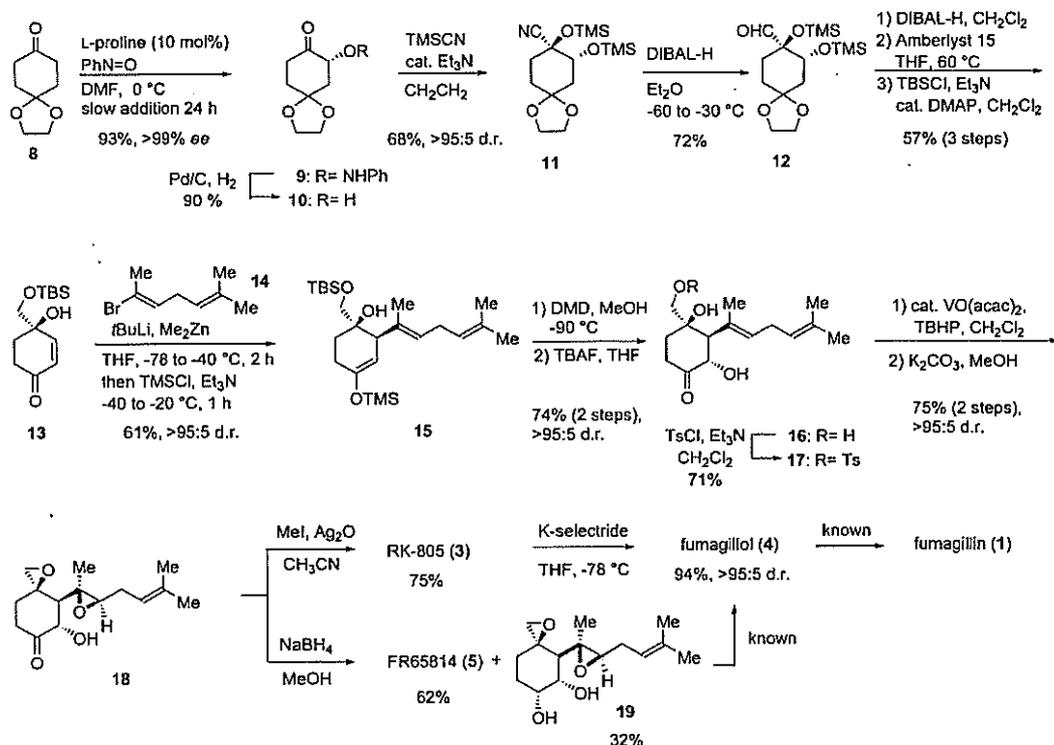


of angiogenesis and is more stable than fumagillin and TNP-470, while 5-demethylovalicin (**7**)<sup>[6]</sup> was isolated recently and found to be as potent an angiogenesis inhibitor as ovalicin. While these natural products are anti-angiogenesis agents, FR65814 (**5**),<sup>[9]</sup> which has a similar structure, displays completely different biological activity and is an immunosuppressant.

Systematic comparison of the biological properties of these natural products and their derivatives is highly desirable.<sup>[10]</sup> These compounds comprise a cyclohexane framework, two epoxides, and five or six contiguous stereogenic centers, three or four of which are situated on the cyclohexane ring. As a result of their unique structure and important biological properties, they have proved attractive synthetic targets. Four racemic syntheses<sup>[11]</sup> including Corey's first excellent total syntheses of fumagillin (**1**)<sup>[11a]</sup> and ovalicin (**6**) have been reported.<sup>[11b]</sup> The optically active compounds have been prepared from a chiral pool, starting from quinic acid<sup>[12]</sup> and quebrachitol<sup>[13]</sup> for ovalicin, glycido<sup>[14]</sup> for fumagillin, allose<sup>[15]</sup> and mannitol<sup>[16]</sup> for fumagillin, and glucose<sup>[17]</sup> for FR65814, while diastereoselective syntheses of fumagillin using chiral auxiliaries have been reported by Sorensen<sup>[18]</sup> and Eustache<sup>[19]</sup> and their respective co-workers. However, only one catalytic asymmetric synthesis has been reported for any of these compounds, namely, Corey's synthesis of ovalicin (**6**)<sup>[20]</sup> through substrate-enhanced asymmetric dihydroxylation. Moreover, there is no single, flexible method to access

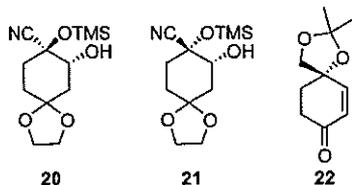
both families. Herein, we disclose the concise, flexible, and highly diastereoselective asymmetric, catalytic total syntheses of compounds of both families, including RK-805 (**3**), fumagillol (**4**), FR65814 (**5**), ovalicin (**6**), and 5-demethylovalicin (**7**) using our recently developed proline-mediated  $\alpha$ -aminooxylation of carbonyl compounds<sup>[21]</sup> as a key step.

Synthesis of the fumagillin family started from 1,4-cyclohexanedione monoethylene ketal (**8**; Scheme 1).  $\alpha$ -Aminooxylation of **8** (1.2 equiv) using 10 mol % of L-proline with slow addition of nitrosobenzene (1.0 equiv) over 24 h proceeded efficiently at 0 °C to afford nearly optically pure *R*- $\alpha$ -aminooxylated cyclohexanone **9** (>99% *ee*) in 93% yield, in a reaction that can be scaled up to 25 g of **8** without compromising the yield or enantioselectivity.<sup>[21b,c]</sup> Reductive cleavage of the N–O bond was performed under an atmosphere of H<sub>2</sub> in the presence of Pd/C<sup>[21e]</sup> for 3 h in THF (90%). Diastereoselective construction of the epoxide moiety from the ketone carbonyl was found to be troublesome because of easy racemization and low selectivity: Racemic epoxide was obtained, albeit in good yield, when **10** was treated at room temperature with a sulfur ylide such as dimethylsulfonium methylide.<sup>[22]</sup> The epoxide was generated with low diastereoselectivity (2.5:1–3.4:1) by conventional two-step procedures such as vinylidene formation with Ph<sub>3</sub>P=CH<sub>2</sub> and successive epoxidation with either TBHP in the presence of VO(acac)<sub>2</sub><sup>[23]</sup> at room temperature or MCPBA at 0 °C. After some experimentation, it was found that cyano bis(trimethyl-



**Scheme 1.** Total syntheses of fumagillol (**4**), RK-805 (**3**), FR65814 (**5**), and the formal total synthesis of fumagillin (**1**). DMF = *N,N*-dimethylformamide; TMS = trimethylsilyl; DIBAL-H = diisobutylaluminum hydride; TBS = *tert*-butyldimethylsilyl; DMAP = 4-(dimethylamino)pyridine; DMD = dimethyldioxirane; TBAF = tetra-*n*-butylammonium fluoride; Ts = *p*-toluenesulfonyl; acac = acetylacetonate; TBHP = *tert*-butylhydroperoxide; selectride = tri-*sec*-butylborohydride.

silyl) ether **11** could be obtained with high diastereoselectivity in moderate yield, although the diastereoselectivity of the initial cyanation was low. When hydroxy ketone **10** was treated with TMS-CN (2.5 equiv) in the presence of Et<sub>3</sub>N (0.1 equiv)<sup>[24]</sup> at 0 °C, cyano mono(trimethylsilyl) ethers **20** and **21** were obtained in low diastereoselectivity (3.5:1) after



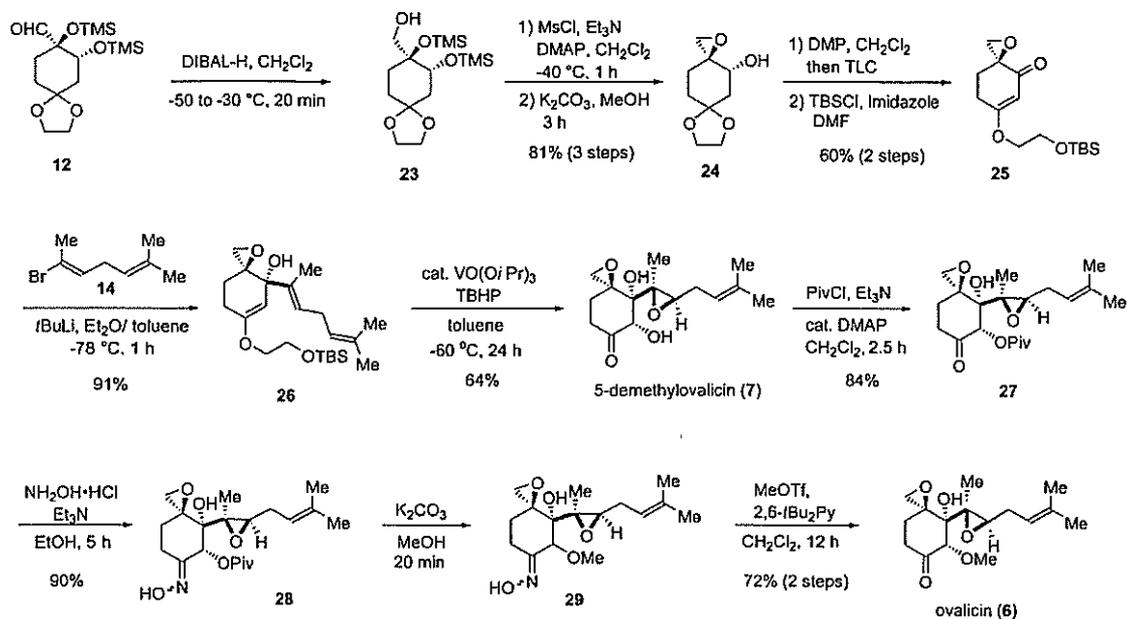
0.5 h. After 2.5 h, however, bis(trimethylsilyloxy) cyanocyclohexane **11** was obtained with high diastereoselectivity (> 95:5) in 68 % yield along with cyano mono(trimethylsilyl) ether **21** in 20% yield because of kinetic discrimination between the diastereomers during the formation of the second TMS ether. The two-step transformation of the cyanide to the alcohol was cleanly performed by repeated reductions with DIBAL-H. Acid treatment with Amberlyst in THF/H<sub>2</sub>O at 60 °C for 2 days led to removal of all the protecting groups, with concomitant dehydration affording a cyclohexenone diol. Selective protection of the primary alcohol with TBSCl using Et<sub>3</sub>N at room temperature for 12 h afforded TBS ether **13** in 57 % yield over three steps. The optical purity (> 99 % *ee*) of **13** was checked by chiral HPLC analysis of its acetate, which indicated that no racemization had occurred during these transformations. The absolute stereochemistry was confirmed by the conversion of **13** into the enantiomer of Taber's intermediate **22**.<sup>[14]</sup>

The next task was diastereoselective introduction of the side chain which was also found to be troublesome. The choice of protecting group for the cyclohexenone and the metal cation of the nucleophile are both important for achieving the desired conjugate addition: The tertiary alcohol should be free,<sup>[25]</sup> and vinyl zincate was found to be the reagent of choice.<sup>[26]</sup> Thus,  $\alpha,\beta$ -enone **13** reacted with vinyl zincate prepared from **14**<sup>[27]</sup> at -78 °C to afford the Michael addition product, which was trapped with TMS-Cl as its trimethylsilyl enol ether **15**. This ether was obtained in 61 % yield as a single isomer, in which the side chain had been introduced stereoselectively from the same side as the hydroxy group. Protection of the tertiary alcohol or use of divinyl cuprate instead of vinyl zincate led to unsatisfactory results. Epoxidation<sup>[28]</sup> of the silyl enol ether with dimethyldioxirane (DMD) at low temperature (-90 °C) in acetone proceeded diastereoselectively without oxidation of the other trisubstituted double bonds and  $\alpha$ -hydroxy cyclohexanone **16** was obtained after treatment with TBAF as a single isomer in 74 % yield over two steps. Though the reaction sequence of conjugate addition, silyl trapping, and Rubottom oxidation was also employed in Taber's synthesis of fumagillin to install a side chain and the hydroxy group at the C5 position, the stereochemistry of the conjugate addition was completely different.<sup>[14]</sup> Taber et al. reported that intermediate **22** containing an acetal group reacted stereoselectively with a divinyl

cuprate derivative in the undesired fashion, that is, *anti* to the oxygen atom of the spirocyclic ether, necessitating several additional steps to correct the stereochemistry. In our synthesis, direct introduction of the side chain with the correct stereochemistry by exploiting the free hydroxy group in combination with a zincate makes the total synthesis efficient and straightforward.

