HIF-1α controls keratinocyte proliferation by up-regulating p21(WAF1/Cip1)

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Abstract

The cyclin-dependent kinase inhibitor p21WAF1/Cip1 plays a central role in a spatial and temporal balance of epidermal keratinocyte proliferation and growth arrest. However, what controls p21 expression in keratinocytes remains uncertain. Hypoxia-inducible factor 1α (HIF-1α) does not only express a variety of genes essential for hypoxic adaptation, but also up-regulates p21 so as to slow down cell cycle under hypoxic conditions. In the present study, we examined the role of HIF-1α in p21-mediated growth arrest of keratinocyte. Keratinocyte proliferation was arrested in the G1 phase at a high cell density. p21 was also up-regulated in a cell density-dependent manner and was found to be highly expressed in epidermal keratinocytes of normal human skins. In addition, in the same specimens and cells, we noted robust HIF-1α expression. HIF-1α siRNAs inhibited p21 expression and released the G1 arrest. In vivo, moreover, the intradermal injection of HIF-1α siRNA attenuated p21 expression in rat epidermis and induced skin hyperplasia. Mechanistically, we propose that the production of mitochondrial reactive oxygen species and the activation of the MEK/ERK pathway are involved in the HIF-1α stabilization in keratinocytes. These results imply that HIF-1α functions as an up-stream player in the p21-mediated growth arrest of keratinocytes.
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1. Introduction

In normal epidermis, the proliferation and differentiation of keratinocytes occur continuously throughout life. Keratinocytes are newly generated from stem cells in basal layers, and undergo a sequential multi-step differentiation, i.e., growth arrest – migration to suprabasal layers – apoptosis — and finally cornification. Moreover, the rates of proliferation and growth arrest are precisely balanced by desquamation of the cornified layer, and this process maintains the epidermal thickness [1–3]. Thus, an increase in proliferation or low growth arrest may cause hyperproliferative skin disorders. Then which molecular entities are key players in epidermal homeostasis? The answer to this question may provide a clue to the prevention and treatment of hyperproliferative skin disorders. In this work, we focus on the mechanism underlying the growth arrest of keratinocytes.

The cyclin-dependent kinase inhibitor p21WAF1/Cip1 has been shown to play an important role in the control of keratinocyte growth and differentiation, as it does in many terminally
Differentiating cells [4]. p21 expression is induced at the onset of keratinocyte growth arrest and differentiation by Ca$$^{2+}$$ [5,6], TPA [6] or interferon-γ [7]. Although the p21-mediated cell cycle arrest is well known in many cell types, the contribution made by p21 to epidermal differentiation remains ambiguous. In studies by Paramio et al. [8], p21 knock-out failed to affect keratinocyte growth rates. In contrast, in different studies using similar culture models, p21 knock-out showed significantly increased proliferative potential of keratinocytes [9,10]. In addition, it was also demonstrated that Ras-transformed, p21-knocked-out keratinocytes exhibit highly aggressive tumorigenic behavior in nude mice and that p21 knock-out mice are more susceptible to carcinogen-induced tumorigenesis in the skin. Therefore, p21 up-regulation in keratinocytes is believed to contribute to growth arrest. However, the factors that control p21 expression in keratinocytes have not been determined.

HIF-1α is an up-stream factor that induces p21 transcription. Originally, HIF-1α was found to be a key transcription factor of the hypoxic induction of erythropoietin. As 60 or more genes that are essential for hypoxic adaptation are up-regulated by HIF-1α, it is now viewed as a master protein that directs angiogenesis and cell survival [11]. HIF-1α belongs to the basic-helix–loop–helix Per-Arnt-Sim family, and is O2-dependently regulated at the post-translational level [12]. Under normoxic conditions, HIF-1α is hydroxylated by HIF-1-prolyl hydroxylases, and then ubiquitinated and degraded by Von

