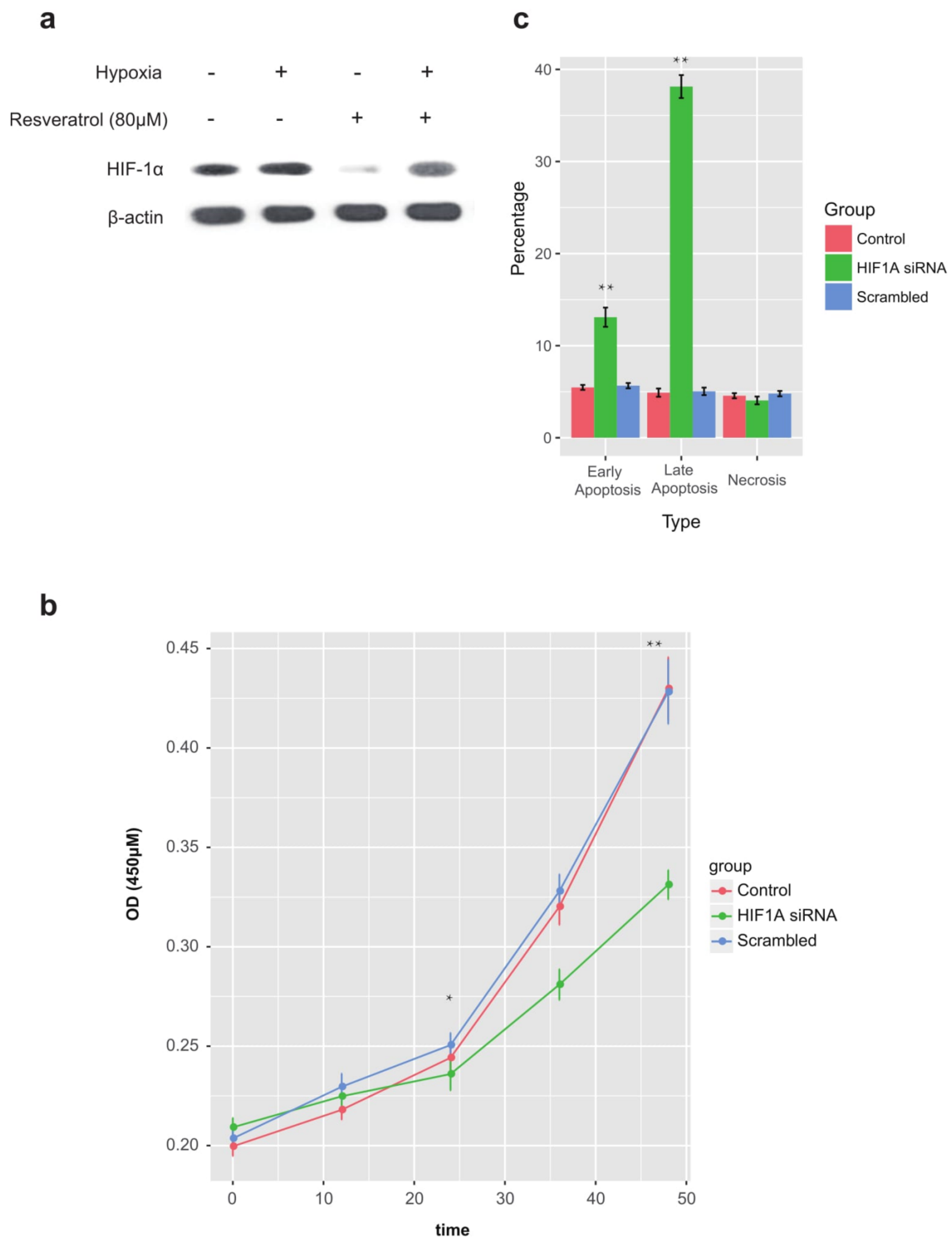


Figure 3: Suppression of HIF-1α accounts for attenuation on proliferation and induction of apoptosis in hypoxia-challenged keloid fibroblasts. a). Western blot of HIF-1α of keloid fibroblasts treated/untreated with resveratrol under hypoxia/normoxia. b). Cell growth curves of HIF-1α deficient keloid fibroblasts. HIF-KD: HIF-1α deficient,*: p<0.05, **: p<0.01. c). Apoptosis rates of HIF-1α deficient keloid fibroblasts. HIF-KD: HIF-1α deficient, *: p<0.05, **: p<0.01. c). y-axis represents percentage of cells in early apoptosis, late apoptosis or necrosis.

