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Ascochlorin Inhibits Growth Factor-Induced HIF-1α Activation and Tumor-Angiogenesis Through the Suppression of EGFR/ERK/p70S6K Signaling Pathway in Human Cervical Carcinoma Cells

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ABSTRACT

Ascochlorin, a non-toxic prenylphenol compound derived from the fungus *Ascochyta viciae*, has been shown recently to have anti-cancer effects on various human cancer cells. However, the precise molecular mechanism of this anti-cancer activity remains to be elucidated. Here, we investigated the effects of ascochlorin on hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression in human epidermoid cervical carcinoma CaSki cells. Ascochlorin inhibited epidermal growth factor (EGF)-induced HIF-1α and VEGF expression through multiple potential mechanisms. First, ascochlorin selectively inhibited HIF-1α expression in response to EGF stimulation, but not in response to hypoxia (1% O_2) or treatment with a transition metal ($CoCl_2$). Second, ascochlorin inhibited EGF-induced ERK-1/2 activation but not AKT activation, both of which play essential roles in EGF-induced HIF-1α protein synthesis. Targeted inhibition of epidermal growth factor receptor (EGFR) expression using an EGFR-specific small interfering RNA (siRNA) diminished HIF-1α expression, which suggested that ascochlorin inhibits HIF-1α expression through suppression of EGFR activation. Finally, we showed that ascochlorin functionally abrogates in vivo tumor angiogenesis induced by EGF in a Matrigel plug assay. Our data suggest that ascochlorin inhibits EGF-mediated induction of HIF-1α expression in CaSki cells, providing a potentially new avenue of development of anti-cancer drugs that target tumor angiogenesis. J. Cell. Biochem. 113: 1302–1313, 2012.

KEY WORDS: EGFR; HIF-1α; VEGF; ANGIOGENESIS; ASCOCHLORIN

Angiogenesis, the formation of new blood capillaries from the existing blood vascular network, is essential for the delivery of nutrients and oxygen to cells that are distant from existing blood vessels [Folkman and Shing, 1992]. Angiogenesis is an essential component of normal physiological processes (e.g., embryonic development and wound healing) [Breier, 2000] as well as many pathological processes (e.g., cardiac ischemia, diabetic retinopathy, and tumor growth and metastasis) [Iruela-Arispe and Dvorak, 1997].

Ji-Hak Jeong and Yun-Jeong Jeong contributed equally to this work.

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In general, the limit of diffusion between spheroidal tumor tissue and existing blood vessels is estimated as no more than 100 μm in order for the tumor to be supplied with nutrients and oxygen via diffusion [Guillemin and Krasnow, 1997]. Furthermore, tumor blood vessels are typically disorganized and lack structural integrity, making them prone to collapse and creating areas of inadequate perfusion and transient hypoxia [Folkman and Shing, 1992]. Angiogenesis is the most effective mechanism of overcoming the lack of nutrients and oxygen, and accumulation of waste products, in a growing tumor.

Hypoxia is one of the major drivers of tumor progression. Under hypoxic conditions, most tumor cells trigger angiogenesis through an oxygen sensing mechanism by increasing synthesis of angiogenic factors and decreasing the synthesis of anti-angiogenic factors [Semenza, 2000]. The adaptation of tumor cells to hypoxia also involves metabolic changes, such as increased glucose transport and glycolysis, and a shift in the balance between pro- and anti-apoptotic factors to promote survival. Although several transcription factors have been implicated in the cellular response to hypoxia, such as AP-1, NF-κB, and hypoxia-inducible factor-1 (HIF-1) [Faller, 1999], HIF-1 is the most potent inducer of expression of genes involved in angiogenesis. HIF-1 regulates the transcription of a number of genes that are crucial for tumor survival under hypoxic conditions, including vascular endothelial growth factor (VEGF), erythropoietin and several glycolytic enzymes [Semenza et al., 1996; Iyer et al., 1998; Ryan et al., 1998; Semenza, 2000]. HIF-1 also associates with c-Myc and counteracts its transcription repression against genes including $p21^{CIP1}$, hTERT, and BRCA1, or its transcription activation for mismatch repair genes, affecting genetic stability of tumor cells [Koshiji et al., 2004]. Experimental and clinical studies implicate relationship of HIF-1 expression to poor prognosis and chemotherapy resistance of cancer.

HIF-1 is a heterodimeric protein complex consisting of HIF-1α and HIF-1β subunits, which are basic helix-loop-helix-PAS domain proteins [Wang and Semenza, 1995]. HIF-1β is constitutively expressed and its expression is not affected by changes in oxygen partial pressure (ρO_2). In contrast, HIF-1α expression is tightly regulated by O_2 pressure and HIF-1α accumulates rapidly in cells exposed to hypoxic conditions [Wang et al., 1995; Jewell et al., 2001]. HIF-1α is regulated at the post-translational level by hydroxylation, acetylation, and phosphorylation [Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Jeong et al., 2002]. Under normoxic conditions, HIF-1α is hydroxylated by a set of closely related Fe^{2+} and 2-oxoglutarate-dependent prolyl-4-hydroxylase domain containing protein (PHD) 1–3 at two proline residues (Pro^{402} and Pro^{564}) within the HIF-1α oxygen-dependent degradation (ODD) domain, and is subsequently recognized by the von Hippel–Lindau (VHL) ubiquitin ligase complex, which results in ubiquitination and degradation of HIF-1α through the 26S proteasome pathway. Under hypoxic conditions, prolyl hydroxylation of HIF-1α is impaired, leading to a decrease in pVHL-ubiquitination and increased HIF-1α stability [Ivan et al., 2001; Jaakkola et al., 2001]. In addition, hypoxia activates transactivating activity of HIF-1α through the recruitment of p300 histone acetylase via the C-terminal transactivation domain (CTAD), which is suppressed through the hydroxylation of Asn^{803} in normoxic condition, which is mediated by another Fe^{2+} and 2-oxoglutarate-dependent prolyl-4-hydroxylase, factor inhibiting HIF-1 (FIH-1) [Guillemin and Krasnow, 1997; Semenza, 2000; Bruick and McKnight, 2001; Jaakkola et al., 2001; Maxwell and Salnikow, 2004]. Transcription activation of HIF-1α is also regulated through cooperative binding of VHL and FIH-1, which recruit histone-deacetylase (HDAC) to HIF-1α.

Expression of HIF-1α is induced by growth factors that stimulate tyrosine kinase receptors including ErbB family receptors and insulin like growth factor receptor. Growth factor stimulation induces HIF-1α protein synthesis via a signal transduction pathway involving phosphotidylinositol-3-kinase (PI3K), mammalian homologue target of rapamycin (mTOR) and AKT, which are essential for the phosphorylation and regulation of protein translational regulators such as p70S6K and 4E-BP1 [Semenza, 2003]. Eukaryotic translation initiation factor (eIF-4F) performs the rate-limiting function of recruiting the 40S ribosome subunit to mRNA, with the eIF-4E subunit binding directly to the 5′ cap structure. 4E-BP-1 binds eIF-4E and inhibits its activity. Phosphorylation of 4E-BP-1 decreases its ability to bind eIF-4E, and thus positively regulates translation. While the p70 and p85 kinase (S6K), which phosphorylate the S6 protein of 40S ribosomal subunit, have been shown to control the translation of mRNAs that containing polypyrimidine tracts within their 5′-UTR, the HIF-1α 5′-UTR contains tracts of 8, 9, and 17 pyrimidines downstream of nucleotide +32. S6K contains multiple site phosphorylated by RAS/RAF/ERK and PI3K/AKT/mTOR pathways, those phosphorylation events contribute to the multistep activation of S6K. Activation of PI3K/mTOR/AKT signal pathway also suppresses expression of HSP70 and HSP90, stabilizers of HIF-1α protein, subsequently reducing cellular HIF-1α activity.