During epoxidation at the side chain, the order of the next two procedures was very important to obtain high diastereoselectivity (Scheme 1). The diastereoselectivity of epoxidation of dihydroxy tosylate **17** with TBHP in the presence of VO(acac)<sub>2</sub><sup>[23]</sup> was excellent, and bis(epoxide) **18** was obtained as a single isomer after treatment with K<sub>2</sub>CO<sub>3</sub> in MeOH, whereas reversal of the order of reaction led to low diastereoselectivity (2:1).<sup>[29]</sup> Formation of the methyl ether with MeI and Ag<sub>2</sub>O in CH<sub>3</sub>CN gave RK-805, which was stereoselectively reduced with K-selectride at -78 °C to give fumagillol as a single isomer in good yield. The conversion of fumagillol into fumagillin in a single step is known,<sup>[11a,14]</sup> thus, the formal total synthesis of fumagillin was also accomplished. When **18** was reduced with NaBH<sub>4</sub> in MeOH at -50 °C to -10 °C, FR65814 was obtained predominantly in 62 % yield along with **19** in 32 % yield. The conversion of **19** into fumagillol in a single step is known.<sup>[11c,18]</sup>

Next, the syntheses of 5-demethylovalicin and ovalicin were examined (Scheme 2). The intermediate **12** used in our synthesis of the fumagillin family was employed here also, first to generate the epoxide **24**. Oxidation of **24** with the Dess-Martin periodinane (DMP),<sup>[30]</sup> followed by acid treatment with thin layer chromatography (TLC) to generate a 3-(2-hydroxyethoxy)cyclohex-2-enone derivative, and treatment with TBSCl afforded cyclohexenone **25**. The side chain was introduced in a highly diastereoselective manner by using a vinyl lithium reagent.<sup>[20]</sup> As the side chain, the 6-methylhepta-2,5-dien-2-yl substituent was found to be unstable, easy to isomerize, and prone to decomposition, thus its epoxidation had to be carried out immediately.<sup>[31]</sup> Though conventional epoxidation with VO(acac)<sub>2</sub> and TBHP,<sup>[23]</sup> or *m*-chloroperbenzoic acid gave a complex mixture owing to the instability of the side chain, and the other double bond of the side chain was selectively epoxidized with DMD, VO-(O*i*Pr)<sub>3</sub><sup>[32]</sup> was found to be an efficient catalyst, promoting the epoxidation of both the silyl enol ether and the desired side chain alkene at low temperature (-60 °C) to afford 5-demethylovalicin (**7**) as a single isomer with the creation of three chiral centers. The last task necessary to convert 5-demethylovalicin into ovalicin (**6**) was transformation of the alcohol to its methyl ether. Although conventional reagents<sup>[33]</sup> such as NaH and MeI, Ag<sub>2</sub>O and MeI, or MeOTf and 2,6-di-*tert*-butylpyridine, failed, a modification of Corey's method<sup>[11b]</sup> through the corresponding oxime gave ovalicin stereoselectively. Thus, protection of the alcohol as an ester, formation of the oxime, treatment with base in MeOH, and conversion of the oxime into a ketone gave ovalicin (**6**) as a single isomer, with the ovalicin intermediates **28** and **29** each obtained also as single isomers.<sup>[34]</sup> Note that oxime **29** was converted into ovalicin (**6**) in good yield without affecting the two epoxides or the trisubstituted alkene under alkylation conditions, when this conversion is usually performed under



**Scheme 2.** Total syntheses of ovalicin (6) and 5-demethylovalicin (7). Ms = methanesulfonyl; DMP = Dess–Martin periodinane; Piv = pivaloyl; OTf = trifluoromethanesulfonate, Py = pyridine.

oxidative, reductive, or acid-hydrolysis conditions.<sup>[33]</sup> Though the synthetic scheme from 25 is similar to that in Corey's elegant synthesis,<sup>[11b]</sup> in which introduction of the side chain followed by various functional group manipulations are key transformations, there is an important difference; namely, the effective epoxidation catalyst (VO(OiPr)<sub>3</sub>), which allows diastereoselective double epoxidation without affecting the labile side chain, saves a couple of steps and improves the efficiency of the synthesis.

The <sup>1</sup>H and <sup>13</sup>C NMR spectra, IR spectra, and optical rotations of the synthetic samples of 3,<sup>[4]</sup> 4,<sup>[14]</sup> 5,<sup>[9]</sup> 6,<sup>[13b,35]</sup> and 7<sup>[6]</sup> are in complete agreement with those previously reported.

In summary, the concise enantio- and diastereoselective total syntheses of fumagillol, RK-805, FR65814, 5-demethylovalicin, and ovalicin in 11–15 steps from commercially available compounds have been demonstrated. These are some of the shortest syntheses reported for these chiral natural products and demonstrate clearly the power of the proline-mediated asymmetric catalytic  $\alpha$ -aminoxylation. The initial aminoxylation reaction controls both the absolute and the relative stereochemistry of the subsequently generated stereogenic centers, which are formed by the following transformations: 1) a highly diastereoselective formation of bis(trimethylsilyl ether) cyanide 11 involving kinetic discrimination; 2) a diastereoselective Michael reaction by the use of vinyl zincate (13→15); 3) a stereoselective double epoxidation catalyzed by VO(OiPr)<sub>3</sub> at low temperature (26→7); and 4) an alkylation deprotection of an oxime (29→6). Corey's asymmetric total synthesis of ovalicin (6)<sup>[20]</sup> using asymmetric dihydroxylation is a landmark chiral synthesis of a member of the fumagillin and ovalicin families. The present route using an  $\alpha$ -aminoxylation catalyzed by inexpensive proline is as efficient as Corey's synthesis;<sup>[20]</sup> it allows the synthesis to be performed on a large scale and allows easy derivatization, as

well as being the first strategy applied to both fumagillin and ovalicin families, thus demonstrating the flexibility of the present synthetic method.

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## PTEN/Akt Signaling through Epidermal Growth Factor Receptor Is Prerequisite for Angiogenesis by Hepatocellular Carcinoma Cells That Is Susceptible to Inhibition by Gefitinib

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### Abstract

Hepatocellular carcinoma (HCC) is one of the most common tumor-related causes of death worldwide for which there is still no satisfactory treatment. We previously reported the antiangiogenic effect of gefitinib, a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that has been used successfully to treat lung cancer. In this study, we investigated the effects of gefitinib on tumor-induced angiogenesis by using HCC cell lines (HCC3, CBO12C3, and AD3) *in vitro* as well as *in vivo*. Oral administration of gefitinib inhibited angiogenesis induced by HCC3 and CBO12C3, but not by AD3 in the mouse dorsal air sac model. Production of both vascular endothelial growth factor (VEGF) and chemokine C-X-C motif ligand 1 (CXCL1) by EGF-stimulated HCC was more markedly inhibited by gefitinib in HCC3 and CBO12C3 cells than in AD3 cells. EGF stimulated the phosphorylation of EGFR, Akt, and extracellular signal-regulated kinase 1/2 (ERK1/2) in HCC3 and CBO12C3 cells, whereas EGF stimulated phosphorylation of EGFR and ERK1/2, but not Akt in AD3 cells. In fact, Akt was constitutively activated in the absence of EGF in AD3 cells. Gefitinib inhibited Akt phosphorylation in all three cell lines, but it was about five times less effective in AD3 cells. The concentration of PTEN in AD3 cells was about a half that in HCC3 and CBO12C3 cells. Transfection of HCC3 cells with PTEN small interfering RNA reduced their sensitivity to gefitinib in terms of its inhibitory effect on both Akt phosphorylation and the production of VEGF and CXCL1. In conclusion, effect of gefitinib on HCC-induced angiogenesis depends on its inhibition of the production of angiogenic factors, probably involving a PTEN/Akt signaling pathway. (Cancer Res 2006; 66(10): 5346-53)

### Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide (1) and the prognosis for HCC patients is still very poor. Members of the epidermal growth factor receptor (EGFR) family have emerged as critical factors in the development and growth of various types of cancer, including HCC (2). These receptors are part of a complex network of signal transduction

casades that modulate tumorigenic processes, such as proliferation, adhesion, migration, differentiation, angiogenesis, and escape from apoptosis (3-6). High EGFR expression in human cancers is often correlated with advanced disease, metastasis, and poor clinical outcome, for example in non-small cell lung carcinoma (NSCLC), breast, cervical, and head and neck carcinomas (7-9).

Gefitinib (Iressa, ZD1839) is an orally active and selective EGFR tyrosine kinase inhibitor that blocks EGFR-mediated signal transduction pathways involved in cancer growth (10, 11). Gefitinib has antiproliferative activity in various human cancers *in vivo* as well as *in vitro* (12, 13). It is now approved as a monotherapy for patients with locally advanced or metastatic NSCLC (14-16) and is under investigation for the treatment of prostate, breast, head and neck, gastric, and colorectal cancer (17). An important recent discovery is the close association between a clinical response to gefitinib in patients with NSCLC and somatic mutations in the *EGFR* gene (18, 19). In NSCLC cells carrying such mutations, gefitinib treatment markedly inhibited phosphorylation of EGFR and its downstream signaling kinases, Akt and extracellular signal-regulated kinase 1/2 (ERK1/2). Consistent with this observation, we have reported that the sensitivity of NSCLC cell lines to gefitinib under basal growth condition is closely correlated with their dependence for survival and proliferation on Akt and ERK1/2 activation in response to EGFR signaling (20). Cappuzzo et al. (21) have reported that patients with phosphorylated Akt-positive tumors who received gefitinib had a better response rate and time to progression. Moreover, increased *EGFR* gene copy number evaluated by fluorescent *in situ* hybridization (FISH) was significantly associated with higher response rates and lower progression rates in lung cancer (22) and in colon cancer (23).

HCC is a typical hypervascular tumor and tumor angiogenesis is a prerequisite for both its growth and metastasis (24, 25). Angiogenesis and vascular invasion are common characteristics of malignant tumors in patients with HCC (26, 27). Enhanced expression of vascular endothelial growth factor (VEGF) and chemokine C-X-C motif ligand 1 (CXCL1) is often seen both in HCC cells in culture and at sites of angiogenesis in the livers of HCC patients (26, 28-33). Ishikawa et al. (34) have previously reported antitumor effect by antiangiogenesis gene therapy using *angiostatin* gene on HCCs in the xenograft model. A recent study by Liu et al. (35) has also shown that administration of a VEGFR tyrosine kinase inhibitor induced both antitumor and antiangiogenesis effects against HCCs in the xenograft model. On the other hand, we have previously reported the inhibition of EGF-induced migration of vascular endothelial cell and neovascularization in mouse corneas by gefitinib, suggesting that its antitumor effect

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may be mediated in part through its antiangiogenic activity (36, 37). Overexpression of EGFR was also observed in 60% to 85% of tumor tissue of HCC (38), and a phase II trial of gefitinib has been proceeding against patients with advanced unresectable HCC in the United States. In this study, we asked whether EGFR inhibition could modulate tumor angiogenesis induced by HCC cells and examined possible mechanisms underlying the effects of gefitinib.

## Materials and Methods

**Cell culture and reagents.** Three murine HCC cell lines HCC3, CBO12C3, and AD3 were established as described previously (39, 40) and were cultured in William's E (Life Technologies, Inc., Grand Island, NY) supplemented with 10 ng/mL EGF, 10% fetal bovine serum (FBS), 320 mg/L L-glutamine, and 2 g/L glucose, at 37°C in 5% CO<sub>2</sub> in a humid environment. Gefitinib was provided by Astra Zeneca Pharmaceuticals (Macclesfield, United Kingdom) and was dissolved in DMSO for *in vitro* studies as described previously (20, 27). LY294002, a selective inhibitor of phosphatidylinositol 3-kinase (PI3K), and U0126, an ERK inhibitor, were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant EGF, anti-phospho-EGFR (Tyr<sup>1173</sup>), anti-EGFR, and anti-PI3K p85 antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-Akt (Ser<sup>473</sup>), anti-Akt, anti-phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), and anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-PTEN and anti-PI3K p110 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin antibody was from Abcam, Ltd. (Cambridge, United Kingdom).