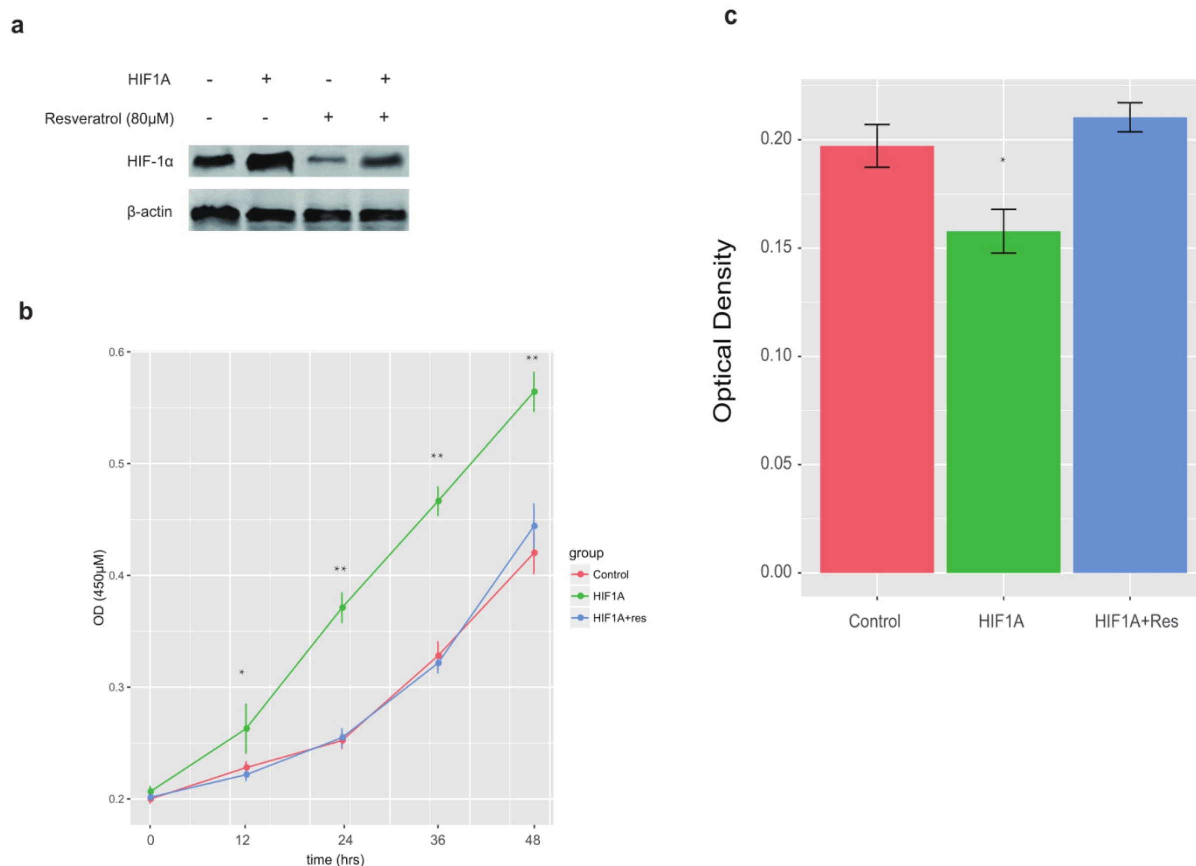


Figure 4: Overexpression of HIF-1α reverses the inhibition of proliferation and induction of apoptosis by resveratrol. a). Western blot of HIF-1α in HIF1A overexpressing keloid fibroblasts. b). Growth curves of keloid fibroblasts overexpressing HIF1A treated/untreated with resveratrol for 2 days. Y-axis represents OD values under 450nm and x-axis represents time points when the OD values were taken. Error bars indicate standard deviation. *: $p<0.05$, **: $p<0.01$. c). Caspase-3 activity of HIF1A overexpressing keloid fibroblasts treated/untreated with resveratrol. *: $p<0.05$.

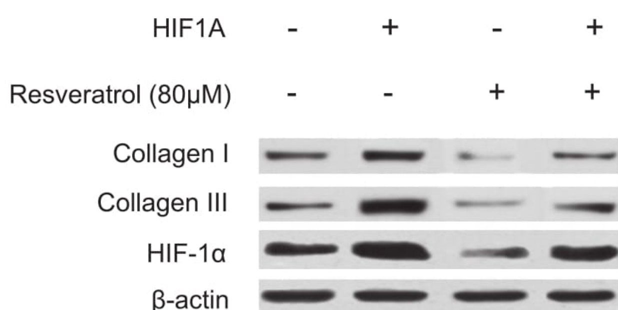


Figure 5: HIF-1α promotes collagen synthesis and resveratrol inhibits it. Western blot of HIF-1α, Collagen I, and Collagen III of HIF1A overexpressing keloid fibroblasts.

In the present study, overexpression of HIF-1α was found in hypoxia challenged keloid fibroblasts, suggesting its potential role in promoting cell growth and suppressing apoptosis of keloid fibroblasts. Resveratrol treatment downregulated HIF-1α, the effect of which was attenuated by overexpressing HIF-1α, suggesting that HIF-1α may be the mediator of effect of resveratrol on keloid fibroblasts. On the other hand, since glycolysis can be blocked by resveratrol and *Sirt1* would be upregulated accordingly. The deacetylation of HIF-1α by excess of *Sirt1* could further inhibit activity of HIF-1α. Therefore, resveratrol may be a potent therapeutic agent for inhibiting and suppressing HIF-1α.