Ascochlorin (Fig. 1A) is a prenyl-phenol compound isolated from the fungus *A. viciae* [Tamura et al., 1968]. Although ascochlorin-related compounds were originally characterized as antiviral antibiotics [Tamura et al., 1968], they have a variety of physiological functions, including hypolipidemic activity [Sawada et al., 1973], suppression of hypertension [Hosokawa et al., 1981], amelioration of type I and II diabetes [Hosokawa et al., 1985], immunomodulation [Magae et al., 1986], and antitumor activity [Magae et al., 1988]. Ascochlorin has been shown to possess anti-tumor activity, making it a compound of special interest in the prevention and/or treatment of cancer, but the precise mechanism for the antitumor activity is unknown. We recently demonstrated that ascochlorin activates p53 and enhances transcription of p53 downstream targets, including $p21^{WAF1/CIP1}$ and Hdm2 [Jeong et al., 2009], and the induction of G1 arrest by ascofuranone is associated with the p53-independent activation of $p21^{WAF1/CIP1}$ through disruption of c-Myc [Jeong and Chang, 2010; Jeong et al., 2010]. In human renal carcinoma cells, ascochlorin and related compounds selectively suppress AP-1 activity and AP-1 downstream targets, such as matrix metalloproteinase-9 (MMP-9), through suppression of the ERK-1/2 signaling pathway [Hong et al., 2005; Cho et al., 2007]. Moreover, proteome analysis of ascochlorin-treated human osteosarcoma cells indicated that this compound decreases the expression of several genes in the MAP kinase signaling cascade, including epidermal growth factor receptor (EGFR) and ERK-1/2 [Kang et al., 2006]. These findings suggest the possibility that ascochlorin suppresses tumor

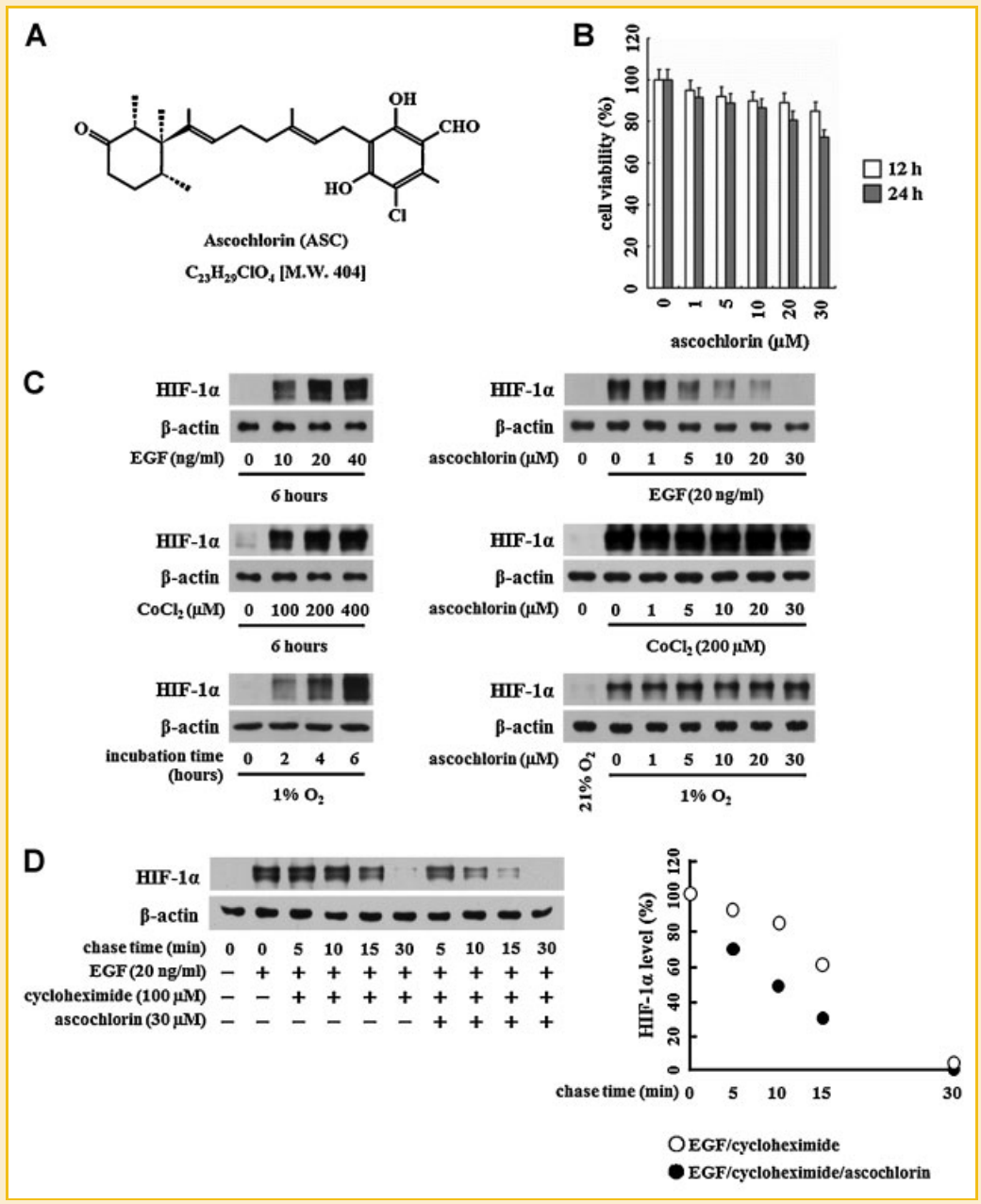

Fig. 1. Effect of ascochlorin on expression and stability of HIF-1α in CaSki cells. A: Chemical structure of ascochlorin. B: Dose-dependent effect of ascochlorin on the viability of CaSki cells. Cells were treated with the indicated concentrations of ascochlorin for 12 or 24 h. Viability was determined by MTT assay. Values represent the means ± SD of triplicate assays. C: HIF-1α expression in the presence of ascochlorin in CaSki cells. Cells were pretreated with the indicated concentrations of ascochlorin for 1 h, and then stimulated by hypoxia, $CoCl_2$ treatment, or EGF treatment. Nuclear extracts were subjected to Western blot using antibodies against HIF-1α or β-actin. (D) Effect of ascochlorin on HIF-1α protein stability. EGF-treated CaSki cells were cultured in the presence or absence of ascochlorin for 1 h, followed by incubation with 100 μM cycloheximide from 0 to 30 min. Expression of HIF-1α and β-actin in nuclear extracts was analyzed by Western blot (*left panel*). Intensity of HIF-1α protein signals obtained by Western blot was quantified by densitometry. HIF-1α expression levels were normalized to β-actin. Data represents means ± SD of three independent experiments (*right panel*).

angiogenesis, because downstream signaling cascade from EGFR regulates expression and activity of HIF-1α, one of major regulators of angiogenesis implicated in poor prognosis in various cancers.