**Cell proliferation assay.** HCC cells were suspended at  $5.0 \times 10^3$ /mL in medium with or without 10 ng/mL EGF and seeded into 48-well plates for the indicated periods. The following day, cell numbers were counted using a Z1 Series Coulter Counter (Beckman Coulter, Inc., Fullerton, CA).

**Mouse dorsal air sac assay.** This assay was carried out in 7- to 10-week-old male mice as previously described (41). Male BALB/c mice were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). HCC cells ( $2 \times 10^6$ ) were suspended in 150 μL PBS and injected into a chamber that consisted of a ring (Millipore Corp., Bedford, MA) covered with Millipore filters (0.45 μm pore size) on each side. This was implanted into an air sac produced by injecting 10 mL of air s.c. on the back of an anesthetized mouse (50 mg/kg pentobarbital, i.v.) on day 0. Gefitinib was administered p.o. from day 0 to day 4. On day 5, the chambers were removed from the s.c. fascia and replaced with black rings of the same inner diameter as the chambers. Photographs of these sites were assessed by counting the number of newly formed vessels >3 mm in length within the area of the rings.

**Quantification of VEGF and CXCL1 in conditioned medium.** The concentrations of VEGF and CXCL1 in conditioned medium from HCC cells were measured using ELISA kits (R&D Systems, Minneapolis, MN) as described previously (42). Briefly, HCC cells were seeded in 24-well dishes at  $2.5 \times 10^4$ /2 mL/well and, when subconfluent, the medium was replaced with serum-free medium for 24 hours, containing different concentrations of kinase inhibitors, with or without 10 ng/mL EGF at 37°C. Results are presented as means ± SD.

**Western blot analysis.** After culture for 12 hours in serum-free medium without EGF, HCC cells were treated with the indicated concentration of gefitinib for 3 hours and then stimulated with 10 ng/mL EGF for 15 minutes at 37°C. After rinsing with ice-cold PBS, the cells were lysed in Triton X-100 buffer [50 μmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L Na<sub>2</sub>VO<sub>4</sub>]. Lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore). Blots were incubated with primary antibodies and visualized with a secondary antibody coupled to horseradish peroxidase (Amersham, Piscataway, NJ), and enhanced chemiluminescence system, as described previously (20). Bands on Western blots were analyzed densitometrically using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD).

**PTEN gene silencing using small interfering RNA.** The ability of 25-nucleotide duplexes of RNA to interfere with PTEN expression was tested. The specific small interfering RNA (siRNA) sequence used was nucleotide 58 to 82, relative to the first nucleotide of the start codon, of PTEN (Genbank accession number NM\_008960). Mock siRNA duplexes were obtained from Invitrogen (Carlsbad, CA). siRNA duplexes were transfected using LipofectAMINE 2000 and Opti-MEM medium (Invitrogen) according to the recommendations of the manufacturer. Reduction in PTEN expression was confirmed by Western blot analysis.

**FISH analysis.** FISH analysis was done as described previously (43). Mouse EGFR cDNA (BC-023729-EGFR) and Mere Mouse BAC clone 43L24 were obtained from Open Biosystems (Huntsville, AL). The probes for EGFR and chromosome 11 were labeled with digoxigenin and biotin by nick translation methods, respectively. The slides were incubated at 75°C for 10 minutes to codenature the EGFR and chromosome 11 probes and allowed to hybridize overnight at 37°C. After stringency wash, digoxigenin-labeled EGFR and biotin-labeled chromosome 11 probes were detected using antidigoxigenin-Cy3 and anti-biotin-Cy5. The chromatin was counterstained with 4',6-diamino-2-phenylindole. Analysis was done with a Leica DMRA2 fluorescence microscope (Deerfield, IL) equipped with the Leica CW4000 FISH software. Average numbers of chromosome were determined by scoring 30 to 40 metaphase spreads.

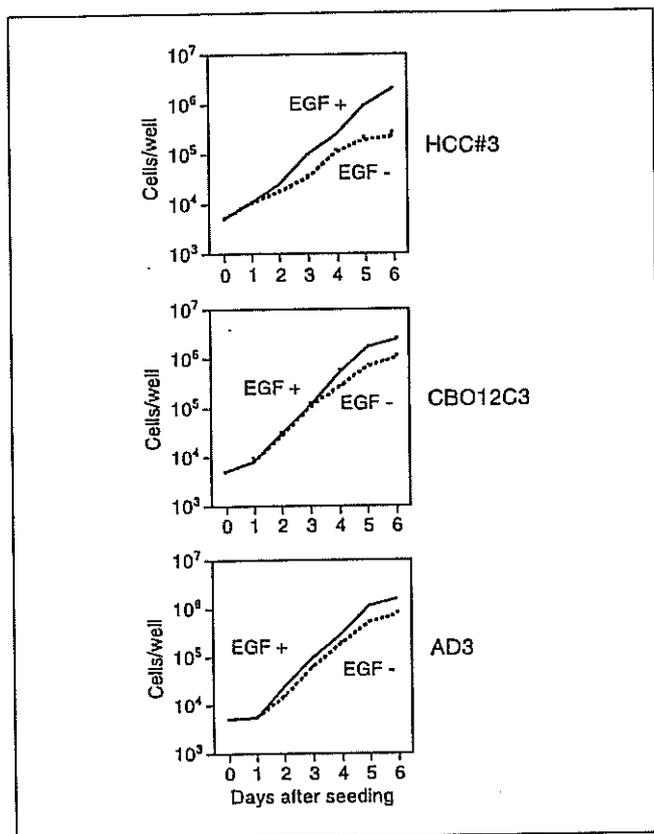
**Statistical analysis.** Statistical analysis used the Mann-Whitney *U* test and Student's *t* test, with *P* < 0.05 considered statistically significant, and was done using JMP 5.01 software (SAS Institute, Inc. Cary, NC).

## Results

**Effect of EGF on the cell growth of HCC cell lines.** The growth rates of HCC cell lines were measured under normal growth conditions with 10% FBS, in the presence or absence of 10 ng/mL EGF. Doubling times for HCC3, CBO12C3, and AD3 were 13.5, 12.1, and 12.6 hours, respectively, in the presence of EGF and 22.4, 14.6, and 14.6 hours, respectively, in the absence of EGF (Fig. 1). Growth rates for the three lines were comparable, although EGF affected the growth of HCC3 cells rather more than that of the other two cell lines.

**Gefitinib inhibits tumor-induced angiogenesis *in vivo*.** We next investigated whether gefitinib could inhibit angiogenesis induced by HCC cells *in vivo*, using the dorsal air sac assay. In the absence of gefitinib, implantation of chambers containing each of the three HCC cell lines resulted in the development of microvessels with thin curled structures and tiny bleeding spots, in addition to the preexisting vessels (Fig. 2A), consistent with our previous studies (41). The oral administration of gefitinib at 150 mg/kg/d markedly reduced the development of microvessels induced by HCC3 and CBO12C3 cells, but not by AD3 cells (Fig. 2A). About a 60% reduction in angiogenesis was seen in mice implanted with HCC3 cells and given 75 mg/kg/d gefitinib and a similar reduction was seen in mice implanted with CBO12C3 cells and given 150 mg/kg/d gefitinib (Fig. 2B).

**Inhibitory effect of gefitinib on production of VEGF and CXCL1 in HCC cells.** To understand how gefitinib modulates angiogenesis, we examined the effect of this EGFR-selective drug on the production of two potent inducers of angiogenesis, VEGF and CXCL1 [interleukin-8 (IL-8) homologue/KC/Gro-α] in HCC cell lines (Fig. 3A). EGF increased the production of both VEGF and CXCL1 by 1.6- to 3.2-fold in all three cell lines. At 0.1 to 5.0 μmol/L, gefitinib inhibited EGF-stimulated production of VEGF and CXCL1 by HCC3, CBO12C3, and AD3 to different extents. At 1 μmol/L, gefitinib blocked the production of CXCL1 by >80% in HCC3 and CBO12C3 cells and by 60% in AD3 cells in the presence of EGF. At the same concentration, gefitinib also blocked VEGF production by



**Figure 1.** Effect of EGF on the cell growth of three HCC cell lines, HCC3, CBO12C3, and AD3:  $5.0 \times 10^3$ /well were incubated with or without 10 ng/mL EGF in 48-well plates. The cells were counted at the times indicated. Points, mean of triplicate wells; bars, SD.

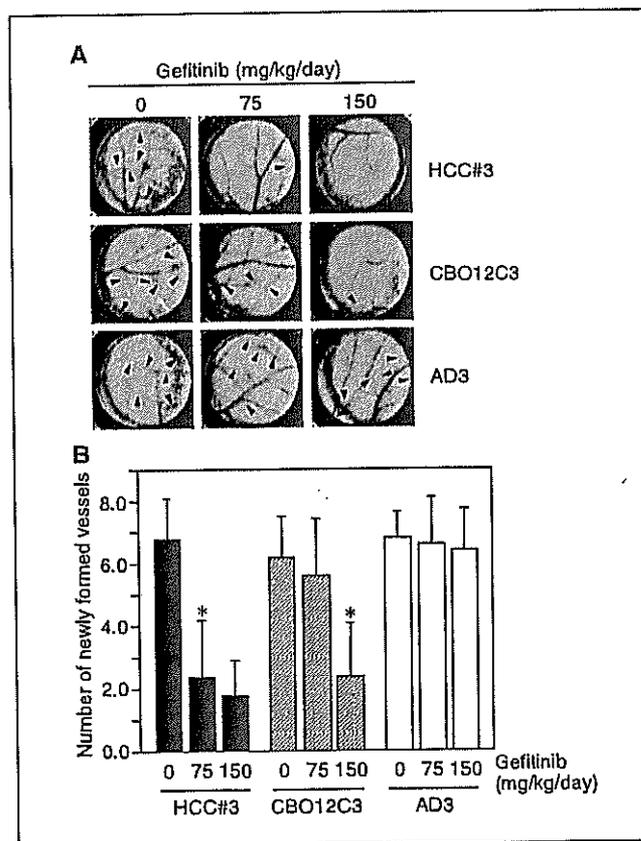
HCC3 and CBO12C3 cells by 60% to 80%, whereas VEGF production by AD3 cells was only inhibited by 20% in the presence of EGF (Fig. 3A). The production of VEGF and CXCL1 in response to exogenous EGF was rather more sensitive to inhibition by gefitinib in HCC3 and CBO12C3 cells compared with AD3 cells.

**Effect of gefitinib on phosphorylation of EGFR, Akt, and ERK1/2 in HCC cells.** When EGFR binds EGF or transforming growth factor- $\alpha$ , it is autophosphorylated and activates a number of downstream signaling molecules, such as Akt and mitogen-activated protein kinase (ERK1/2; ref. 42). We examined the effect of gefitinib on the phosphorylation of EGFR, Akt, and ERK1/2 in the three cell lines *in vitro*. Exogenous EGF enhanced the phosphorylation of EGFR, Akt, and ERK1/2 in HCC3 and CBO12C3 cells. By contrast, EGF enhanced the phosphorylation of EGFR and ERK1/2, but not Akt, in AD3 cells; Akt was constitutively phosphorylated in AD3 cells when EGF was added exogenously (Fig. 3B). Phosphorylation of Akt was also inhibited by gefitinib to different extents in the three cell lines: 0.1  $\mu$ mol/L gefitinib inhibited Akt phosphorylation in HCC3 and CBO12C3 by 50% compared with EGF alone, but had no effect at this concentration in AD3 cells (Fig. 3B). Although Akt was constitutively phosphorylated in the absence of EGF in AD3 cells, phospho-Akt was rather less sensitive to the inhibitory effect of gefitinib in AD3 than in the other two cell lines.