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**Fig. 1. p21 expression and cell cycle arrest in human keratinocytes.** A) Cell density-dependent G1 arrest. After HaCaT cells had been cultured to reach the indicated cell densities, cell cycle was analyzed by propidium iodide staining and flow cytometry. Each bar represents the mean ± SD of 9 separate experiments. *: p < 0.05 vs. either the 20% or 40% confluence group. B) mRNA levels of cell cycle inhibitors. Total RNAs were extracted and the mRNA levels of p21, p27, p53, and β-actin were determined by semiquantitative RT-PCR. C) Cell density-dependent expression of p21 in keratinocytes. In HaCaT cells and primary keratinocytes, p21 and β-actin protein levels were analyzed by immunoblotting. D) p21 expression in human epidermal keratinocytes. Biopsy sections from human normal skin were incubated with anti-p21 antiserum or preimmune rabbit serum (-antibody). p21 (arrow) was identified at a magnification of 100×. [E], epidermis; [D], dermis. E) p21 is not expressed in proliferating keratinocytes. Immunofluorescence staining for p21 (Alexa Fluor 488 green) and Ki67 (Alexa Fluor 568 red) was performed in combination with DAPI blue staining (for labeling all nuclei). Keratinocytes expressing p21 (green) do not overlap with proliferating keratinocytes expressing Ki67 (red).
Hippel–Lindau protein and 26S proteasome. However, hypoxia limits this modification of HIF-1α, and thereby stabilizes HIF-1α, which associates with ARNT and transactivates genes [13,14]. In addition to this role, HIF-1α is known to induce p21 expression by inhibiting c-Myc, a repressor of the p21 gene. HIF-1α down-regulates c-Myc by disorganizing β-catenin/Tcf4 transcription factor for c-Myc expression [15], and also derepresses p21 by displacing c-Myc binding from p21 promoter [16]. However, since all of these findings have been reported only in cancer cells, it is uncertain whether HIF-1α-dependent cell cycle arrest occurs in non-cancerous cells. In the present study, we demonstrated that HIF-1α functions as an up-stream player in the p21-mediated growth arrest of keratinocytes.

2. Materials and methods

2.1. Reagents and antibodies

Desferrioxamine (DFO), cycloheximide (CHX), N-acetyl cysteine (NAC), dithiothreitol (DTT), rotenone (ROT), 4, 4′-diisothiocyanatostilbene-2, 2′-disulfonic acid (DIDS), 2′,7′-dichlorofluorescein diacetate (DCFDA), and other chemicals were purchased from Sigma-Aldrich Corp (St. Louis, MO). Protein kinase inhibitors PD98059, U0126, wortmannin, LY294002, SB202190, calphostin C, and herbimycin A were purchased from Alexis Biochemicals (Lausen, Switzerland). [α-32P]CTP (500 Ci/mmol) was from NEN Life Science (Boston, MA), and serum-free keratinocyte media (KSFM) from Cambrex Bio Sci. (Walkersville, MD). Other culture media, Hanks’ balanced salt solution, and fetal calf serum were purchased from Invitrogen Corp. (Carlsbad, CA). For an effective transfer of O2 and CO2 in some experiments, cells were cultured in gas-permeable, Lumox™ culture dishes purchased from Greiner Bio-One (Frickenhausen, Germany). Anti-human HIF-1α and anti-rat HIF-1α antisera were generated in rats or in mice against bacterially expressed fragments encompassing amino acids 418–698 of human HIF-1α or rat HIF-1α, as previously described [17]. Antibodies against MEK-1, ERK, and their phosphorylated forms were purchased from Cell Signaling Technology (Danvers, MA). Other antibodies, rabbit anti-p21^WAF1^CIP1, goat anti-ARNT, mouse anti-β-actin, mouse anti-c-Myc, rabbit anti-Ki67, and HRP-conjugated secondary antibodies were purchased from SantaCruz Biotechnology Inc. (Santa Cruz, CA). Fluorescence probes (Alexa Fluor 488 green and Alexa Fluor 568 red) were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell culture