In the present study, we investigated the molecular mechanism of inhibition of angiogenesis by ascochlorin in cervical epidermoid carcinoma CaSki cells. Ascochlorin significantly inhibited epidermal growth factor (EGF)-induced HIF-1α stabilization via the EGFR/ERK/p70S6K signaling pathway and suppressed angiogenesis in vivo. Our results provide important clues to the molecular basis of the anticancer effects of ascochlorin, and contribute to the development of novel EGFR-targeted anticancer agents as well as effective HIF-1-targeted cancer therapeutics.

MATERIALS AND METHODS

CELLS AND MATERIALS

Cervical epidermoid carcinoma CaSki cells were grown in RPMI 1640 medium supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. For hypoxia treatment, the cells were incubated in a chamber containing 1% oxygen, 5% carbon dioxide, and 94% nitrogen at 37°C. Recombinant human EGF was purchased from R&D Systems (Minneapolis, MN). Cobalt(II) chloride ($CoCl_2$) and cycloheximide were obtained from Sigma Chemicals (St Louis, MO). Specific EGFR, mTOR, p38-MAPK, p42/44 MAPK, c-Jun N-terminal kinase, and phosphoinositide 3-kinases inhibitors (AG1478, rapamycin, SB203580, PD98059, SP600125, and wortmannin, respectively) were obtained from Calbiochem (San Diego, CA). Ascochlorin was supplied by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan).

CYTOTOXICITY ASSAYS

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Roche Applied Science, Indianapolis, IN) assays were performed as described in the supplier's protocol to evaluate the cytotoxicity of ascochlorin.

FRACTIONATION OF CELL EXTRACTS

Following experimental treatment of cells, nuclear and cytosolic extracts were prepared based on the method of Molitor et al. [1990]. Cells were harvested by centrifugation for 5 min at 10,000 rpm, rinsed with ice-cold PBS, and then transferred to a microfuge tube. Cells were resuspended in buffer A (10 mM Hepes, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 300 mM sucrose, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 5 min. The crude nuclear pellet was isolated by a pulsed microcentrifugation spin and the supernatant was immediately placed on dry ice. The nuclear pellet was resuspended in buffer B (20 mM Hepes, pH 8.0, 20% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), and nuclei were vortexed and lysed for 20 min and then clarified by pulsed microcentrifugation.

WESTERN BLOT ANALYSIS

Western blotting of all samples was performed as described previously [Hong et al., 2005] using the indicated primary antibodies and corresponding secondary antibodies specific for whole immunoglobulin from mouse or rabbit (Amersham Biosciences, Buckinghamshire, UK). Immunoreactive proteins were detected using an enhanced chemiluminescence Western blotting kit (Roche Diagnosis, Mannheim, Germany) according to the manufacturer's instructions. The anti-HIF-1α antibody was purchased from BD Transduction Laboratories (San Diego, CA). Specific antibodies for EGFR, phosphorylated p70 S6 kinase (Thr 421/Ser 424), phosphorylated 4E-BP1 (Thr 70), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific antibodies for phosphorylated EGFR (Tyr 1068), phosphorylated p44/42 MAP kinase (Thr 202/Tyr 204), AKT, phosphorylated AKT (Ser 473), mTOR, and phosphorylated mTOR (Ser 2448) were purchased from Cell Signaling Technology (Danvers, MA). The Anti-VEGF antibody was purchased from Abcam (Cambridge, UK).

REVERSE TRANSCRIPTASE (RT)-PCR ASSAY

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using a commercial kit (Superscript II RNase H-reverse transcriptase, Invitrogen) and total RNA (1 μg) from CaSki cells, according to the manufacturer's protocol. The sequences of the primers were as follows: for *VEGF*, 5′-CTACCTCCACCATGCCAAGT-3′ (sense) and 5′-TCTCTCCTATGTGCTGGCCT-3′ (antisense); for *MAPK1*, 5′-AGCAGGAAGCAAAGTGGGAT-3′ (sense) and 5′-CAAGACACACAACCCTTGCC-3′ (antisense); for *EGFR*, 5′-AGGGCTCCACAGCTGAAAAT-3′ (sense) and 5′-CTTGCTGGATGCGTTTCTGT-3′ (antisense); for *β-actin*, 5′-GCCATCGTCACCAACTGGGAC-3′ (sense) and 5′-CGATTTCCCGCTCGGCCGTGG-3′ (antisense). Amplified products were resolved by 1.0% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. We quantified the actual VEGF mRNA level by using Eagle Sight densitometry software (Version 3.21; Stratagene, La Jolla, CA).

VEGF ELISA

VEGF levels in cell culture supernatants were determined by ELISA using the Quantikine human VEGF ELISA kit from R&D Systems. Culture supernatants were collected and cells were counted. VEGF concentrations in the supernatants were determined and normalized to cell number in each well.

RNA INTERFERENCE

CaSki cells at 50% confluency were transfected with 50 nM negative control small interfering RNA (siRNA) or EGFR specific siRNA duplexes, and ERK specific siRNA duplexes (Dharmacon Inc., Chicago, IL) using Trans IT-TKO (Mirus Bio Corp., Madison, WI), according to the manufacturer's instructions.

LUCIFERASE ASSAY

The ability of ascochlorin to inhibit HIF-1α transcription was determined by hypoxia response element (HRE)-dependent reporter assay. In brief, at 50–80% confluency, CaSki cells were co-transfected with pGL3-HRE-Luciferase [Maxwell et al., 1999], which contained six copies of an HRE derived from the human VEGF gene, and pRL-CMV (Promega, Madison, WI), which encoded renilla luciferase (*Rluc*) under the control of a constitutive promoter, using Lipofectamine plus reagent (Invitrogen), according to the manufacturer's instructions. To analyze luciferase expression, cells were washed twice with PBS and then lysed in 200 μl of 1× Reporter lysis buffer (Promega). Luciferase activity in lysate (50 μl) was determined, and the results were normalized to the activity of renilla luciferase.

MATRIGEL PLUG ASSAY

C57BL/6N mice (female, 5-weeks-old) were purchased from Samtako (Osan, Korea) and maintained in pathogen-free conditions. CaSki cells at subconfluence were harvested, washed with PBS, and re-suspended in serum-free medium. Aliquots of cells (3×10^6) were mixed with 0.5 ml of Matrigel in the presence or absence of EGF

(500 ng/ml) and ascochlorin (10 or 30 μM). Immediately, the mixture was subcutaneously injected into mice. The mice were sacrificed when tumors were visible, and the Matrigel plugs were carefully separated from adjacent tissue and removed. The excised plugs were placed in cold PBS at 4°C overnight to liquefy the matrigel. Specimens were subjected to centrifugation at 14,000 rpm and supernatants were collected. Hemoglobin content was determined using Drabkin's reagent kit (Sigma Chemicals), as described previously [Passaniti et al., 1992].

STATISTICAL ANALYSIS

All in vitro results are representative of at least three independent experiments performed in triplicate. A *P*-value of <0.05 between experimental and control groups were considered statistically significant. Differences between experimental and control values were analyzed by analysis of variance using one-way ANOVA.