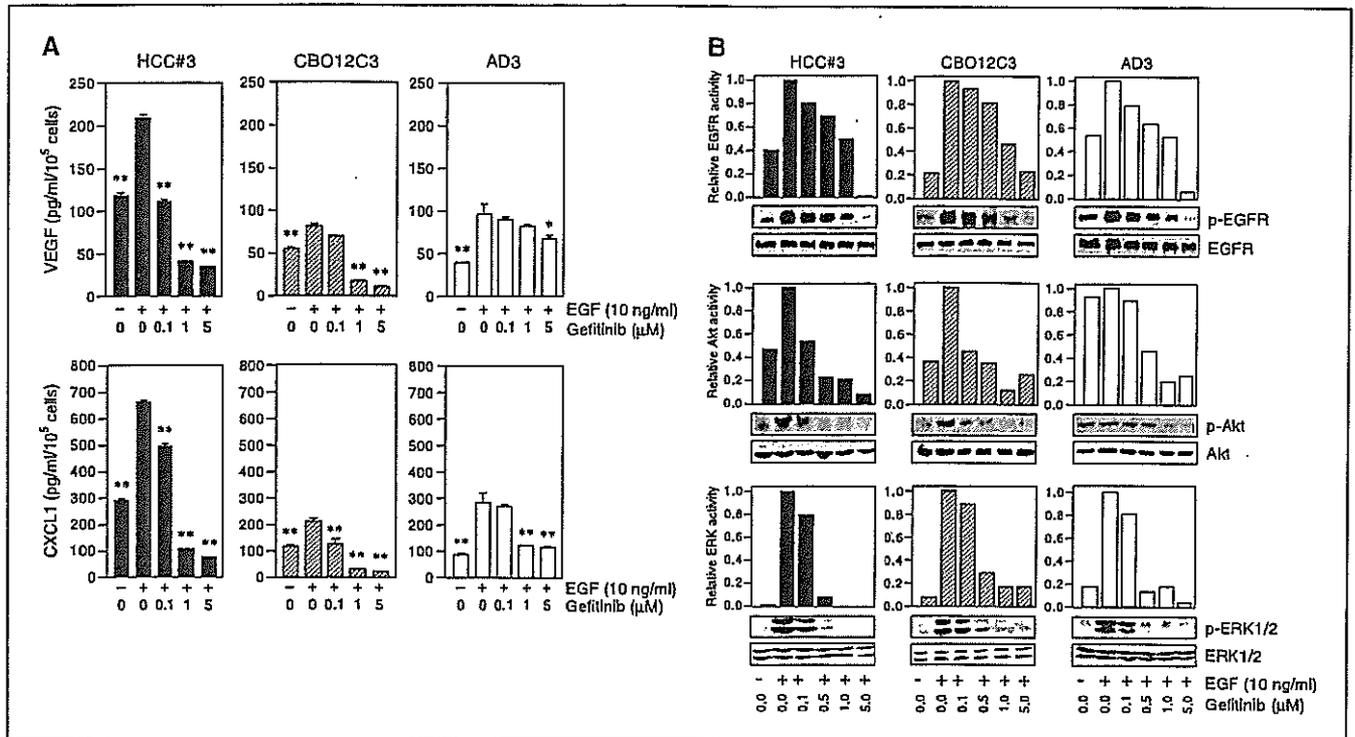
**Involvement of Akt and ERK1/2 in EGF-induced production of angiogenic factors, VEGF and CXCL1.** To identify the EGF/

EGFR-activated signal pathways involved in the production of angiogenic factors, we looked at the effects of LY294002, a selective inhibitor of PI3K, and U0126, an inhibitor of ERK1/2, on the production of VEGF and CXCL1 by HCC3 and AD3 cells. LY294002 inhibits Akt activation by specifically blocking PI3K, a positive regulator of Akt kinase. EGF increased VEGF and CXCL1 production in both these cell lines 2- to 3-fold (see Fig. 3A) and treatment with LY294002 resulted in a significant decrease in the production of both factors in HCC3 cells compared with EGF alone (Fig. 4A and B). However, LY294002, tested up to a concentration of 5  $\mu$ mol/L, had no effect on the EGF-induced production of VEGF and CXCL1 in AD3 cells (Fig. 4A and B). Treatment with U0126 also affected cellular production of VEGF in both cell lines but there was no significant difference in the inhibitory effect of U0126 on cellular production of VEGF between two cell lines (Fig. 4C). U0126 showed only a slight, if any, inhibition of CXCL1 production, but there was no significant difference in its inhibition between two cell lines (Fig. 4D).

**Role of PTEN in gefitinib-mediated inhibition of Akt activation and production of angiogenic factors.** Akt activation is regulated by both phosphorylation at serine-473 and PIP<sub>3</sub>



**Figure 2.** Effect of gefitinib on angiogenesis induced by HCC cell lines in the mouse dorsal air sac assay. A, representative photographs of assay chambers containing HCC3, CBO12C3, or AD3 cells, with or without gefitinib at 75 or 150 mg/kg/d. Arrowhead, newly formed vessels, with characteristic zigzagging lines. Gefitinib was given p.o., daily, for 5 days. B, quantitative analysis of tumor angiogenesis induced by three HCC cell lines and its inhibition by gefitinib. The angiogenic responses were determined by counting the number of new blood vessels >3 mm in length as shown in (A). Columns, mean of five or six mice; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with controls using the two-tailed Mann-Whitney *U* test.



**Figure 3.** Effect of gefitinib on the EGF-induced production of angiogenic factors and phosphorylation of EGFR, Akt, and ERK1/2 in HCC cell lines. **A**,  $2.5 \times 10^4$  cells per well were incubated in the presence or absence of 10 ng/mL EGF, with or without different doses of gefitinib, in 24-well plates for 24 hours. VEGF and CXCL1 were measured in culture supernatants with an ELISA kit. Columns, mean for triplicate wells; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with treatment with EGF alone using the two-tailed Student's *t* test. **B**, the cells were stimulated with 10 ng/mL EGF for 15 minutes with or without gefitinib at the concentrations indicated. Protein extracts were resolved by Western blot analysis and probed with various antibodies. Activities of phosphorylated EGFR, Akt, and ERK1/2 were normalized to their nonphosphorylated form when each activity of phospho-EGFR, phospho-Akt, and phospho-ERK1/2 in the presence of EGF without gefitinib in three cell lines was normalized as 1.0.

binding to pleckstrin homology domain of Akt. The level of cellular PIP<sub>3</sub> is controlled by PI3K and the lipid phosphatase, PTEN (44). We compared the expression of PI3K and PTEN in the three cell lines in the presence or absence of EGF. In the absence of EGF, all three cell lines expressed similar levels of the PI3K catalytic subunit, p110, but HCC3 and CBO12C3 expressed rather higher levels of the regulatory subunit, p85, than AD3 (Fig. 5A). Quantitative analysis showed that PTEN was expressed in AD3 at about half the level in HCC3 or CBO12C3, both in the absence and presence of EGF (Fig. 5A). This difference in PTEN expression between AD3 and the other two cell lines was consistently found in repeated experiments with independently cultured cell lines (data not shown).

Because gefitinib had been found to be a much less effective inhibitor of Akt activation and angiogenic factor production in AD3 cells than in HCC3 cells (Fig. 3B), we next examined whether the lower expression of PTEN in AD3 could be related to this. In HCC3 cells transfected with PTEN siRNA, the *PTEN* gene was very effectively silenced (see Fig. 5B); Akt was found to be phosphorylated in the absence of EGF, but was not in mock-transfected cells; and Akt phosphorylation was much less sensitive to inhibition by gefitinib (Fig. 5C). We then looked at cellular levels of CXCL1 and VEGF. The cellular levels of CXCL1, but not VEGF, in PTEN siRNA-transfected HCC3 cells were about half those in mock-transfected cells (Fig. 5D) and the production of both VEGF and CXCL1 in response to EGF became insensitive to inhibition by gefitinib.

**Increased EGFR gene copy number in HCC cells.** Cappuzzo et al. (22) reported that gene amplification and copy number of *EGFR* gene in association with Akt activation is closely associated with drug sensitivity on therapeutic efficacies of gefitinib in NSCLC. We examined if *EGFR* gene was amplified in liver cancer cell lines, HCC3 and AD3 by FISH analysis. HCC3 and AD3 cells showed increased copy number of *EGFR* gene compared with normal spleen cells (Fig. 6). However, *EGFR* gene amplification was not observed in both HCC3 and AD3. HCC3 cells were near triploid with  $2.7 \pm 0.9$  copies of chromosome 11 per cells and  $61.3 \pm 18.4$  of chromosome per cells. AD3 cells were near tetraploid with  $3.7 \pm 0.9$  copies of chromosome 11 per cells and  $77.8 \pm 18.3$  of chromosome per cells. We found that both cell lines increased copy number of *EGFR* gene; however, there was no apparent difference in both gene amplification and copy number of EGFR in HCC3 and AD3.

**Discussion**

We have previously reported that gefitinib inhibits EGF-induced angiogenesis both *in vitro* and *in vivo* (36, 37). In this study, we have shown for the first time that tumor-induced angiogenesis in the mouse dorsal air sac assay can be blocked by gefitinib treatment. However, when different HCC cell lines were used in this model to induce angiogenesis, its susceptibility to inhibition by this drug was found to vary. Angiogenesis induced by HCC3 cells was most susceptible to inhibition by gefitinib. Angiogenesis induced by

CBO12C3 cells was also inhibited by gefitinib, but at higher doses of the drug. In contrast, angiogenesis induced by AD3 was relatively resistant to inhibition by gefitinib.

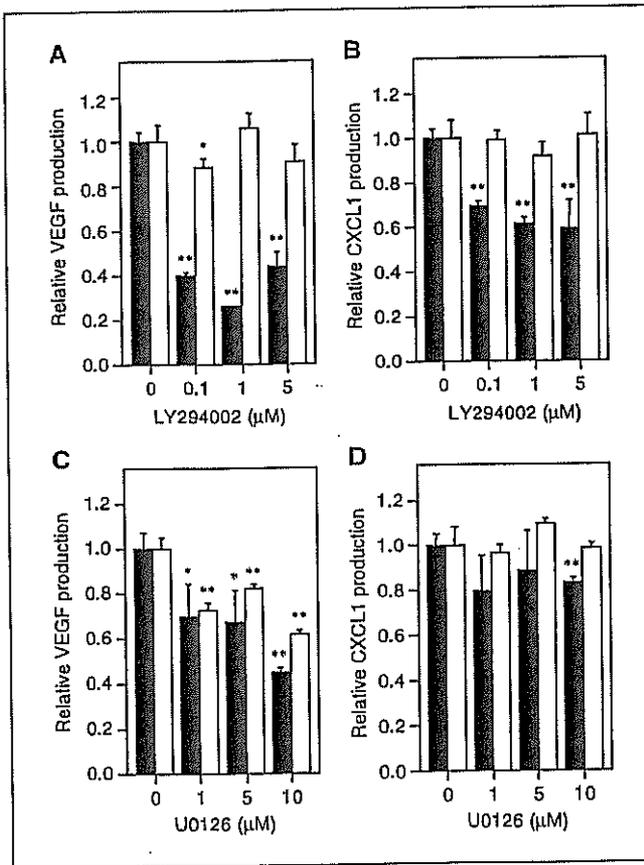
We first examined expression of which angiogenic factor was augmented by exogenous addition of EGF in HCC cell lines by membrane-bound antibody array technology. Of various angiogenesis-related factors, we observed up-regulation of *VEGF* and *CXCL1* genes by EGF (data not shown), and we focused on these two potent angiogenic factors. In all HCC lines, EGF was found to enhance cellular production of both VEGF and CXCL1. The production of these potent angiogenic factors was also more effectively inhibited by gefitinib in HCC3 and CBO12C3 cells than that in AD3 cells. This suggests that the inhibitory effect of gefitinib on HCC-induced angiogenesis might be due to its effect on the production of such angiogenic factors. The production of angiogenic factors, including VEGF and IL-8, is often enhanced by EGF, TGF- $\alpha$ , and other cytokines in tumor cell lines (25, 38, 45-48). Consistent with these previous studies, the production of both VEGF and CXCL1 increased 1.6- to 3.2-fold with EGF treatment in all three HCC cell lines. The fact that gefitinib was a much less effective inhibitor of VEGF and CXCL1 production in

AD3 than in HCC3 and CBO12C3 cells suggests that the EGF induction mechanism in AD3 cells may be refractory to gefitinib. Our previous studies suggested two pathways by which gefitinib could exert its antiangiogenic effects: (a) EGF/TGF- $\alpha$  up-regulates the expression of angiogenic factors by cancer cells themselves, resulting in the induction of angiogenesis by a paracrine mechanism, and this process is inhibited by gefitinib; or (b) EGF/TGF- $\alpha$  induces angiogenesis through direct interaction with EGFRs on endothelial cells and this process is inhibited by gefitinib (36, 37). In our studies of EGF-induced neovascularization in the mouse cornea, we obtained evidence supporting the latter pathway as gefitinib inhibited activation of EGFR in the new vessels themselves (37). The results of our present study suggest that the former pathway is a more likely candidate because gefitinib inhibited the production of both VEGF and CXCL1 by HCC cells.