Primary human keratinocytes were cultured from human foreskin samples. The skin specimens were incubated in Hank’s balanced salt solution containing 10 mg/ml dispase to separate the epidermis and dermis [18]. Sheets of epidermis were dissociated using 0.25% trypsin and 1 mM EDTA, and isolated cells were plated in KSFM at 1–3 × 10^6 cells per 100-mm dish. The cells were then grown at 37 °C in a 5% CO2 atmosphere, split in a 1:4 ratio when 60% confluent, and used for experiments at the third passage. HaCaT human keratinocytes, HEK293 human embryonal kidney, and Hep3B human hepatoma cell-lines were cultured in RPMI-1640 medium, Dulbecco’s modified Eagle’s medium, or α-modified Eagle’s medium. All culture media were supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were grown in a humidified 5% CO2 atmosphere at 37 °C in an incubator. When
plating cells 1 day before experiments, cell numbers were adjusted to reach 20–100% of confluence at harvest. After determining cell densities under an inverted microscope, cells were used for experiments.

2.3. Preparation of siRNAs and transfection

To knock-down HIF-1α, synthesized siRNA duplexes were obtained from Invitrogen. The sequences targeting HIF-1α (GenBank number NM_001530) corresponded to nucleotides 994–1018 (siRNA-I), 1204–1228 (siRNA-II), and 360–384 (siRNA-III) of the coding region. For transient transfection, about 40% confluent cells in 60 mm cell culture dishes were transfected with 40 nM siRNA using Lipofectamine (Invitrogen). Cells were allowed to stabilize for 48 h before being used in experiments. To in vivo knock-down HIF-1α in rat skins, a rat HIF-1α siRNA was designed, based on nucleotides 370–387 of the coding region of the rat HIF-1α gene (NM_024359). The HIF-1α siRNA solutions (80 nM) were made in 50 μl of Lipofectamine and injected intradermally into sole skins of six rats, and the control siRNA solutions were injected into the contra-lateral sole skins. Six rats were treated with siRNAs 4 times (3 day intervals), and the sole skins were removed at day 12 after injection started. All animal procedures were performed according to the established procedures described in the Seoul National University Laboratory Animal Maintenance Manual.

2.4. Immunoblotting

Total proteins were separated on 6.5% or 10% SDS/polyacrylamide gels, and transferred to Immobilon-P membranes (Millipore, Bedford, MA), which were then blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature for 1 h, and incubated overnight at 4 °C with primary antibodies diluted 1:1000 in 5% nonfat milk in TTBS. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 in 5% nonfat milk in TTBS. Antibody–antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (Amersham Biosciences, Piscataway, NJ).

2.5. Semiquantitative RT-PCR

To quantify mRNA levels, we used a highly sensitive, semiquantitative RT-PCR method, as previously described [17]. Total RNAs were isolated from cultured cells using TRIZOL (Invitrogen) and reverse-transcribed at 48 °C for 30 min. cDNAs were amplified over 18 PCR cycles (94 °C for 30 s, 53 °C for 30 s, and 68 °C for 30 s) in a 20 μl of reaction mixture containing 5 μl of dCTP and 250 nM of each primer. PCR products (5 μl) were electrophoresed on 4% polyacrylamide gels, and dried gels were autoradiographed. The nucleotide sequences (5′ to 3′) of our primer pairs are: GATGGCAACCTCGAATTTGGTCT and GAAATCTGTACAGCTGCTGCT for p21, CAGAATCCACAAAACCCCTAGA and CCGCTGAAAAACATTTCTTCC for p27, and ATGGAGGAGGCGGAGTGTGAGAT and TGGTAGGTTTCTGGAAG for p53. Primers for VEGF, PGK 1, enolase 1, and β-actin were constructed as previously described [17].

2.6. Immunohistochemistry

HIF-1α and p21 were analyzed by immunohistochemistry staining in formalin-fixed, paraffin-embedded biopsy sections of human or rat skin. Six-micrometer serial sections were cut from each paraffin block, and immunostained by deparaffinizing and rehydrating through a graded alcohol series. Antigens were retrieved by heating the sections in a microwave for 5 min in 10 mM sodium citrate (pH 6.0). After blocking nonspecific sites, sections were incubated overnight at 4 °C with anti-p21 (1:100 dilution) or anti-HIF-1α (1:100) antibodies. Control slides were not treated with primary antibodies. To visualize HIF-1α, sections were stained using a standard method [19], avidin–biotin–horseradish peroxidase complex was used to localize bound antibody, and diaminobenzidine was used as the final chromogen. To visualize p21, sections were stained using the Tyramide Signal Amplification Biotin system (PerkinElmer Life Sciences, Boston, MA) and diaminobenzidine. For histological assessment, HIF-1α and p21 were identified at magnifications of 100×.