RESULTS

ASCOCHLORIN INHIBITS HIF-1α EXPRESSION AND STABILITY IN CaSki CELLS IN RESPONSE TO EGF

To understand the mechanism by which ascochlorin (Fig. 1A) exerts its anti-tumor activity, we first tested the cytotoxic effects of ascochlorin on CaSki cells in complete medium using the MTT assay. Treatment of cells with ascochlorin (1–30 μM) resulted in 8–15% decrease in cell viability after 12 h and 8–18% decrease after 24 h (Fig. 1B). As previously reported [Hong et al., 2005; Jeong and Chang, 2010; Jeong et al., 2009], ascochlorin did not exhibit significant cytotoxicity in CaSki cells. To determine the effect of ascochlorin on HIF-1α expression, we examined HIF-1α protein levels in CaSki cells under various conditions (hypoxia (1% O_2), $CoCl_2$, and EGF treatment) that have been shown to stabilize HIF-1α [Zhong et al., 2000; Maxwell and Salnikow, 2004]. As shown in Fig. 1C, pretreatment of CaSki cells with increasing concentrations of ascochlorin resulted in a dose-dependent decrease in HIF-1α protein levels in the nuclear extract induced by EGF, but had no effect on those induced by hypoxia or $CoCl_2$. Based on these results, we chose a dose of 30 μM ascochlorin for all subsequent experiments.

To further analyze the effect of ascochlorin on HIF-1α, we treated CaSki cells with cycloheximide, a de novo protein synthesis inhibitor, and then measured HIF-1α protein half-life in the presence or absence of ascochlorin. Cells were treated with EGF (20 ng/ml) for 6 h to induce HIF-1α accumulation, and then cycloheximide (100 μM) was added alone or in combination with ascochlorin (30 μM). Ascochlorin treatment significantly reduced the half-life of HIF-1α in CaSki cells (control 8.8 min vs. ascochlorin-treatment 21.3 min, calculated by the kinetics of first 15 min).

ASCOCHLORIN SUPPRESSES HRE-MEDIATED REPORTER GENE AND VEGF EXPRESSION IN CaSki CELLS IN RESPONSE TO EGF

To determine whether ascochlorin inhibited HRE-dependent transcription, CaSki cells were transiently co-transfected with an HRE-responsive luciferase reporter gene, as described in the Materials and Methods Section, and then treated with 30 μM ascochlorin. Ascochlorin significantly inhibited HRE-dependent luciferase reporter activity induced by EGF, while it inhibited those induced by $CoCl_2$ or hypoxia in much lesser degree (Fig. 2A). Under these experimental conditions, ascochlorin had no effect on cell viability (data not shown), in agreement with previous reports [Hong et al., 2005; Jeong et al., 2009]. We next investigated whether inhibition of HIF-1α resulted in decreased expression of typical target gene, VEGF. Ascochlorin inhibited not only VEGF mRNA level (Fig. 2B), as assessed by RT-PCR, but also VEGF protein level (Fig. 2C), as assessed by Western blot assay, in a dose-dependent manner under EGF-stimulated conditions. These results are correlated with the effect of ascochlorin on HIF-1α protein levels. Hypoxia or $CoCl_2$ treatment did not induce transcription of VEGF in our system, and ascochlorin inhibited the basic expression of VEGF only slightly. We also examined the effect of ascochlorin on the secretion of VEGF by ELISA in CaSki cells. The concentration of VEGF in culture supernatants was increased more than twofold under EGF-stimulated conditions, but not under hypoxic or $CoCl_2$ treatment conditions (Fig. 2D). Ascochlorin decreased the levels of secreted VEGF in a dose-dependent manner under all three conditions tested, even though VEGF secretion was not increased by hypoxia or $CoCl_2$.

ASCOCHLORIN INHIBITS ERK AND p70S6K PHOSPHORYLATION BY INTERFERING WITH EGFR ACTIVITY

As ascochlorin dramatically decreased EGF-induced HIF-1α protein levels, we were interested in identifying the signaling molecules that were involved in this inhibitory effect. Binding of growth factors to tyrosine kinase receptors, such as EGFR and IGF1R, activates intracellular signaling pathways including PI3K/AKT/mTOR [Laughner et al., 2001; Semenza, 2002], which results in the induction of HIF-1α protein synthesis [Semenza, 2002, 2003]. Unexpectedly however, ascochlorin did not affect the phosphorylation levels of AKT (Ser^{473}), mTOR (Ser^{2448}), and 4E-BP1 (Thr70) (Fig. 3A), while significantly decreased those of EGFR (Tyr^{1068}), ERK (Thr^{202}/Tyr^{204}), and p70S6K (Thr^{421}/Ser^{424}) in EGF-stimulated CaSki cells (Fig. 3B). These results suggested that ascochlorin inhibits EGF-mediated HIF-1α expression through the specific inhibition of EGFR/ERK signal transduction pathway, leading to suppression of $p70^{S6K}$-dependent protein synthesis of HIF-1α.

EFFECTS OF VARIOUS KINASE INHIBITORS ON EGF-INDUCED HIF-1α EXPRESSION IN CaSki CELLS

To determine whether the EGFR signaling pathway was required for EGF-induced HIF-1α expression, CaSki cells were exposed to various kinase inhibitors that targeted individual components in the pathway. AG1478 inhibits EGFR tyrosine kinase activity, and PD98059 selectively blocks the activity of MAPK kinase (MEK), an activator of ERK kinases. Before stimulation with EGF, cells were incubated with AG1478 (10 μM), PD98059 (50 μM), or ascochlorin (30 μM) for 1 h. Cells were then incubated for 6 h in the presence of EGF (20 ng/ml). As expected, AG1478 and PD98059 caused a decrease in EGF-mediated HIF-1α expression, similar to ascochlorin (Fig. 4A). We tested other kinase inhibitors, including SB203580 (100 nM), a specific inhibitor of p38 kinase, SP600125 (10 μM), an inhibitor of c-Jun N-terminal kinase (JNK), rapamycin (20 nM), a