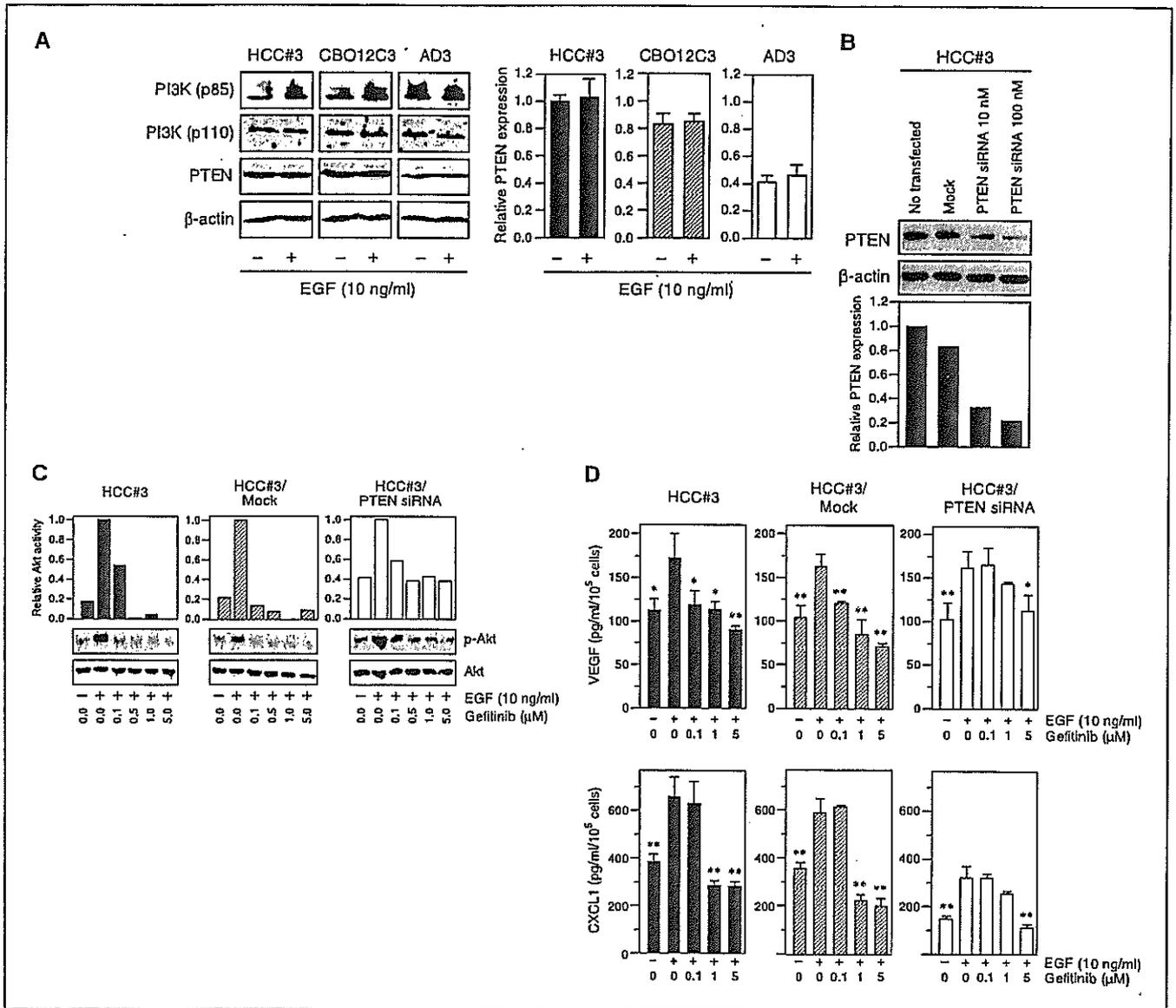
While studying the mechanism underlying the different inhibitory effects of gefitinib on VEGF and CXCL1 production by HCC cell lines, we found that the drug had differential effects on Akt activation. Whereas EGF treatment of HCC3 and CBO12C3 cells resulted in the phosphorylation of EGFR, Akt, and ERK1/2, and this was highly susceptible to inhibition of gefitinib. In AD3 cells, gefitinib also inhibited the phosphorylation of EGFR and ERK1/2 in response to EGF, but Akt seemed to be constitutively active in the absence of EGF, and phospho-Akt levels in AD3 cells were less susceptible to the effects of gefitinib. Interestingly, LY294002, a selective inhibitor of PI3K, blocked production of VEGF and CXCL1 in HCC3, but not that in AD3, whereas U0126, an ERK1/2 inhibitor, did not have this selective inhibitory effect. Taken together, these different effects of gefitinib on the induction of angiogenesis and the production of angiogenic factors suggest an important role for the Akt pathway. However, as ERK1/2 and PI3K inhibitors also reduced the production of angiogenic factors, these signaling pathways may also play a role in the antiangiogenic effects of gefitinib.

Recent studies have shown that a mutation in exons 18 to 21 of the *EGFR* gene is closely associated with a clinical response to gefitinib in NSCLC patients (18, 19) and that the proliferation of NSCLC cells carrying such *EGFR* mutations is highly sensitive to gefitinib *in vitro* (18). We, however, could not observe any mutation in exons 18 to 21 of the *EGFR* gene in three lines used in this study (data not shown). Cappuzzo et al. (22) have reported that *EGFR* gene amplification and increased copy number are important to limit therapeutic efficacy of gefitinib in lung cancer. However, there was no difference in gene amplification and copy number of *EGFR* in both HCC3 and AD3 cells. Therefore, altered expression of angiogenic factors and Akt activations between two lines might not be directly associated with gene amplification of *EGFR*.

In this study, AD3 cells showed both constitutive Akt activation and reduced levels of PTEN compared with HCC3 and CBO12C3 cells (Figs. 3B and 5A). It has been shown that phospho-Akt was recognized as a risk factor for early disease recurrence and poor prognosis of HCC (49). We have previously reported that sensitivity to gefitinib in NSCLC cell lines is associated with *EGFR* signaling pathways involving Akt and ERK1/2 and is closely linked with cell growth and apoptosis; activation of Akt and/or ERK1/2 was most susceptible to inhibition by gefitinib in one of the nine NSCLC cell lines, which carries a mutation in the *EGFR* catalytic domain (20). Both PI3K and PTEN are closely associated with activation of Akt, and reducing PTEN levels might be expected to change both Akt activation in response to EGF and its



**Figure 4.** Effect of kinase inhibitors, LY294002 and U0126, on the production of VEGF and CXCL1 by HCC3 and AD3 cells cultured in the presence of EGF. Cells ( $2.5 \times 10^4$ /well) were incubated with or without kinase inhibitors in 24-well plates for 24 hours. VEGF and CXCL1 were measured in culture supernatants using ELISA kits. Concentrations of the angiogenic factors in the presence of inhibitors were normalized to those in the absence of inhibitors. Columns, mean of triplicate wells for HCC3 (closed) and AD3 (open); bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with treatment with EGF alone using the two-tailed Student's *t* test.



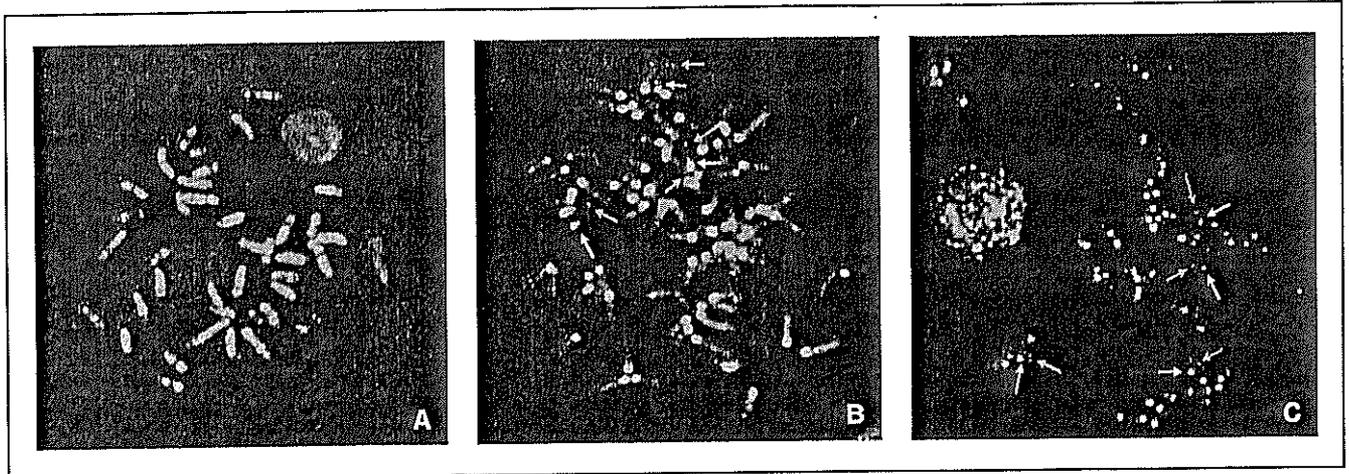
**Figure 5.** Comparison of PI3K and PTEN protein levels in three HCC cell lines and effects of depleting PTEN using siRNA in HCC3. **A**, HCC cells were cultured with or without 10 ng/mL EGF for 24 hours. Protein extracts were resolved by Western blot analysis with anti-PI3K p110, anti-PTEN, or anti-β-actin antibodies. Levels of PTEN expression were measured by densitometry and normalized to the β-actin levels in each cell line. *Columns*, mean of triplicate experiments; *bars*, SD. **B**, HCC3 cells were transfected with PTEN siRNA, and after 24 hours PTEN levels were determined by Western blot analysis. Levels of PTEN expression were normalized to β-actin levels in each cell line. **C**, effect of PTEN siRNA on Akt phosphorylation in HCC3. Twenty-four hours after transfection with siRNA (100 nmol/L), HCC3 cells were stimulated with 10 ng/mL EGF for 15 minutes, with or without gefitinib at the concentrations indicated. Phosphorylated Akt levels were normalized to nonphosphorylated Akt levels in the presence of EGF but without gefitinib. **D**, effect of PTEN siRNA on inhibition of the EGF-induced production of VEGF and CXCL1 by gefitinib in HCC3. Cells ( $2.5 \times 10^5$ /well) were incubated in the presence or absence of 10 ng/mL EGF with or without different doses of gefitinib in 24-well plates for 24 hours. *Columns*, mean of triplicate wells for nontransfected (*closed*), mock-transfected (*hatched*), and PTEN siRNA-transfected cells (*open*); *bars*, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with treatment with EGF alone using the two-tailed Student's *t* test.

sensitivity to gefitinib. Reducing *PTEN* gene expression in HCC3 cells using PTEN siRNA indeed decreased the sensitivity of both Akt activation and the production of VEGF and CXCL1 to gefitinib (Fig. 5). Another study has shown that loss of PTEN expression contributed to resistance to gefitinib by permitting Akt activation independently of receptor kinase signaling, in EGFR-expressing tumor cells (50). These findings support our current evidence for a role of constitutive Akt activation in the gefitinib resistance shown by angiogenesis induced by AD3 cells. However, further studies will be required to understand exactly how constitutive Akt

activation is associated with the sensitivity or resistance of EGFR signaling to gefitinib.

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**Figure 6.** Detection of gene amplification and copy number of *EGFR* gene in HCC3 and AD3 cells by FISH analysis. The digoxigenin-labeled *EGFR* and biotin-labeled chromosome 11 probes were detected using antidigoxigenin-Cy3 (yellow) and anti-biotin-Cy5 (red). Representative metaphase spreads from normal spleen cells (A), HCC3 cells (B), and AD3 cells (C). The hybridized probe was detected by fluorescence microscopy.