2.7. Dual fluorescence staining for p21 and Ki67

Skin biopsy slides were deparaffinized, rehydrated, and heated in 10 mM sodium citrate (pH 6.0). After blocking nonspecific sites, sections were incubated overnight at 4 °C with rabbit anti-Ki67 and mouse anti-p21 antisera (1:100). The slides were incubated for 1 h with Alexa Fluor 568 (red)-conjugated goat anti-rabbit IgG (1:1000) for Ki67 staining. The slides were further incubated with biotinylated goat anti-mouse IgG (1:1000) for 1 h, treated with streptavidin–HRP (1:100) (Perkin Elmer, Torrance, CA) for 30 min, incubated with the biotinylated tyramide amplification reagent (1:50) for 10 min, and finally reacted with Alexa Fluor 488 (green)-conjugated streptavidin (1:1000) for 1 h. All nuclei were counterstained with 0.1 μg/ml of DAPI (blue). Fluorescence images were observed using an IX71 microscope (Olympus, Hamburg). Images were captured using an UIS 2 Olympus camera running Image pro Plus 5.1 software (Olympus).

2.8. Cell cycle analysis

HaCaT cells were cultured at densities of 20%, 40% or 80% of confluence, harvested, and fixed in 75% ethanol for 30 min on ice. After washing with PBS, cells were labeled with propidium iodide (0.05 mg/ml) in the presence of RNase A (0.5 mg/ml), and incubated at room temperature in the dark for 30 min. Propidium iodide incorporated into DNA was excited at 488 nm and detected at 650 nm, using a FACStar flow cytometer (BD Biosciences).

2.9. ROS detection

HaCaT cells were cultured at densities of 40% or 80% of confluence. After washing cells in pre-warmed PBS, culture media were replaced with a Hank’s Balanced Salt Solution that had been pre-incubated at 37 °C in a 5% CO2 atmosphere. Cells were then treated with 50 μM DCFDA for 30 min in the dark, and detached with trypsin–EDTA solution. After a brief washing, the oxidized form of DCFDA, fluorescent dichlorofluorescein was excited at 488 nm and detected at 530 nm, using a FACStar flow cytometer.

2.10. Statistical analysis

All data were analyzed using Microsoft Excel 2002 software, and results are expressed as means and standard deviations. The Mann–Whitney U test (SPSS 10.0 for Windows, Chicago, IL) was used to compare cell cycle phases, protein levels, and reporter activities. Differences were considered statistically significant at the p<0.05 level. All statistical tests were two-sided.

3. Results

3.1. Cell cycle arrest and p21 expression depend on keratinocyte cell density

The transition from highly proliferative cell at low cell density to stationary cell at high cell density is the earliest event in the program of keratinocyte terminal differentiation [20]. As expected, at a cell density of 80%, the G1 population significantly increased at the expense of the S and G2/M populations in HaCaT cells (Fig. 1A). We next examined the mRNA levels of the cyclin inhibitors p21, p27, and p53, and found that p21 mRNA was induced in a cell density-dependent manner (Fig. 1B). The cell density-dependent expression of p21 protein was also identified in both HaCaT cells and primary epidermal keratinocytes (Fig. 1C). However, p21 was barely detected in human normal skins by conventional immunohistochemistry using avidin–biotinylated enzyme complex (ABC). To increase the sensitivity of immunohistochemistry, we stained p21 with the Tyramide Signal Amplification (TSA) Biotin system, which was
reported to enhance the antigen detection sensitivity up to \( \times 100 \) fold versus the conventional ABC system [21]. As a result, p21 was successfully detected in the nuclei of epidermal keratinocytes in human normal skins (Fig. 1D), as previously reported [22]. To examine the role of p21 in keratinocyte growth arrest, p21 and Ki67 (a marker for proliferating cell) were co-stained in the human skin tissues and visualized under fluorescent microscopy. Fig. 1E shows that red Ki67 and green p21 were rarely merged in keratinocytes, indicating that p21 was expressed only in non-proliferating keratinocytes. This result further supports that p21 expression is associated with keratinocyte growth arrest.