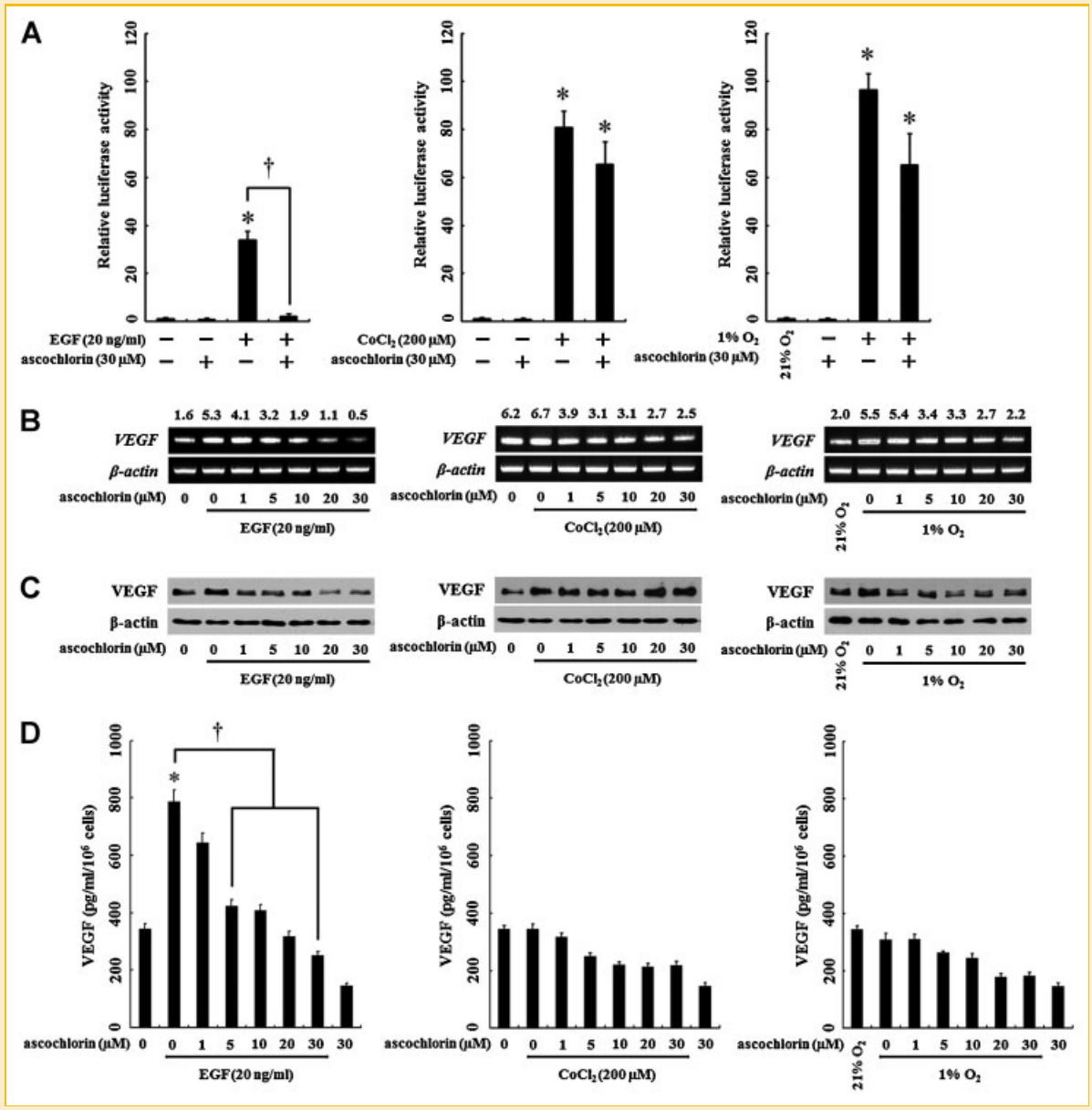

Fig. 2. Effect of ascochlorin on HRE-mediated reporter gene and VEGF expression in CaSki cells. A: CaSki cells were transiently co-transfected with a reporter gene, pGL3-HRE-Luciferase, and pRL-CMV as a reference. Following incubation for 24 h, the cells were incubated under various conditions (hypoxia, $CoCl_2$ treatment, and EGF treatment) in the presence or absence of 30 μM ascochlorin. Luciferase activities were determined as described in Materials and Methods Section. Data represents the means ± SD of three independent experiments. $^{*}P < 0.05$ as compared to untreated control; $^{\dagger}P < 0.01$ as compared to EGF only treatment. Results were analyzed using one-way ANOVA. B: RT-PCR analysis of VEGF mRNA was carried out using total RNA prepared from CaSki cells incubated under various conditions (hypoxia, $CoCl_2$ treatment, and EGF treatment) in the presence or absence of the indicated concentrations of ascochlorin. And the VEGF mRNA expressions were quantified by using Eagle Sight densitometry software (Version 3.21; Stratagene, La Jolla, CA). C: VEGF protein expressions were analyzed by Western blot assay by using total lysates prepared from CaSki cells incubated under various conditions (hypoxia, $CoCl_2$ treatment, and EGF treatment) in the presence or absence of the indicated concentrations of ascochlorin. D: VEGF protein levels in culture supernatants of CaSki cells incubated under various conditions (hypoxia, $CoCl_2$ treatment, and EGF treatment) in the presence or absence of the indicated concentrations of ascochlorin were evaluated by ELISA. The data represents the means ± SD of three independent experiments. $^{*}P < 0.05$ as compared to untreated control; $^{\dagger}P < 0.01$ as compared to EGF only treatment. Data was analyzed using one-way ANOVA.

inhibitor of raptor/mTOR complex, and wortmannin (20 nM), a specific inhibitor of PI3K under similar experimental conditions, and found no effect on HIF-1α expression in EGF-stimulated CaSki cells (Fig. 4A).

To verify these results, we examined the effect of the kinase inhibitors on HRE promoter activity and VEGF secretion in culture supernatants in EGF-stimulated CaSki cells. CaSki cells were incubated with kinase inhibitors for 1 h, and then incubated for 18 h in the presence of EGF. The inductions of HRE promoter activity and VEGF secretion by EGF stimulation were almost completely suppressed by AG1478, PD98059, and ascochlorin, whereas SB203580, SP600125, rapamycin, and wortmannin had no effect

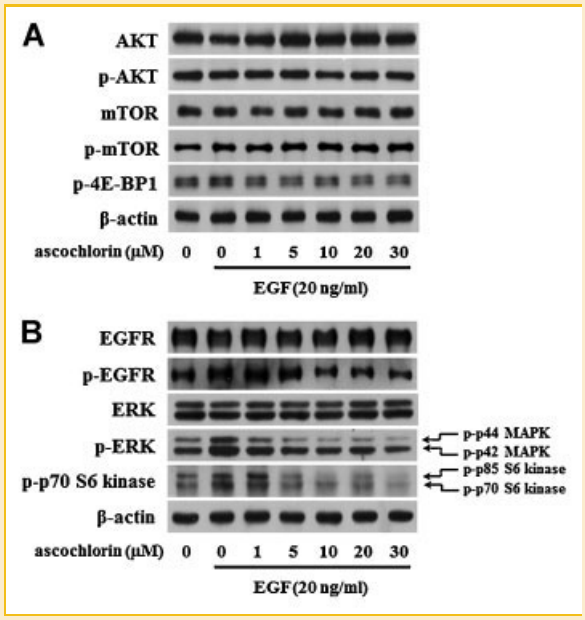

Fig. 3. Effect of ascochlorin on EGFR-mediated signaling leading to the expression of HIF-1α in CaSki cells. CaSki cells were pretreated with the indicated concentrations of ascochlorin for 1 h, followed by incubation with 20 ng/ml EGF for 15 min. The levels of PI3K/AKT/mTOR (A) and RAS/RAF/ERK (B) signaling components were determined by Western blot analysis.

(Fig. 4B,C). These results were consistent with the effects of these inhibitors on HIF-1α expression, and suggested that major signaling pathway for EGF-mediated HIF-1α and VEGF expression in CaSki cells is RAS/RAF/ERK signaling pathway, while PI3K/AKT/mTOR-, p38 kinase-, and JNK-mediated pathways are not involved.