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## 17 $\beta$ -Estradiol Induces Down-Regulation of *Cap43/NDRG1/Drg-1*, a Putative Differentiation-Related and Metastasis Suppressor Gene, in Human Breast Cancer Cells

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**Abstract.** Purpose: Cap43 is known as a nickel- and calcium-inducible gene. In the present study, we examined whether 17 $\beta$ -estradiol (E<sub>2</sub>) could affect the expression of Cap43 in breast cancer. Experimental Design: Real-time PCR, immunoblotting, and immunocytochemistry were used to examine the expression of Cap43 and estrogen receptor- $\alpha$  (ER- $\alpha$ ) in breast cancer cell lines. MDA-MB-231 and SK-BR-3 cell lines were transfected with ER- $\alpha$  cDNA to establish cells overexpressing ER- $\alpha$ . Immunohistochemistry was used to evaluate the expression of the Cap43 protein in breast cancer patients ( $n = 96$ ) and the relationship between Cap43 expression and clinicopathologic findings was examined. Results: Of the eight cell lines, four expressed higher levels of Cap43 with very low levels of ER- $\alpha$ , whereas the other four expressed lower levels of Cap43 with high ER- $\alpha$  levels. Treatment with E<sub>2</sub> decreased the expression of Cap43 dose-dependently in ER- $\alpha$ -positive cell lines but not in ER- $\alpha$ -negative lines. Administration of antiestrogens, tamoxifen and ICI 182780, abrogated the E<sub>2</sub>-induced down-regulation of Cap43. Overexpression of ER- $\alpha$  in both ER- $\alpha$ -negative cell lines SK-BR-3 and MDA-MB-231 resulted in down-regulation of Cap43. Immunostaining studies showed a significant correlation between Cap43 expression and the histologic grade of tumors ( $P = 0.0387$ ). Furthermore, Cap43 expression was inversely correlated with the expression of ER- $\alpha$  ( $P = 0.0374$ ). Conclusions: E<sub>2</sub>-induced down-regulation of Cap43 seems to be mediated through ER- $\alpha$ -dependent pathways in breast cancer cells both in culture and in patients. Cap43 has potential as a molecular marker to determine the therapeutic efficacy of antiestrogenic anticancer agents in breast cancer.

Cap43 has been identified as a nickel- and calcium-inducible gene (1). The Cap43, a 43-kDa protein, has three unique 10-amino acid tandem-repeat sequences at its carboxyl terminus and is phosphorylated by protein kinase A (2). The *Cap43* gene is identical to the *N-myc* downstream regulated gene 1 (*NDRG1*), a human homocysteine-inducible gene (3), and to the differentiation-related gene-1 (4). Cap43 expression is reduced in tumor cells (*RTP/Rit42*; ref. 5). Furthermore, the

expression of Cap43 is markedly influenced by several stimuli, including oxidative stress, metal ions, hypoxia, phorbol esters, vitamins A and D, steroids, homocysteine, and tunicamycin as well as oncogenes (*N-myc* and *C-myc*) and tumor suppressor genes (p53 and VHL; refs. 1, 3, 5–10).

Although several studies have elucidated various characteristics of the Cap43, its exact function remains unclear. Cap43 is expressed in various organs, including the prostate, ovary, colon, and kidney, and its expression is dynamically changed during postnatal development in the kidney, brain, liver, and nerves (3, 11–13). These studies, which suggest the involvement of Cap43 in organ maturation and differentiation, have recently prompted detailed investigations of its role in the neuronal system. Cap43 was originally shown to be responsible for Charcot-Marie-Tooth disease type 4D; mutations in *Cap43* are commonly identified in this hereditary neuropathy of the motor and sensory systems. Okuda et al. have recently established Cap43 knockout mice that exhibit Schwann cell dysfunction, suggesting that Cap43 is essential for the maintenance of the myelin sheaths in peripheral nerves (14). Consistent with this study, Hirata et al. have reported that Cap43 plays an important role in the terminal differential of Schwann cells during nerve regeneration (15).

In contrast with these studies, Stein et al. have identified *Cap43* as a gene that is up-regulated by p53 and have also

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shown that Cap43 is required for p53-dependent apoptosis, thereby indicating that Cap43 is a p53 target gene (16). Furthermore, Kim et al. have reported that the Cap43 is associated with microtubules in the centrosome and participates in the spindle checkpoint in a p53-dependent manner, suggesting that Cap43 may play a key role in the regulation of microtubule dynamics (17). Overexpression of the Cap43 gene also inhibits growth in colon cancers as well as metastasis in prostate and colon cancer cells in an animal model (18, 19), suggesting that Cap43 suppresses metastasis. Low levels of Cap43 expression in breast cancer cells are closely correlated with poor clinical outcomes (20). Cap43 thus seems to be closely associated with the differentiation and/or malignant states of human cancers.

In the present study, we examine how the expression of the Cap43 gene could be modulated during therapeutic treatment by antiestrogenic drugs and discuss the potential of Cap43 as a molecular target for the therapeutic efficacy of antiestrogenic anticancer agents in breast cancer.

## Materials and Methods

**Cells and cell culture.** Human breast cancer cells SK-BR-3, MDA-MB-231, T47D, MCF-7, and CRL1500 were obtained from the American Type Culture Collection (Manassas, VA). YMB-1 and OCUB-M were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) and Riken/Wako (Osaka, Japan), respectively. A tamoxifen-resistant cell line, R-27, was established from MCF-7 (21). All cell lines were grown in phenol red-free McCoy's (PromoCell GmbH, Heidelberg, Germany) and Leibovitz's L-15, RPMI 1640, and  $\alpha$ -MEM containing 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100  $\mu$ g/mL streptomycin sulfate.

**Estrogen receptor- $\alpha$  overexpression in estrogen receptor-negative cell lines.** Two estrogen receptor- $\alpha$  (ER- $\alpha$ )-negative cell lines, SK-BR-3 and MDA-MB-231, were transfected with either pRC/CMV vector or ER- $\alpha$  plasmid DNA (a kind gift from Dr. Shin-ichi Hayashi, Department of Molecular Medical Technology, Faculty of Medicine, Tohoku University, Sendai, Japan) using LipofectAMINE Plus (Invitrogen Life Technologies, Inc., Gaithersburg, MD). Three days after transfection, 500  $\mu$ g/mL G418 disulfate (Nacalai Tesque, Kyoto, Japan) was added to the growth medium. The resulting G418-resistant cells were propagated to generate stable cell lines for use in further studies.

**Cell proliferation assay.** Cells were plated at  $2 \times 10^4$  per well in 12-well dishes. After 48 hours, cells were rinsed and incubated for a further 24 hours in same medium supplemented with 5% double charcoal-stripped serum CSS. Cells were then washed with serum-, estrogen-, and phenol red-free medium and then incubated in medium supplemented with 5% CSS in the presence or absence of 17 $\beta$ -estradiol E<sub>2</sub> (Sigma-Aldrich Co., St. Louis, MO) with or without tamoxifen (Calbiochem, La Jolla, CA). The cells were trypsinized and counted at 0, 2, 4, and 6 days after incubation with a coulter counter (Beckman Coulter, Miami, FL).

**Immunocytochemistry.** Cells were trypsinized and plated on glass coverslips in six-well plates. Then, cells were rinsed with PBS and fixed in 4% paraformaldehyde/PBS for 30 minutes at room temperature. Cells were permeabilized with solution containing 5% bovine serum albumin, 0.2% Triton X-100 in PBS for 90 minutes at room temperature. After 1 hour of blocking with 2% goat serum, the cells were incubated overnight with rabbit polyclonal anti-Cap43 (1:1,000; developed in our laboratory; ref. 10). Cells were then rinsed and incubated with goat anti-rabbit IgG and 1  $\mu$ g/mL Alexa Fluor 546 (Molecular Probes, Eugene, OR) for 60 minutes at room temperature. Nuclear staining was carried out using 4',6-diamidino-2-phenylindole (1:1,000, Dojindo, Kumamoto, Japan). Coverslips were mounted on

slide glasses using gel mount and viewed using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan).

**Western blot analysis.** After treatment with E<sub>2</sub>, tamoxifen, ICI 182780 (Nacalai Tesque), or nickel chloride (NiCl<sub>2</sub>; Nacalai Tesque), 200  $\mu$ L lysis buffer [0.2% NP40, 225 mmol/L NaCl, 25 mmol/L Tris (pH 7.4)] was added. The cells were harvested, and the cell slurry was sonicated briefly before centrifugation at 15,000  $\times$  g for 15 minutes at 4°C. The supernatant was collected, and 50  $\mu$ g aliquots of protein were loaded into each well, separated using SDS-PAGE, and transferred to Immobilon membranes (Millipore, Bedford, MA). After transfer, blots were incubated with blocking solution and probed with antibodies. The antibodies used were as follows: rabbit polyclonal antibody directed against Cap43 (produced in our laboratory; ref. 10), rabbit polyclonal anti-ER- $\alpha$  antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti- $\beta$ -actin (Sigma-Aldrich). The relative expression of each protein was calculated using the NIH Image Analysis Program version 1.62 (NIH, Bethesda, MD).

**RNA extraction and cDNA synthesis.** Total RNA was extracted using ISOGEN-LS reagent (Nippon Gene, Toyama, Japan) and digested with DNase I (Sigma-Aldrich). Total RNA (1  $\mu$ g) was then reverse transcribed using random hexamer priming and SuperScript II reverse transcriptase (Toyobo, Osaka, Japan).

**Real-time PCR.** cDNA (100 ng) was amplified in a real-time PCR using SYBR Green Mix (PE Applied Biosystems, Warrington, United Kingdom) and 200 nmol/L primer for Cap43. The real-time PCR reactions were done in an ABI PRISM Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA) under the following conditions: 50°C for 2 minutes, 95°C for 1 minute followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The sequences of primers were as follows: Cap43 forward primer 5'-AGCCGGACATTCTGGAATG-3' and reverse primer 5'-CGGTACTTCCCCAGCACACTT-3', pS2 forward primer 5'-TTTGGAGCAGAGAGGAGGCAATGG-3' and reverse primer 5'-TGGTATTAGGATAGAACACCAGGG-3', and C-myc forward primer 5'-AGCCGGCCGTAGTTAATTCAT-3' and reverse primer 5'-CGCCC-TCTGCTTTGGGA-3'. Glyceraldehyde-3-phosphate dehydrogenase and  $\beta$ -actin were used as reference genes.

**Immunohistochemistry.** Tissue sections were taken from 96 breast cancer patients who underwent radical surgery (Department of Surgery, Kurume University Hospital, Kurume, Japan) between 1995 and 1999. The 4- $\mu$ m tissue sections were deparaffinized for 15 minutes at 85°C and the slides were heated in a microwave oven in a CCl<sub>4</sub> buffer for 60 minutes. The sections were stained using the Benchmark (IHC Automated Systems, Tucson, AZ) with rabbit polyclonal anti-Cap43 (9, 10), anti-ER- $\alpha$ , anti-progesterone receptor (PgR), anti-epidermal growth factor receptor (EGFR), and anti-HER-2 antibody. All antibodies else than Cap43 (produced in our laboratory) were purchased from Ventana Medical Systems (Tucson, AZ). The samples were viewed using an Olympus BX51 fluorescence microscope.

The extent to which Cap43 proteins were stained in immunohistologic studies was analyzed to compare the strength of Cap43 expression in cancer cells with that in normal glands: cancer cells that expressed Cap43 stronger than normal glands appeared positive for staining and normal glands expressing Cap43 stronger than cancer cells were negative for staining. The extent to which ER- $\alpha$  or PgR proteins were stained in immunohistologic studies was defined by the percentage of cells with strongly stained nuclei:  $\geq 10\%$  defined a gland as positive for ER- $\alpha$  or PgR and  $\leq 9\%$  defined it as negative. The immunohistochemical expression of EGFR and HER-2 was categorized into four groups: score 0, no staining at all or membrane staining in  $< 10\%$  of the tumor cells; score 1+, faint/barely perceptible partial membrane staining in  $> 10\%$  of the tumor cells; score 2+, weak to moderate staining of the entire membrane in  $> 10\%$  of the tumor cells; score 3+, strong staining of the entire membrane in  $> 10\%$  of the tumor cells. The extent of immunohistologic staining for EGFR and HER-2 was defined as follows: scores of 2+ or 3+ were regarded as positive and scores of 0 or 1+ were regarded as negative. The positive cells were counted by two experienced observers who were blinded to the condition of the

patients. The relationship between Cap43 and each clinicopathologic finding (age, tumor size, menopausal status, histologic grade, lymph node metastasis, and expression of EGFR, HER-2, ER- $\alpha$ , and PgR) and postoperative survival was examined.