3.2. HIF-1\( \alpha \) is expressed in keratinocytes and mediates growth arrest

As was observed for p21 expression, HIF-1\( \alpha \) was induced in a cell density-dependent manner in both primary epidermal keratinocytes and HaCaT cells (Fig. 2A). Although non-keratinocyte cell-lines HEK293 and Hep3B are capable of expressing HIF-1\( \alpha \) in response to desferrioxamine, they did not express HIF-1\( \alpha \) at high cell density (Fig. 2B). To rule out the possibility that the 80% confluence makes the medium hypoxic and consequently induces HIF-1\( \alpha \), oxygen dissolved in the

![Fig. 2. HIF-1\( \alpha \) expression in human keratinocytes. A) Cell density-dependent expression of HIF-1\( \alpha \) in human keratinocytes. Keratinocytes isolated from human normal skin and HaCaT cells were cultured to reach the indicated cell densities, or treated with 130 \( \mu \)M desferrioxamine (DFO) for 8 h. HIF-1\( \alpha \) and \( \beta \)-actin expressions were analyzed by immunoblotting. B) HIF-1\( \alpha \) expression in other human cells. HEK293 and Hep3B cells cultured to reach the indicated cell densities, HIF-1\( \alpha \) was analyzed by immunoblotting. C) Oxygen tension in culture media. HaCaT cells were cultured to reach the indicated cell densities, and partial pressure of oxygen dissolved in the media was measured using Dissolved Oxygen Meter with oxygen electrode provided from World Precision Instruments (Sarasota, FL). The mean (% O₂) and SD values were calculated from 3 experiments. D) Cell density-dependent expressions of HIF-1\( \alpha \) and p21 in gas-permeable dishes. HaCaT cells were cultured to reach the indicated cell densities in Lumox oxygen permeable dishes, and HIF-1\( \alpha \) and p21 were analyzed by immunoblotting. E) Expression of hypoxia-induced genes. In HaCaT cells reaching the indicated cell densities, mRNA levels of enolase 1 (Enol 1), phosphoglycerate kinase 1 (PGK 1), and \( \beta \)-actin were determined by semiquantitative RT-PCR. F) Hypoxic responses of HaCaT cells. HaCaT cells in 40% or 80% confluence were incubated under normoxic (N) or hypoxic (H) conditions for 16 h, and then proteins and mRNAs were analyzed by Western blotting (WB) and RT-PCR, respectively.](image-url)
medium was measured using an oxygen meter. Oxygen levels tended to decrease in cell density-dependent manner, but the oxygen drop in 80% confluence was not significant (−1.4%, \( p = 0.25 \)) compared to that in 40% confluence (Fig. 2C). To prevent the oxygen drop during sub-confluent or confluent culture, HaCaT cells were cultured in gas-permeable dishes, and it was found that HIF-1\( \alpha \) and p21 expressions were still induced in a cell density-dependent manner (Fig. 2D). Compared to those in 80% confluence, both expressions further increased in 100% confluence. Moreover, mRNAs of hypoxia-induced genes, such as enolase 1 and PGK 1, were not induced in 80% confluence (Fig. 2E), whereas they were noticeably induced by hypoxia in both 40% and 80% confluence (Fig. 2F). These results suggest that the cell density-dependent HIF-1\( \alpha \) expression is a unique feature of epidermal keratinocyte, rather than a hypoxic event.