EGFR OR ERK KNOCKDOWN BY siRNA ABROGATES EGF-INDUCED HIF-1α STABILIZATION IN CaSki CELLS

To confirm that EGFR activity is necessary for HIF-1α expression in EGF-stimulated CaSki cells, we designed a siRNA that targeted the EGFR gene. CaSki cells were transfected with a siRNA for EGFR, or a non-specific siRNA as a negative control. Although it was previously reported that an EGFR-targeted siRNA inhibits cell proliferation and induces apoptosis in epidermoid carcinoma cells [Nagy et al., 2003], siRNA-mediated knockdown of EGFR in CaSki cells sustained more than 70% viability. Cells were pretreated with ascochlorin (30 μM) for 1 h, followed by stimulation with EGF (20 ng/ml) for 6 h, and then the expression of EGFR in total lysate and HIF-1α in nuclear extracts was analyzed by Western blot. Knockdown of EGFR resulted in a decrease in EGFR protein levels, and abrogated EGF-induced HIF-1α expression, although the siRNA did not completely knocked down the EGFR mRNA expression (Fig. 5A). Suppressive effect of ascochlorin on HIF-1α expression observed in untransfected cells or cells transfected with control siRNA, was diminished in EGFR-knockdown cells. We also tested another siRNA, targeted the ERK gene to further confirm that ERK activity is necessary for the HIF-1α expression under similar experimental conditions, and found the effects of ERK-targeted siRNA on HIF-1α expression similar with EFGR-targeted siRNA in EGF-stimulated CaSki cells (Fig. 5B). These results were further confirmed with EGF-induced VEGF secretion in EGFR-knockdown or ERK-knockdown CaSki cells incubated with ascochlorin (30 μM) for 1 h, and then treated with EGF (20 ng/ml) for 18 h. VEGF secretion of EGFR- or ERK-knockdown cells was not affected by EGF or ascochlorin treatment (Fig. 5C,D). Taken together, these results indicated that *bona fide* target for the ascochlorin-mediated suppression of EGF-induced HIF-1α activation is EGFR/ERK itself in CaSki cells.

ASCOCHLORIN INHIBITS TUMOR ANGIOGENESIS IN VIVO

Above results suggest that ascochlorin can be clinically used for a potent angiogenesis inhibitor in cancer therapy. To verify this possibility, we assessed the anti-angiogenic effects of ascochlorin in C57BL/6N (female) mice by Matrigel plug assay. In the presence of EGF (500 ng/ml), new blood vessel formation from nearby tissue was induced in CaSki-implanted Matrigel plugs 7 days post injection (positive control). By comparison, treatment with vehicle alone failed to induce the formation of blood vessels in the plug (Fig. 6A). Ascochlorin at a concentration of 10 μM partially inhibited EGF-induced angiogenesis; at a concentration of 30 μM, the effect on angiogenesis was more apparent, and the Matrigel plugs appeared more transparent as compared to the positive control (Fig. 6A). Blood vessel formation in the plugs was also analyzed by measuring hemoglobin contents as a relative angiogenesis index. Hemoglobin content was dramatically elevated in CaSki-implanted plugs in the presence of EGF (500 ng/ml), and ascochlorin treatment (30 μM) decreased the hemoglobin levels increased by EGF in a dose-dependent manner, consistent with the visual observation (Fig. 6B). These results indicated that ascochlorin is a potent inhibitor of tumor angiogenesis in vivo.

DISCUSSION

In the present study, we utilized three different experimental conditions that stimulate expression of HIF-1α expression to implicate HIF-1α-mediated angiogenesis as a target of ascochlorin. The first strategy was to target growth factor receptor tyrosine kinases that regulate the translation of HIF-1α [Semenza, 2002, 2003]. Second, we tested the effect of ascochlorin on HIF-1α under hypoxia (1% O_2). For the third strategy for HIF-1α activation, we used a transition metal that can mimic hypoxia by replacing iron in hypoxia sensor hydroxylases, PHD1-3, or FIH-1. Ascochlorin selectively inhibited expression and activity of HIF-1α induced by EGF treatment, but not those induced by hypoxia or $CoCl_2$. While hypoxia or $CoCl_2$ activate HIF-1α mainly through protein stabilization, growth factors enhance HIF-1α translation by stimulating intracellular signaling pathways including PI3K/AKT/mTOR and RAS/RAF/ERK. We asked which pathway is involved in the inhibitory effect of ascochlorin on EGF-induced HIF-1α accumulation in CaSki cells? and found that ascochlorin significantly inhibits EGF-induced phosphorylations of ERK and S6K in RAS/RAF/ERK pathways, while the phosphorylations of AKT, mTOR, and 4E-BP1 were not significantly increased by EGF treatment, nor was it inhibited by ascochlorin in CaSki

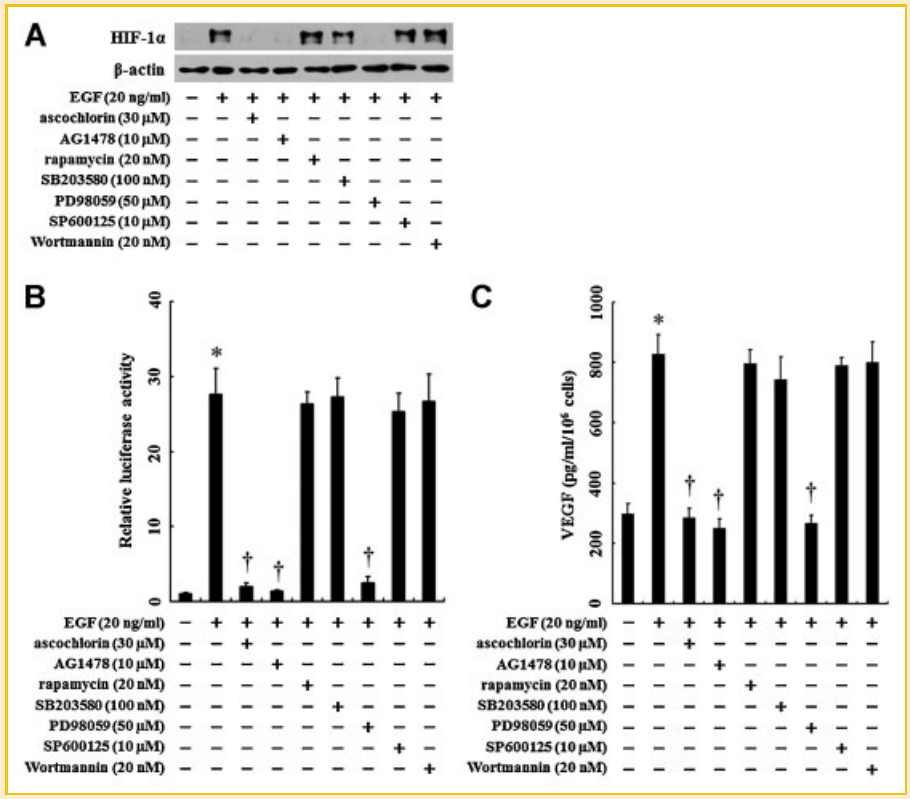

Fig. 4. HIF-1α expression in the presence of ascochlorin and various kinase inhibitors in CaSki cells. A: Western blot analysis of HIF-1α expression in nuclear extracts from CaSki cells pretreated with ascochlorin and the indicated kinase inhibitors for 1 h, followed by stimulation with 20 ng/ml of EGF for 6 h. B: CaSki cells were transiently co-transfected with pGL3-HRE-Luciferase and pRL-CMV. Following 24 h incubation, cells were pretreated with ascochlorin or indicated kinase inhibitors for 1 h followed by stimulation with 20 ng/ml of EGF for 6 h. Luciferase activities were determined as described in the Materials and Methods Section. C: VEGF protein levels in culture supernatants of CaSki cells pretreated with ascochlorin and indicated kinase inhibitors for 1 h followed by stimulation with 20 ng/ml of EGF for 18 h. Data represents the means ± SD of three independent experiments. $^{*}P < 0.05$ as compared to untreated control; $^{\dagger}P < 0.01$ as compared to EGF only treatment. Data was analyzed using one-way ANOVA.