**Statistical analysis.** The  $\chi^2$  test, Fisher's exact probability test, and Student's *t* test were used for statistical analyses. In patients undergoing resection, the relationships between Cap43 expression and prognosis were examined by the Kaplan-Meier method (22) and the univariate relationship between prognostic factors and overall survival rate was assessed by the log-rank test. *P* < 0.05 was regarded as statistically significant.

**Results**

**Comparison of cellular levels of Cap43 and ER- $\alpha$  in human breast cancer.** All eight breast cancer cell lines used in this study were screened for Cap43 expression using immunoblotting and Immunocytochemistry (Fig. 1). SK-BR-3, MDA-MB-231, CRL1500, OCUB-M, and R-27 showed relatively high levels of Cap43 protein expression, whereas its expression was decreased in T47D, MCF-7, and YMB-1 (Fig. 1A). ER- $\alpha$  was expressed in T47D, MCF-7, YMB-1, and R-27, but its expression was negligible in SK-BR-3, MDA-MB-231, CRL1500, and OCUB-M. Consistent with the results from Western blot analysis (Fig. 1A), immunocytochemical analysis of the eight cell lines showed the expression of Cap43 in SK-BR-3, MDA-MB-231, CRL1500, OCUB-M, and R-27 (Fig. 1B). A tamoxifen-resistant cell line, R-27, showed a much higher expression of Cap43 protein than its parental counterpart cell

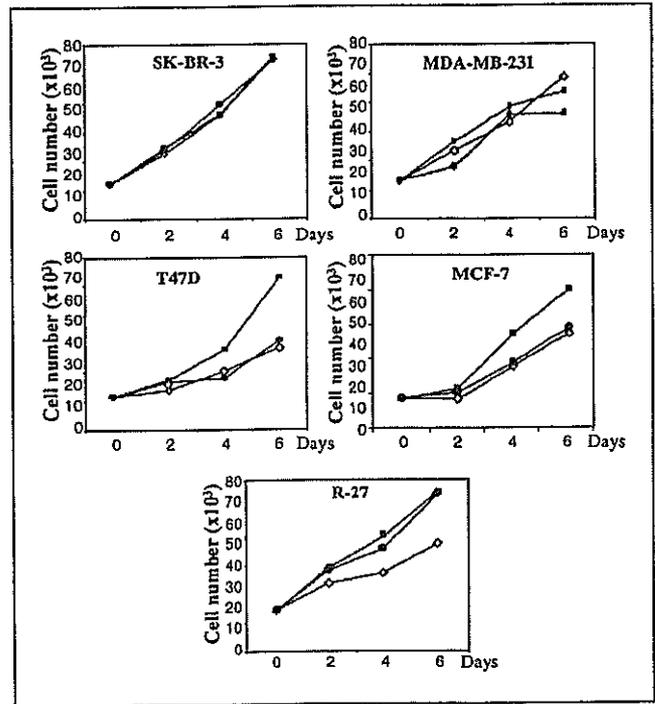


Fig. 2. Effect of E<sub>2</sub> (10<sup>-8</sup> mol/L) with or without tamoxifen (10<sup>-6</sup> mol/L) on cell growth. The cells were trypsinized and counted with a Coulter counter at the indicated times. ○, E<sub>2</sub> (-); ■, E<sub>2</sub> (10<sup>-8</sup> mol/L); ●, E<sub>2</sub> (10<sup>-8</sup> mol/L) + tamoxifen (10<sup>-6</sup> mol/L).

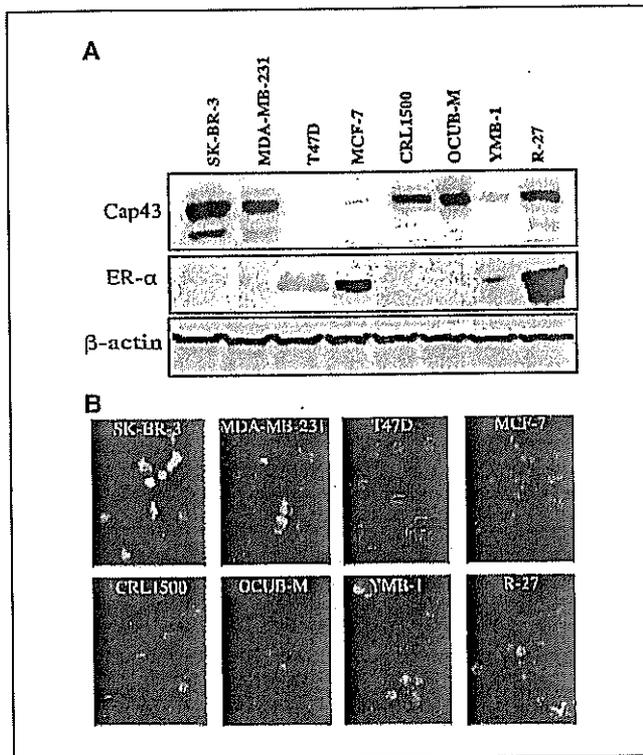


Fig. 1. Protein expression of Cap43 and ER- $\alpha$  in eight breast cancer cell lines. A, cellular protein levels of Cap43 and ER- $\alpha$  were determined by Western blot analysis. B, cells were viewed using an Olympus BX51 fluorescence microscope and photographed with an Olympus DP-70 digital camera. Magnification,  $\times 200$ . Green, Cap43.

line, MCF-7 (Fig. 1A and B). We conducted further studies to examine whether the expression of ER- $\alpha$  in these cell lines, and the addition of E<sub>2</sub>, tamoxifen, and ICI 182780, to the culture could modulate the expression of Cap43. Three ER- $\alpha$ -positive (T47D, MCF-7, and R-27) and two ER- $\alpha$ -negative (SK-BR-3 and MDA-MB-231) cell lines were selected for further study.

**Cell growth dependence on E<sub>2</sub> and the effect of tamoxifen.** We used an E<sub>2</sub>-depleted culture condition to examine whether the addition of E<sub>2</sub> to the five breast cancer cell lines tested in this experiment promoted E<sub>2</sub>-dependent growth. Previous studies have shown that the growth of human breast cancer cell lines depends on the presence of 10<sup>-8</sup> to 10<sup>-9</sup> mol/L E<sub>2</sub> in the culture medium (23, 24). Of the five cell lines, the addition of 10<sup>-8</sup> mol/L E<sub>2</sub> enhanced growth in the ER- $\alpha$ -positive cell lines (T47D, MCF-7, and R-27). However, neither of the ER- $\alpha$ -negative lines, SK-BR-3 and MDA-MB-231, showed enhanced growth on the addition of E<sub>2</sub> to the culture (Fig. 2). Tamoxifen at 10<sup>-6</sup> mol/L inhibited E<sub>2</sub>-induced cell growth in T47D and MCF-7 cell lines, resulting in a degree of growth similar to that observed in the absence of E<sub>2</sub>. However, tamoxifen could not inhibit E<sub>2</sub>-induced cell growth in R-27 cells (Fig. 2).

**Effects of E<sub>2</sub> with or without tamoxifen or ICI 182780 on Cap43 expression.** We next used real-time PCR and Western blotting to examine whether E<sub>2</sub> could modulate expression of Cap43 in cultured breast cancer cells. As shown in Fig. 3A, expression of Cap43 mRNA was markedly down-regulated by exogenous addition of E<sub>2</sub> for 24 hours in the ER- $\alpha$ -positive lines, T47D, MCF-7, and R-27, but not in the ER- $\alpha$ -negative lines, SK-BR-3 and MDA-MB-231 (Fig. 3A). Furthermore, E<sub>2</sub> down-regulated expression of Cap43 in a dose-dependent

manner in the three ER-α-positive cell lines. Tamoxifen at 10<sup>-6</sup> mol/L or ICI 182780 at 10<sup>-7</sup> mol/L almost completely abrogated the E<sub>2</sub>-induced down-regulation of the Cap43 gene in two ER-α-positive lines, T47D and MCF-7, in the presence

of various doses of E<sub>2</sub> (10<sup>-12</sup> to 10<sup>-6</sup> mol/L). However, the abrogatory effect of tamoxifen was hardly observed in the tamoxifen-resistant R-27 line in comparison with its parental MCF-7 cells (Fig. 3A). ICI 182780 at 10<sup>-7</sup> mol/L almost

