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Knockdown of HIF-1α inhibits the proliferation and migration of outer root sheath cells exposed to hypoxia *in vitro*: An involvement of Shh pathway

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ABSTRACT

Aims: Outer root sheath (ORS) is a highly proliferative component of a hair follicle. This study is performed to investigate whether hypoxia-induced elevation of hypoxia-inducible factor (HIF)-1α, a transcriptional activator, contributes to the outgrowth of ORS cells *in vitro*.

Main methods: Hair follicles with intact ORS collected from 4-month old male American minks were cultured in normoxic or hypoxic condition (3% oxygen) for 7 days. Primary ORS cells isolated from the mink hair follicles were exposed to hypoxia for 12, 24 or 48 h, and their proliferation was analyzed with immunofluorescence assay using anti-proliferating cell nuclear antigen (PCNA) antibody. The migratory ability of ORS cells was detected *via* the transwell chamber. The endogenous HIF-1α was knocked down with its specific siRNA in ORS cells.

Key findings: Hypoxic exposure induced an elevation of HIF-1α in *ex vivo* cultured hair follicles. The mRNA and protein levels of sonic hedgehog (Shh), Shh receptor Patched 1, Smoothened and glioma-associated oncogene homologue 1 were upregulated. *In vitro*, hypoxia induced an increase in HIF-1α in ORS cells. Further, under hypoxic condition, the number of PCNA-positive cells was increased, and more cells migrated towards high serum media. Hypoxia-enhanced proliferation and migration of ORS cells were suppressed either by HIF-1α siRNA or by pharmacological inhibitors of Shh pathway, cyclopamine and GANT61. The activation of Shh pathway was attenuated in HIF-1α-silenced ORS cells under hypoxic condition.

Significance: Our work demonstrates a direct role of activated HIF-1/Shh biological axis in sustaining the development of ORS *in vitro*.

1. Introduction

Hair follicles are notable appendages of epidermis, and undergo cycles of rest (telogen), growth (anagen), and degeneration (catagen) [1,2]. Hair follicle stem cells (HF-SCs) located in the bulge (Bu-SCs) are one of the two essential HF-SC populations (another one is located in the hair germ), which maintain a quiescent state during most of the hair cycle, except the anagen. At anagen onset, the Bu-SCs initially give rise to cells located in the outer root sheath (ORS), a cell population that retains major characteristics of stem cells [3]. As the outermost layer, ORS extends from the base of each mature follicle to the matrix, and surrounds the inner differentiating core [4]. During catagen, apoptosis starts from matrix and expands upward into the retracting epithelial strand, while cells within the ORS are mostly quiescent [4]. ORS is a highly proliferative compartment of hair follicle during the growth phase.

Hypoxia-inducible factors (HIFs) are a family of basic helix loop-helix transcriptional activators, including α and β subunits. Heterodimeric HIF complexes formed by α and β subunits can regulate the transcription of hypoxia-responsive genes and control the oxygen homeostasis [5]. Information illuminating the role of HIFs in cell growth is mainly from studies on the carcinogenesis [6] or on the pathogenesis of diseases related to hypoxia, such as myocardial and cerebral ischemia [7,8]. Of note, the oxygenation in skin epidermis commonly occurs through the oxygen in the atmosphere rather than the tissue vasculature, leading to a hypoxic microenvironment that favors an elevation of HIF-1α [9,10]. During the postnatal hair follicle growth, the expression levels of HIF-1α, -2α and -1β are upregulated in the mouse dorsal skin [11]. HIF-1α-induced genes, such as carbonic anhydrase 9 [12] and glucose transporter 1 [13], have been found to restrictively express to the ORS of anagen follicle [14]. These studies

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suggest a spatial and temporal regulation of HIF signaling during the growth of a hair follicle. However, whether hypoxia-induced elevation of HIF-1α contributes to the outgrowth of ORS is unclear.

Sonic hedgehog (Shh) is a secreted glycoprotein that is initially identified to control the embryonic patterning and axon growth during development [15]. Shh and its cognate receptor Patched express in the epithelial and/or stem cell components of a hair follicle [15,16]. Although the excessive activation of Shh signaling results in hair follicle-derived tumors, such as basal cell carcinomas, Shh is also known to regulate the epithelial growth and morphogenesis of normal hair follicles [17]. The follicle development of skin was blocked in Shh mutant mice [18]. Antibodies neutralizing Shh arrested the hair morphogenesis at many stages [19]. Furthermore, localized transient overexpression of Shh was found to accelerate the initiation of anagen in postnatal mice [20]. The upregulation of Shh activity is required by the resting hair follicles to enter the anagen [21]. Shh signaling pathway can be reactivated by HIF-1α in multiple types of cancer cells, such as neuroblastoma [22] and pancreatic cancer [23], and orchestrates the proliferation and/or mobility of these cells. Nonetheless, whether HIF-1/Shh biological axis contributes the outgrowth of ORS is hitherto unknown and requires further investigation.

2. Materials and methods

2.1. Isolation of hair follicles and culture of ORS cells in vitro

The experiment was performed at the Teaching and Research Farm of the Institute of Special Economic Animal and Plant Science of Chinese Academy Agricultural Sciences (Jilin, China). All animal experiments were accordance with the Guidelines for the care and use of laboratory animals of Chinese Academy Agricultural Sciences. Anagen hair follicles with hair shafts from 4-month old male American minks (*Neovison vison*) were cultured in normoxic or hypoxic condition (3% oxygen) for 7 days. Then the hair follicles were microdissected according to the standard procedures established in out lab [24].