cells. EGF-induced phosphorylation of EGFR itself was also significantly inhibited by ascochlorin. To confirm the crucial role of EGFR in the stabilization of HIF-1α in CaSki cells, we used various kinase inhibitors to block EGFR-mediated signaling pathways, and RNA interference to knockdown EGFR in CaSki cells. Ascochlorin decreased the expression of HIF-1α, comparable to the EGFR and MEK inhibitors, AG1478 and PD98059. EGF treatment failed to increase HIF-1α protein expression in EGFR knockdown cells. These results indicated that the inhibition of HIF-1α protein expression by ascochlorin in EGF-treated CaSki cells is mediated by EGFR-dependent activation of the ERK/p70S6K signaling pathway. These results suggest that p70S6K is a major regulator of HIF-1α translation in CaSki cells, which is suppressed by ascochlorin that prevents the ERK-mediated phosphorylation.

In support of our conclusion, it is reported that IL-6-induced early phosphorylations at Ser^{411} and Thr^{421}/Ser^{424} in C-terminal S6K is mediated by ERK-1/2 and inhibited by PD98059, while subsequent phosphorylation at Thr389 in S6K and phosphorylation of 4E-BP1 is mediated by other kinases including PI3K/AKT/mTOR pathway [Shi et al., 2002]. AKT mutation constructs expressed in human embryonic kidney cells demonstrate that activation of AKT is necessary and sufficient for 4E-BP1 phosphorylation but not for S6K phosphoryation [She et al., 2010]. These reports suggest that phosphorylation and activation of S6K are highly dependent on RAS/RAF/ERK pathway, while those of 4E-BP1 are on PI3K/AKT/mTOR pathway and much less affected by RAS/RAF/ERK pathway.

We also examined the involvement of PI3K or mTOR on EGF-induced HIF-1α accumulation and its transcription activation by using rapamycin, an inhibitor of raptor/mTOR, and wortmannin, an inhibitor of PI3K. mTOR is assumed to be one of kinases phosphorylating 4E-BP-1, and it is also participated directly in activation of S6K by phosphorylation or indirectly in inhibition of phosphatase 2A (PP2A), associated with N-terminal S6K, by phosphorylation [Peterson et al., 1999]. Although it is known that the translation of HIF-1α is affected by on PI3K or mTOR activity [Semenza, 2002; Semenza, 2003], we could not obtain the inhibitory effects of rapamycin or wortmannin, at least in each concentration at 20 nM, in EGF-induced either protein expression or transcriptional activation of HIF-1α (Fig. 4A,B). These results suggested that rapamycin or wortmannin did not converge to inhibition of HIF-1α expression in EGF-treated CaSki cells, which are consistent with the results of ascochlorin that inhibits ERK-mediated HIF-1α expression.

Although the inhibition of S6K phosphorylation suggests that ascochlorin inhibits HIF-1α accumulation through the suppression of S6K-mediated translation activation, we found that ascochlorin

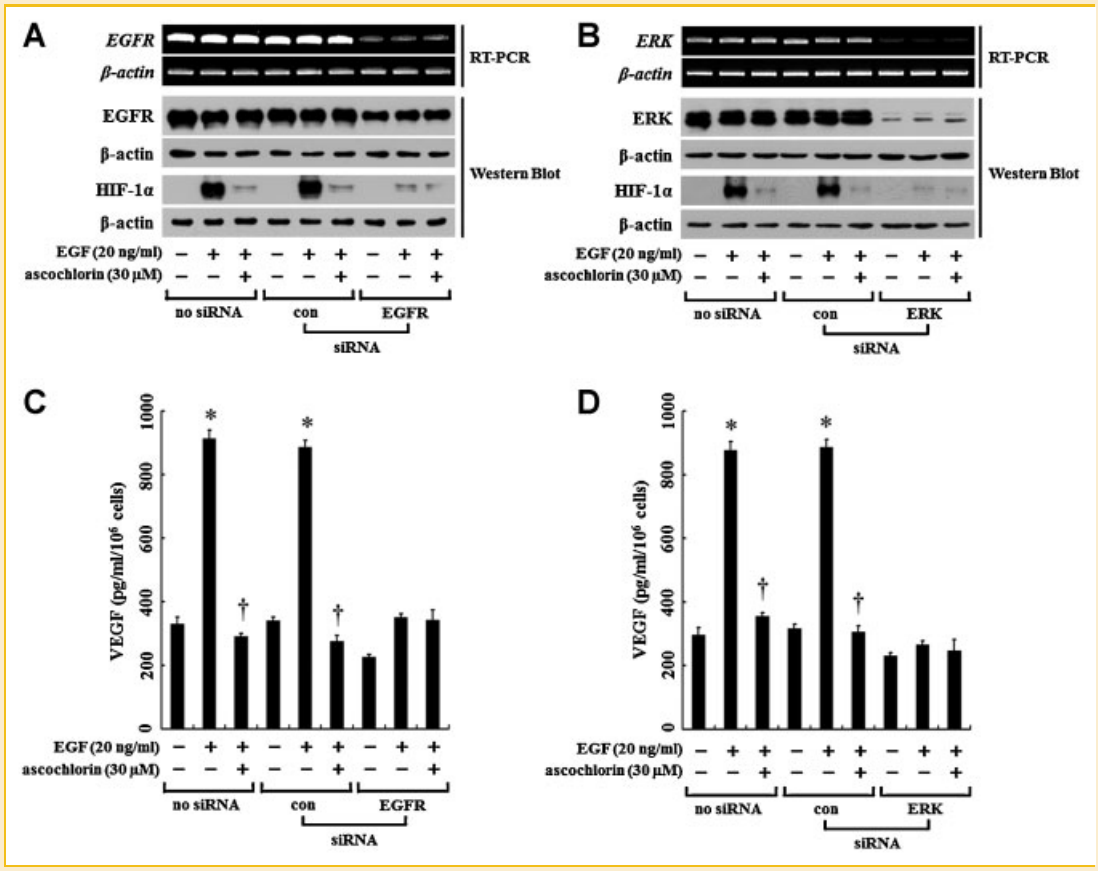

Fig. 5. siRNA-mediated knockdown of EGFR or ERK abrogates EGF-induced HIF-1α stabilization in CaSki cells. A: CaSki cells were transfected with 50 nM of a negative control siRNA, or a siRNA duplex specific for EGFR. B: CaSki cells were transfected with 50 nM of a negative control siRNA, or a siRNA duplex specific for ERK. Before stimulation with EGF, transfected cells were incubated with ascochlorin (30 μM) for 1 h, and then EGF (20 ng/ml) for 6 h. The mRNA levels of EGFR or ERK were determined by RT-PCR, and the protein levels of EGFR or ERK (total lysate) and HIF-1α (nuclear extracts) were determined by Western blot analysis (C) VEGF protein levels in culture supernatant of CaSki cells transfected with EGFR-targeted siRNA. D: VEGF protein levels in culture supernatant of CaSki cells transfected with ERK-targeted siRNA. Cells were pretreated with ascochlorin (30 μM) for 1 h followed by stimulation with 20 ng/ml EGF for 18 h. Data represents the means ± SD of three independent experiments. $^{*}P < 0.05$ as compared to untreated control; $^{\dagger}P < 0.01$ as compared to EGF only treatment. Data was analyzed using one-way ANOVA.