3.3. HIF-1\( \alpha \) mediates keratinocyte growth arrest

To examine whether HIF-1\( \alpha \) mediates p21 expression and growth arrest, we knocked-down HIF-1\( \alpha \) using 3 different siRNAs (I, II and III). All HIF-1\( \alpha \) siRNAs effectively reduced HIF-1\( \alpha \) expression in keratinocytes. Concomitantly, these siRNAs noticeably reduced p21 expression (Fig. 3A) and released G1 arrest at a cell density of 80% (Fig. 3B). These results suggest that HIF-1\( \alpha \) plays an upstream player in p21 expression and growth arrest of keratinocytes. In terms of p21 induction by HIF-1\( \alpha \), two mechanisms have been proposed; c-Myc downregulation due to disruption of the \( \beta \)-catenin/TCF4 complex [15] and c-Myc displacement from p21 promoter [16]. Since the latter event induces p21 without any change in c-Myc expression, c-Myc measurement can provide an indirect evidence in identifying which event contributes to p21 induction in keratinocytes. In spite of p21 suppression, c-Myc levels were not altered by HIF-1\( \alpha \) knock-down (Fig. 3A). When HIF-1\( \alpha \) was induced under normoxic or hypoxic conditions, p21 expression was all induced in HaCaT cells. However, c-Myc expression was not repressed by normoxic HIF-1\( \alpha \) induced by chemical inducers, whereas it was noticeably reduced by hypoxic HIF-1\( \alpha \) (Fig. 3C). Moreover, c-Myc expression was not affected by cell density under normoxic conditions (Fig. 3D). Based on these results, the HIF-1\( \alpha \)-mediated p21 induction in normoxic keratinocytes may be attributed to the c-Myc displacement from p21 promoter, rather
than the β-catenin/TCF4 disorganization. It is also suggested that HIF-1α regulates p21 expression in different ways under normoxic and hypoxic conditions. The exact mechanism underlying the cell density- and HIF-1α-dependent induction of p21 in keratinocytes remains to be further investigated.

3.4. HIF-1α is expressed in the epidermis and associated with epidermal homeostasis

In specimens of human normal skin, HIF-1α was found to be robustly expressed in the nuclei of epidermal keratinocytes (Fig. 4A). To in vivo examine the role of HIF-1α in epidermal homeostasis, we treated a rat HIF-1α siRNA into rat plantar skins for 12 days, and confirmed that keratinocyte HIF-1α expression was inhibited (Fig. 4B, left panel). As shown in cultured keratinocytes, p21 expression in the epidermis of rat plantar skins was also attenuated after HIF-1α siRNA treatment (Fig. 4B, right panel). To examine whether HIF-1α controls epidermal proliferation, the rat plantar skin slides were stained with H&E dyes. The epidermal layers in HIF-1α siRNA-treated soles became significantly thicker, compared to those in the contra-lateral soles treated with the control RNA (Fig. 4C).

Fig. 4. HIF-1α expression and its role in the epidermis. A) HIF-1α expression in human skins. Biopsy sections from human normal skin were incubated with anti-HIF-1α antiserum or preimmune rabbit serum (-antibody). HIF-1α (arrow) was identified at a magnification of 100×. [E], epidermis; [D], dermis. B) p21 suppression in HIF-1α knocked-down skins. Six rats were treated with rat HIF-1α siRNA (siHIF), control RNA (SC), or Lipofectamine (Mock) solutions 4 times (3 day intervals). Rat plantar skins were immunostained with HIF-1α (blue arrow) and p21 (red arrow) antisera, and examined at 100×. Results are representative of 6 experiments. C) Epidermal thickening by knocking-down HIF-1α. The siRNA-treated plantar skins were stained with H&E dyes, and the thickness of the epidermis was analyzed in 12 randomly selected regions from each slide. Measurements were performed at a magnification of 40× with the investigator blinded as to the identity of the samples. Data are presented as the means±SD of samples from 6 rats.
These results strongly support the involvement of HIF-1α in epidermal homeostasis.