For the culture of primary ORS cells, small dorsal skin pieces (1 cm^2) were surgically excised, and the subcutaneous fat and the excess hair shafts outside the skin were removed. After washing with sterile PBS containing 0.1 mg/mL streptomycin and 100 U/mL penicillin (pH = 7.4), the skin samples were disinfected with iodine and then with 75% ethanol for a few seconds. After rinsing with PBS for several times, the tissue samples were incubated with 0.2 mg/mL collagenase IV (Sigma, St. Louis, MO, USA) at 4 °C overnight. Thereafter, the hair follicles with hair shaft were microdissected carefully. The follicle with intact ORS was aligned evenly in a well of the six-well plate, and maintained in DMEM/F12 (Gibco, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS), 5 μg/mL insulin, 10 ng/mL epidermal growth factor (EGF), 0.4 μg/mL hydrocortisone, 100 U/mL penicillin and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO_2. The cell culture medium was replaced every 3 days. Primarily cultured cells were then subjected to immunofluorescence staining using the anti-cytokeratin (CK)-19 antibody (BA2266-1; BOSTER, Wuhan, China). Cyclopamine (a Smoothened/SMO inhibitor; HY-17024, MedChemexpress, Monmouth Junction, NJ, USA) or GANT61 (a glioma-associated oncogene homologue 1 (GLI-1) inhibitor; HY-13901, MedChemexpress) at 5 or 10 μM was used to block the Shh signaling transduction in ORS cells *in vitro*.

2.2. Reverse transcription (RT)-polymerase chain reaction (PCR) and real-time quantitative PCR

Total RNAs were isolated from the microdissected mink hair follicles or the ORS cells with an RNApure Total RNA Extraction Kit (RP1201, BioTeke, Beijing, China). RNA reverse transcription was performed at 42 °C for 50 min by using the Super M-MLV reverse transcriptase (PR6502, BioTeke). For RT-PCR, the obtained cDNAs were amplified with primers listed in Table 1, and analyzed with 1.5% agaropectin. For real-time PCR, the obtained cDNAs were mixed with SYBR Green I Master Mix (SY1020, Solarbio, Beijing, China) and primer pairs, and then subjected to PCR analysis on the Exicycler 96 Quantitative PCR Analyzer (Bioneer, Daejeon, Korea). The fluorescence signals were recorded. Beta-actin was used as the internal reference. The relative mRNA expression levels were calculated with $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences for quantitative real-time PCR.

Gene name	Sequence 5′-3′	Fragment
Shh F	TGGCTGTGGAAGCAGGTTT	239 bp
Shh R	GTCCAGGAAGGTGAGGAAGTCG	
PTCH F	GACTCCCAAGCAAATGTATGAA	180 bp
PTCH R	AGGGTCGTGGTTGTGAAGG	
SMO F	ATCGCTACCCTGCGGTTATT	215 bp
SMO R	CCAGACTACTCCAGCCATCAA	
Gli1 F	CTGTCGGAAGTCCTATTCACGC	123 bp
Gli1 R	CGGTCACTGGCATTGCTAAA	
Beta-actin F	CTGTGCCCATCTACGAGGGCTAT	155 bp
Beta-actin R	TTTGATGTCACGCACGATTTCC	

F, forwards; R, reversed.

2.3. siRNA-mediated gene silencing

The siRNA sequences were as the following: NC siRNA, 5′-UUCUCCGAACGUGUCACGUtt-3′, HIF-1α siRNA, 5′-GCUCACCAUCAG UUAUUUAtt-3′. ORS cells were transfected with a siRNA mixed with RNAiMAX (#13778100; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Migration assay

ORS cell migration was detected by using the transwell chambers (#3422; Corning, NY, USA). In short, cells (1×10^4 cells/200 μL) in presence or absence of Shh pathway inhibitor were added into the upper chamber, with the lower chambers filled with culture media containing 30% FBS (SH30084.03, HyClone, Logan, UT, USA). Then, chambers were exposed to 3% or 21% oxygen for 24 h or 48 h before cell counting. For knockdown of HIF-1α, cells were first transfected with HIF-1α siRNA or NC siRNA, and 24 h later, these cells were subjected to migration assay. The fixed migrating cells were stained with 0.5% crystal violet (#0528, Amresco, Solon, OH, USA) and then counted in 5 random fields on each membrane. Data with three replications were used to measure the average migratory ratios.

2.5. Western blot analysis

In brief, excess hair shafts outside the follicles were cut off, and the follicles were grinded into powder in the liquid nitrogen within 2 min. Then, the samples were mixed with 300 μL RIPA buffer (P0013B, Beyotime, Haimen, China) supplemented with 1% phenylmethanesulfonyl fluoride (ST506, Beyotime) on ice. Ten minutes later, the mixture was centrifuged (10,000*g*) at 4 °C for 10 min, and the cell supernatants were then collected for the following immunoblot analysis. The protein concentration of each sample was approximately 3–5 μg/μL determined *via* a BCA Assay Kit (P0009, Beyotime). Equal protein sample was then separated on the SDS-PAGE, and transferred onto the PVDF membranes. After blocking with 5% non-fat milk solution, the membranes were incubated overnight at 4 °C with rabbit monoclonal antibody to HIF-1α (1:1000; ab179483, Abcam, Cambridge, MA, USA), mouse monoclonal antibodies to Smoothened (SMO; 1:100; sc-166685; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and glioma-associated oncogene homologue 1 (GLI-1; sc-515751; 1: 200; Santa Cruz Biotechnology), rabbit polyclonal antibodies to Shh (1:500; ab19897,