significantly enhanced degradation of HIF-1α. We previously found that ascochlorin activates p53 and increases transcription of its downstream targets including p21 and Hdm2 in a manner distinct from genotoxins [Jeong et al., 2009]. p53 associates with HIF-1α via Hdm2 and facilitates Hdm2-dependent degradation by ubiquitin-dependent mechanism. Consequently, homozygous deletion of p53 in human colorectal cancer promotes neovascularization and growth of tumor xenografts in nude mice. p53 also represses transcription activation of HIF-1α by recruiting HIF-1α to p53 via p300-mediated association [Blagosklonny et al., 1998; Schmid et al., 2004]. Thus, p53 might contribute to suppressive effect of ascochlorin on HIF-1α both through protein destabilization, and through transcription repression. Growth factors activates HIF-1α mediated transcription activity by promoting nuclear translocation through the inhibition of CRM-1-dependent nuclear export [Mylonis et al., 2008]. ERK-dependent phosphorylation at Ser641 and Ser643 is involved in the nuclear translocation, and PD98059 impairs these phosphorylations, nuclear translocation, and transcription activity of HIF-1α. Thus, ascochlorin might suppress nuclear expression and transactivation activity of HIF-1α through inhibition of RAS/RAF/ERK pathway.

Ascochlorin-mediated activation of p53 is related to its effect on mitochondria electron transport because known respiratory inhibitors except oligomycin activates p53 in a manner similar to ascochlorin, and because structure–activity relationship among ascochlorin derivatives suggests close association of p53 activation to respiration inhibition [Jeong et al., 2009]. Consistent with our results, an inhibitor of complex III in mitochondria suppresses HIF-1α induction and angiogenesis [Maeda et al., 2006]. Deletion of mitochondria abolished this effect of antimycin. Antimycin does not affect HIF-1α stability, and suppresses MG132-induced accumulation of HIF-1α, suggesting that antimycin, like ascochlorin, prevents signal transduction cascade regulating HIF-1α translation, although antimycin suppresses HIF-1α activation induced by hypoxia or $CoCl_2$, which were resistant to ascochlorin in our system.

EGFR is overexpressed in a wide range of cancers and aberrant EGFR-mediated signaling is a hallmark of various cancers [Salomon et al., 1995]. EGFR overexpression is frequently found in cervical

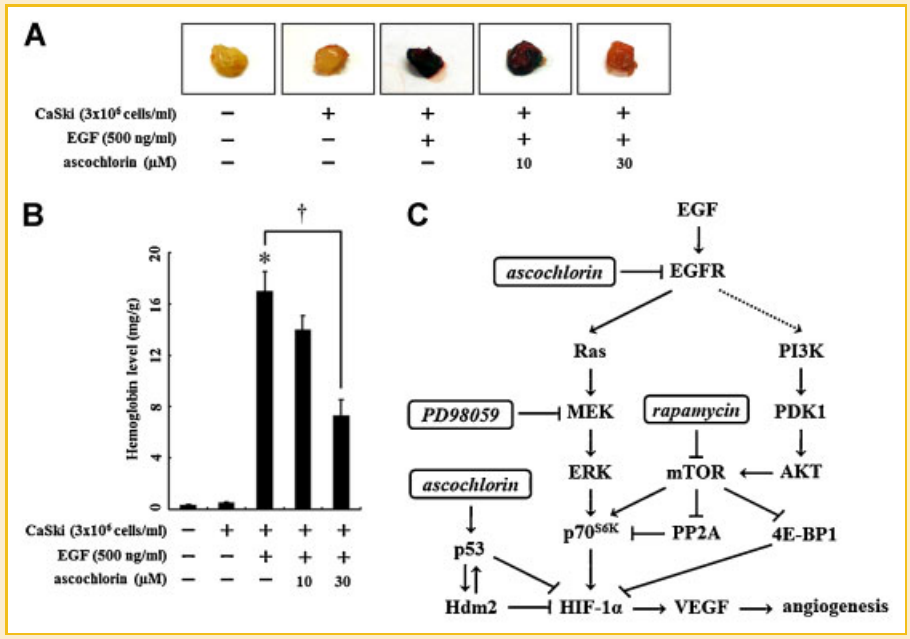

Fig. 6. Ascochlorin inhibits tumor angiogenesis in vivo. A: CaSki cells were re-suspended in serum-free medium at a density of 3×10^6 cells/ml, and aliquots (0.2 ml) were mixed with 0.5 ml of Matrigel in the presence or absence of EGF (500 ng/ml) and ascochlorin (10 or 30 μM). CaSki-implanted Matrigel was injected subcutaneously into C57BL/6N (female) mice. Mice were euthanized 7 days after implantation and Matrigel plugs were removed. Representative images of Matrigel plugs are shown. B: Hemoglobin levels in Matrigel plugs. Hemoglobin levels were quantified using Drabkin's reagent kit (Sigma–Aldrich). Data represents the means ± SD of three independent experiments. $^*P < 0.05$ as compared to untreated control; $^\dagger P < 0.01$ as compared to EGF only treatment. Data was analyzed using one-way ANOVA. C: Proposed molecular mechanism for regulation of HIF-1α and VEGF expression by ascochlorin in EGF-treated CaSki cells. See details in text. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cancer, and is an independent predictor of poor prognosis in advanced stage tumors. Hence, EGFR as a therapeutic target is the focus of intense research. Drugs currently in clinical use that target EGFR include small molecule tyrosine kinase inhibitors (TKIs) such as Gefitinib (Iressa, ZD1839), Erlotinib (Tarceva) and Lapatinib, and monoclonal anti-ErbB1 antibodies such as Cetuximab (Erbitux) and Panitumumab (Vecitibix, ABXEGF) [Bublil and Yarden, 2007]. The TKIs inhibit the action of EGFR through a physical interaction with the ATP- or substrate-binding site on the tyrosine kinase domain of EGFR. Ascochlorin inhibits phosphorylation of EGFR at Tyr1068 and the kinase activity of EGFR in vitro in CaSKi cells. It might be possible that respiratory inhibitors inhibit EGFR phosphorylation, at least in part through reduction of intracellular ATP level.

In summary, we have identified a putative molecular mechanism for suppression of HIF-1α activity and angiogenesis by ascochlorin in EGF-stimulated CaSki cells (Fig. 6C). Stimulation of EGF mainly activates RAS/RAF/ERK signaling pathway in CaSKi cells, which subsequently phosphorylates p70S6K, leading to translation activation of HIF-1α. ERK activation also enhances HIF-1α transactivation by facilitating nuclear translocalization of HIF-1α. Ascochlorin that inhibits EGFR phosphorylation through unknown mechanism as well as an MEK inhibitor PD98059, suppresses ERK-mediated enhancement of translation and transactivation of HIF-1α. And ascochlorin might reduce both protein stabilization and transactivaion of HIF-1α through the induction of p53, as previously reported [Jeong et al., 2009]. Further study concerning pharmacological action of ascochlorin might provide a new strategy for cancer chemotherapy in the inhibition of tumor angiogenesis.

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