3.5. HIF-1α is stabilized density-dependently

How is HIF-1α regulated in keratinocytes? To answer this question, we first measured the mRNA levels of HIF-1α, but found that they did not increase (Fig. 5A). We also examined the de novo synthesis of HIF-1α protein, but observed no differences (Fig. 5B). We next compared the degradation rates of HIF-1α protein, and found that HIF-1α degradation was significantly delayed in 80% confluence vs. that in 40% confluence (Fig. 5C, left panel). Based on protein band intensities, protein half-lives ($t_{1/2}$) were calculated from the slopes of first-order decay curves (Fig. 5C, right panel). HIF-1α half-life was prolonged to 8.47 min in 80% confluence (vs. 3.89 min in 40% confluence). These results suggest that HIF-1α induction in keratinocytes is attributable to stabilization at the protein level.

3.6. HIF-1α stabilization is mediated by mitochondrial ROS and the MEK/ERK pathway

To identify the mechanism of HIF-1α stabilization, we examined the effects of various protein kinase inhibitors on HIF-1α expression. Of these inhibitors, two MEK/ERK inhibitors abolished HIF-1α expression in 80% confluence (Fig. 6A), and MEK and ERK were activated in a cell density-dependent manner (Fig. 6B), suggesting that the MEK/ERK pathway mediates HIF-1α expression in keratinocytes. Thus, we addressed the up-stream regulation of the MEK/ERK pathway. Reactive oxygen species (ROS) are known to activate the MEK/ERK pathway. Attene-
Ramos et al. reported that ROS production increases in overgrown intestinal epithelial cells [23]. Thus, we examined the involvement of ROS in MEK/ERK activation and HIF-1α expression. Intracellular ROS levels were significantly higher in 80% confluence than those in 40% confluence (Fig. 7A). Here, DTT was used to verify ROS-dependent fluorescence. Antioxidants DTT and NAC were found to block MEK/ERK activation (Fig. 7B) and HIF-1α expression (Fig. 7C). We next examined the possibility that mitochondria produce the ROS that underlie HIF-1α expression. Accordingly, we used two inhibitors of mitochondrial ROS generation, rotenone and DIDS, which reduce ROS production from mitochondria by inhibiting the respiratory chain and by blocking the release of superoxide anions through mitochondrial anion channels, respectively. Both inhibitors were found to reduce HIF-1α expression in 80% confluence (Fig. 7D). Moreover, antioxidants and mitochondrial inhibitors all reduced p21 expression in 80% confluence (Fig. 7E). Based on all results, we propose a mechanism of growth arrest of keratinocytes, i.e., ROS generation by mitochondria → MEK/ERK activation → HIF-1α stabilization → p21 induction, and finally cell cycle arrest.

**4. Discussion**

p21 is viewed as a prime contributor to the differentiation-associated growth arrest, but little is known about the mechanism whereby p21 expression is regulated in keratinocytes. In the present study, p21 was found to be expressed in human skins and in cultured keratinocytes. Moreover, p21 expression...
and G1 arrest were found to be induced in a cell density-dependent manner. Similarly, HIF-1α was also expressed in human skins and induced in cultured keratinocytes density-dependently. siRNA experiments revealed that HIF-1α determines p21 expression and keratinocyte growth arrest both in vitro and in vivo. These findings imply that HIF-1α is involved in epidermal homeostasis.

In terms of physiological roles, HIF-1α of keratinocyte is also different from that of other cells. In spite of a noticeable expression of HIF-1α, angiogenesis or glycolysis promoting genes were not activated in keratinocytes, which suggests that HIF-1α expressed at high cell density is not related to the adaptation to hypoxia. Instead, keratinocyte HIF-1α functions as the up-stream player in the p21-mediated growth arrest. Previously, such a function of HIF-1α was described in cancer cells cultured under hypoxic conditions [15,16], and this is considered to underlie the tumor growth inhibition under hypoxic conditions. However, the HIF-1α-dependent growth arrest has not been reported in normal tissues or cells. Our results suggest that HIF-1α participates in the development of the normal skin by controlling keratinocyte proliferation.