Excessive deposition of collagen was a typical character of keloid tissues. Resveratrol was found to markedly reduce levels of fibrotic markers, including fibronectin, collagen I and collagen IV, and decrease ECM deposition via regulation of autophagy in a SIRT1-dependent manner [39]. Karin et al. showed that deletion of HIF-1α disrupted collagen modification, cell migration and metastasis in murine sarcoma models [40]. In the present study, we chose collagen I and collagen III to represent the level of collagen, which constitute 80-90% and 10-15% of total collagen present in the skin, respectively [41,42]. We demonstrated that HIF-1α deficiency impaired expression of pro-collagen type I and type III, and the mRNA level of *COL1A1* and *COL3A1* were also downregulated, indicating that resveratrol not only exerts inhibitory effect on collagen synthesis *via SIRT1* functionality, but also hampers the expression of *COL1A1* and

COL3A1 through downregulation of HIF-1α. Taken together, a complementary mechanism underlying regulation of collagen synthesis by resveratrol was revealed in our study.

In conclusion, our study revealed that hypoxia promotes proliferation and inhibit apoptosis of keloid fibroblasts, which highlights the potential obstacle in treating keloids. Further, we demonstrated resveratrol could reverse the effect of hypoxia on keloids, which involves downregulation of HIF-1α, a stress-responsive protein that was then found to disrupt the collagen synthesis of keloid tissues. These results provide complementary evidence to the multiple molecular signalings controlled by resveratrol and suggest a pathway implicates resveratrol to regulate expression of pro-collagen expression via HIF-1α.

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