Since HIF-1α is degraded in a few minutes after de novo synthesis, in most normal tissues HIF-1α is present at undetectable levels by immunoblotting [24,25]. Only when tissues are subjected to ischemia or hypoxia, HIF-1α can be expressed at detectable levels. Therefore, constitutive HIF-1α expression in the normal human epidermis is a very interesting phenomenon. What stimulates HIF-1α expression in the epidermis? Low oxygen levels in the epidermis could be associated with HIF-1α expression. Indeed, the oxygen status of skin remains controversial despite many studies measuring oxygen partial pressure in animal skins. However, a recent study using EF5 oxygen indicator demonstrated that the epidermis of human skin is moderately hypoxic [26]. Then, the HIF-1α expression in human or rat epidermis may be attributable to the physiologically hypoxic environment in the epidermis. If this is the only reason for HIF-1α expression, HIF-1α could not be expressed in keratinocytes cultured in aerobic conditions. However, even after being isolated from the epidermis, keratinocytes retain the ability to constitutively express HIF-1α. Therefore, keratinocytes seem to have a unique system to regulate HIF-1α. We here propose that in addition to hypoxia, the ROS-MEK/ERK pathway signals the constitutive expression of HIF-1α in the epidermis.

Despite substantial accumulation of HIF-1α in 80% confluence, the transcriptions of genes directly govern by HIF-1α were not induced, which suggests that keratinocyte HIF-1α expressed in normoxia has little transcription activity. In the absence of a hypoxic signal, the transcriptional activity of HIF-1α is known to be repressed by factor-inhibiting HIF (FIH). FIH is an oxygen-dependent enzyme that hydroxylates an asparagine residue (803) within the C-terminal transactivation domain of HIF-1α and in turn blocks the recruitment of p300 co-activator [27]. In hypoxia, FIH is inactivated due to limited oxygen, and the unmodified HIF-1α can recruit p300 and actively express its target genes. According to this regulation, keratinocyte HIF-1α induced under normoxic conditions could be inactive in gene transcription. In contrast, the p21 induction by HIF-1α does not require the activity of the transactivation domain of HIF-1α, or rather it is achieved by its interaction with other proteins such as c-Myc and β-catenin [15,16]. Since the N-terminal of HIF-1α is necessary and sufficient for the protein interaction and p21 induction, keratinocyte HIF-1α having the interacting domain can associate with these proteins and induce p21 expression, despite a low transcriptional activity.

HIF-1α was stabilized in keratinocytes at high cell densities. As cell density increases, the microenvironmental oxygen tension around cells would be expected to fall, which may stabilize HIF-1α. However, other types of cells did not express HIF-1α even in sub-confluent cultures. Therefore, the oxygen tension at a cell density of 80% might not drop to the critical level required for HIF-1α stabilization, which was also confirmed by measuring dissolved oxygen tension. Instead, we found that intracellular ROS production significantly increased in 80% confluent keratinocytes, and that antioxidants and mitochondrial inhibitors prevented the HIF-1α expression. These results suggest that mitochondrial ROS participate in cell density-dependent HIF-1α expression in keratinocytes. Similarly, ROS-dependent HIF-1α expression has also been reported in other types of cells. Indeed, antioxidants including ascorbate, vitamin E, pyrroloidinedithiocarbamate, and NAC block the non-hypoxic induction of HIF-1α by thrombin, PDGF, TGF-β, IGF-1, arsenite, or CoCl2 [28–30]. Although sources of ROS generation and their specific relevance to HIF-1α expression have not been fully resolved, mitochondria have long been implicated in HIF-1α regulation. HIF-1α is destabilized in mitochondria-deficient ρ0 [31] or complex III-deficient cells [32,33], and the mitochondrion-targeting antioxidant MitoQ prevents HIF-1α stabilization [34]. In terms of signal transduction, three pathways have been suggested to be responsible for non-hypoxic HIF-1α expression; phosphatidylinositol 3-kinase (PI3K), p38 MAPK and p44/42 MAPK (ERK) [35]. Of these, ERK is more likely to mediate the cell density- and ROS-dependent stabilization of HIF-1α in keratinocytes, since MEK and ERK are both activated in a cell density-dependent manner and inactivated by antioxidants. Moreover, MEK/ERK inhibitors prevented HIF-1α expression, but PI3K and p38 MAPK inhibitors did not. However, little is known about the mechanism whereby ERK stabilizes HIF-1α.

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References