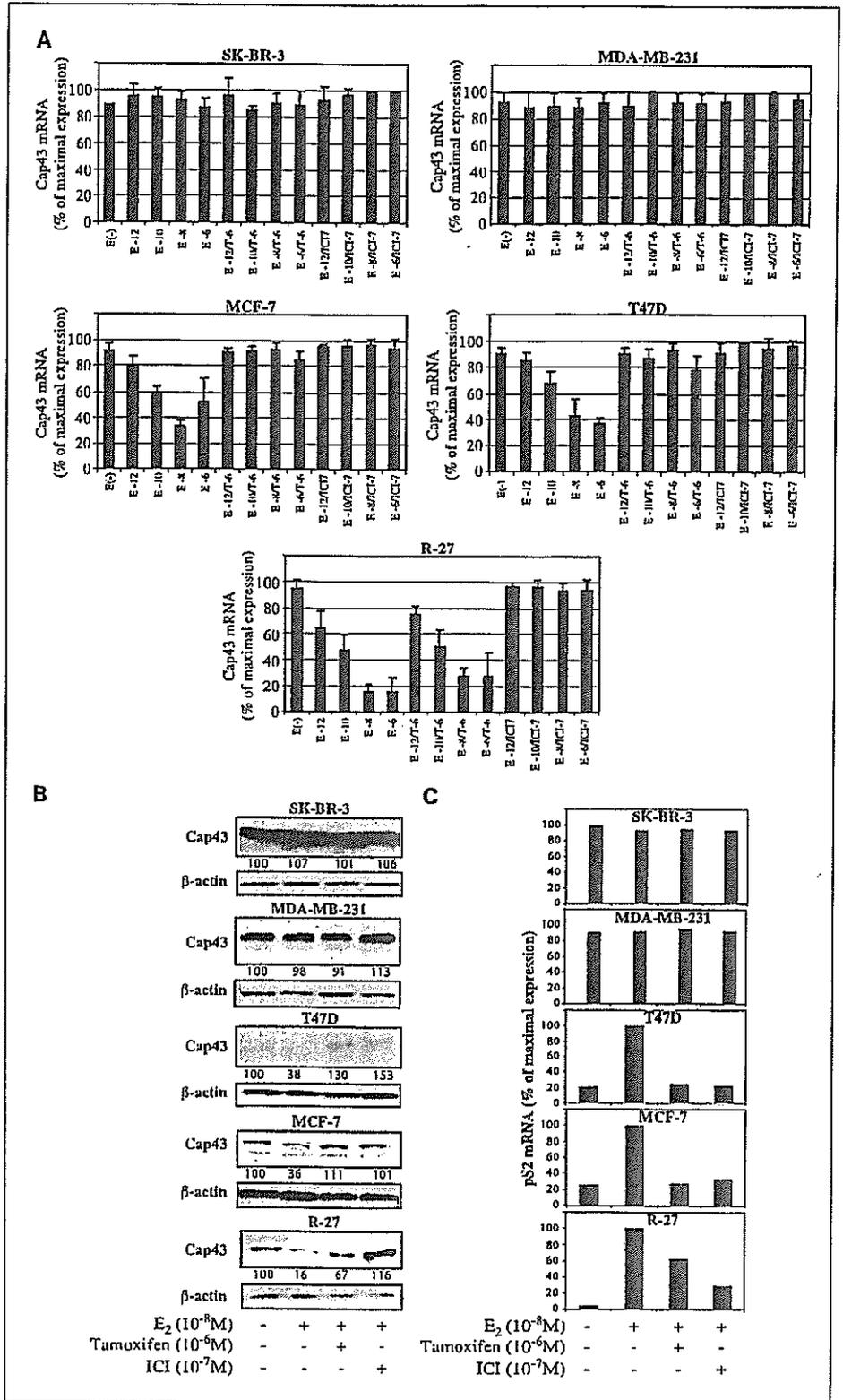


Fig. 3. Effect of E<sub>2</sub> with or without tamoxifen and ICI 182780 on Cap43 expression. **A**, Cap43 mRNA. E(-), E<sub>2</sub> (-); E-12, E<sub>2</sub> (10<sup>-12</sup> mol/L); E-10, E<sub>2</sub> (10<sup>-10</sup> mol/L); E-8, E<sub>2</sub> (10<sup>-8</sup> mol/L); E-6, E<sub>2</sub> (10<sup>-6</sup> mol/L); E-12/T-6, E<sub>2</sub> (10<sup>-12</sup> mol/L) + tamoxifen (10<sup>-6</sup> mol/L); E-10/T-6, E<sub>2</sub> (10<sup>-10</sup> mol/L) + tamoxifen (10<sup>-6</sup> mol/L); E-8/T-6, E<sub>2</sub> (10<sup>-8</sup> mol/L) + tamoxifen (10<sup>-6</sup> mol/L); E-6/T-6, E<sub>2</sub> (10<sup>-6</sup> mol/L) + tamoxifen (10<sup>-6</sup> mol/L); E-12/ICI-7, E<sub>2</sub> (10<sup>-12</sup> mol/L) + ICI 182780 (10<sup>-7</sup> mol/L); E-10/ICI-7, E<sub>2</sub> (10<sup>-10</sup> mol/L) + ICI 182780 (10<sup>-7</sup> mol/L); E-8/ICI-7, E<sub>2</sub> (10<sup>-8</sup> mol/L) + ICI 182780 (10<sup>-7</sup> mol/L); E-6/ICI-7, E<sub>2</sub> (10<sup>-6</sup> mol/L) + ICI 182780 (10<sup>-7</sup> mol/L). **B**, Cap43 protein. Numbers, density of the Cap43 band for each cell line normalized to the density in the absence of any drug (100%). **C**, effects of E<sub>2</sub> with or without tamoxifen and ICI 182780 on pS2 mRNA expression in breast cancer cells. Maximal expression levels of pS2 mRNA in each cell line are normalized as 100%.

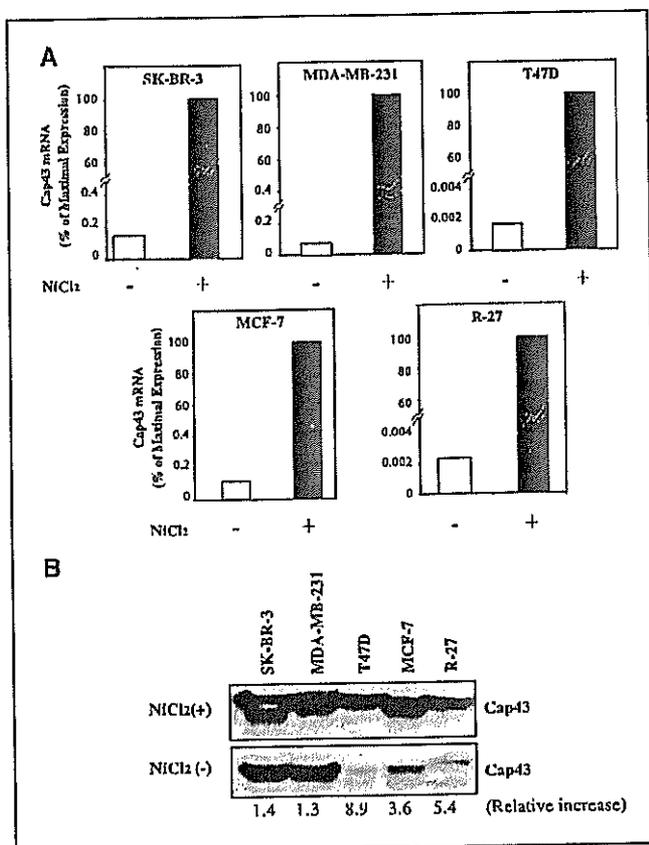


Fig. 4. Enhanced expression of Cap43 gene by nickel in breast cancer cell lines. Expression of Cap43 mRNA (A) and Cap43 protein (B) were analyzed by real-time PCR and Western blotting, respectively. The relative fold increase of Cap43 protein expression is the amount of Cap43 protein detected in the presence of NiCl<sub>2</sub> divided by the amount of Cap43 present in the absence of NiCl<sub>2</sub>.

completely abrogated the E<sub>2</sub>-induced down-regulation of the Cap43 in R-27 cells, whereas tamoxifen at 10<sup>-6</sup> mol/L could not abrogate the E<sub>2</sub>-induced down-regulation of the Cap43 when in the presence of E<sub>2</sub> at 10<sup>-8</sup> and 10<sup>-6</sup> mol/L, respectively. In contrast, no marked change in Cap43 mRNA levels in SK-BR-3 and MDA-MB-231 was evident when these cell lines were treated with E<sub>2</sub> in the absence or presence of tamoxifen and ICI 182780 (Fig. 3A). Consistent with the effects on mRNA levels of Cap43, Western blot analysis also showed that the expression of Cap43 protein was decreased in E<sub>2</sub>-treated T47D, MCF-7, and R-27 lines and tamoxifen at 10<sup>-6</sup> mol/L or ICI 182780 at 10<sup>-7</sup> mol/L abrogated the E<sub>2</sub>-induced down-regulation of Cap43 in these three ER-α-positive cell lines. By contrast, neither treatment with E<sub>2</sub> alone nor E<sub>2</sub> with tamoxifen or ICI 182780 affected cellular Cap43 protein levels in the SK-BR-3 or MDA-MB-231 lines (Fig. 3B).

Previous studies have shown that the expression of the pS2 gene is profoundly affected by E<sub>2</sub> in MCF-7 cells (23-25). The results from the present study are consistent with these findings, showing that administration of E<sub>2</sub> markedly increases expression of pS2 mRNA and that administration of tamoxifen or ICI 182780 blocks this E<sub>2</sub>-induced stimulatory effect in the T47D, MCF-7, and R-27 cell lines (Fig. 3C). The inhibitory effect of tamoxifen on the E<sub>2</sub>-induced stimulation of

pS2 mRNA expression in the R-27 line was much less than that in its parental MCF-7 cells. By contrast, ICI 182780 at 10<sup>-7</sup> mol/L more greatly abrogated the E<sub>2</sub>-induced down-regulation of Cap43 than tamoxifen at 10<sup>-6</sup> mol/L in R-27 cells. Expression of pS2 mRNA was not affected by either E<sub>2</sub> alone or E<sub>2</sub> and tamoxifen in the SK-BR-3 and MDA-MB-231 (Fig. 3C).

**Up-regulation of the Cap43 gene by nickel in all breast cancer cell lines.** Cap43 was originally isolated as a gene induced by nickel compounds and its expression is highly susceptible to the presence of nickel (1, 9). We examined whether exposure to nickel at 1 mmol/L for 24 hours could specifically alter the expression of Cap43 in the five breast cancer cell lines. Exposure to nickel markedly enhanced cellular Cap43 mRNA levels in all five lines (Fig. 4A). The marked increase in the levels of Cap43 mRNA seen in these cell lines was observed irrespective of the expression of ER-α (Fig. 4A). Western blot analysis also showed significant increases in the levels of Cap43 protein in nickel-treated cell lines (Fig. 4B). Cap43 protein levels were higher in the two untreated ER-α-negative lines, SK-BR-3 and MDA-MB-231, than in the ER-α-positive lines. However, expression of Cap43 protein was further enhanced in these ER-α-positive cell lines in response to nickel.

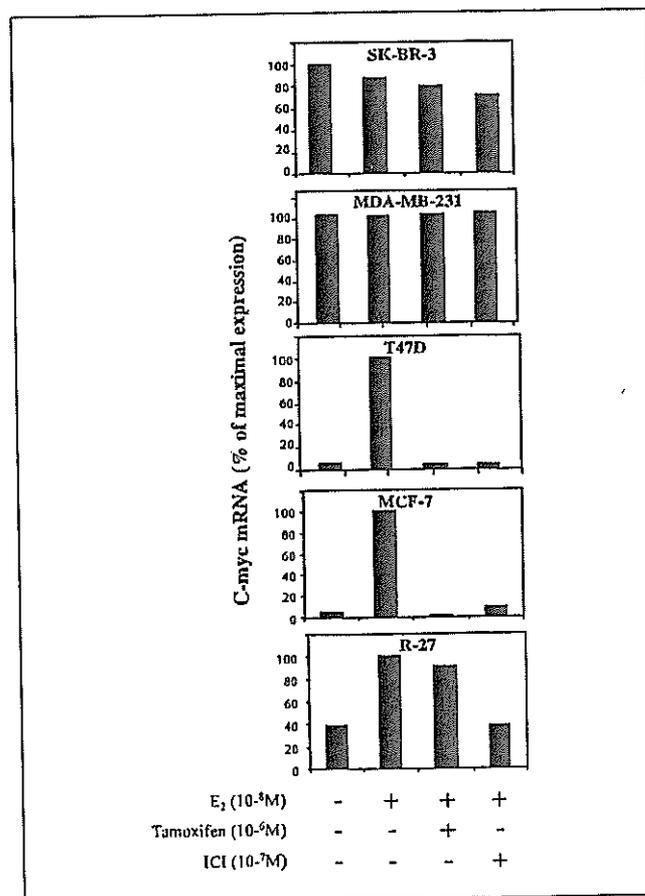


Fig. 5. Effects of E<sub>2</sub> with or without tamoxifen and ICI 182780 on C-myc mRNA expression in breast cancer cell lines. Maximal expression levels of C-myc mRNA in each cell line are normalized as 100%.

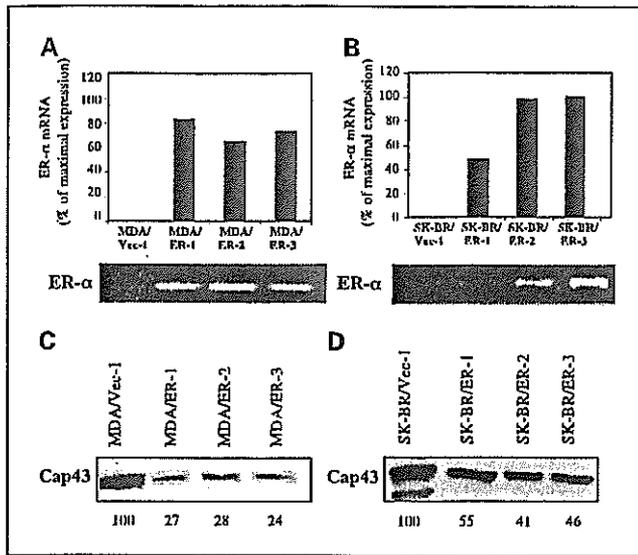


Fig. 6. Overexpression of ER- $\alpha$  in two breast cancer cell lines with low ER- $\alpha$  expression, MDA-MB-231 and SK-BR-3, and Cap43 expression in these cell lines. ER- $\alpha$  mRNA levels were determined by real-time PCR (A and B). Cap43 protein levels in ER- $\alpha$  transfectants were analyzed using Western blotting (C and D). Cap43 protein levels in each transfectant are normalized to the Cap43 protein band in MDA/Vec-1 or SK-BR/Vec-1 as 100%.

**Effect of  $E_2$  with or without tamoxifen or ICI 182780 on C-myc mRNA expression.** C-myc as well as N-myc is known to regulate expression of Cap43 gene (3). Expression of C-myc is highly susceptible to  $E_2$ . As shown in Fig. 5, expression of C-myc mRNA was markedly up-regulated by addition of  $E_2$  in the ER- $\alpha$ -positive lines, T47D, MCF-7, and R-27. However, there was no change in C-myc expression in the ER- $\alpha$ -negative cell lines, SK-BR-3 and MDA-MB-231. Similarly, tamoxifen or ICI 182780 blocks this  $E_2$ -induced stimulatory effect in T47D and MCF-7 cells (Fig. 5). Compared with the inhibitory effect of tamoxifen on the  $E_2$ -induced C-myc up-regulation in MCF-7 cells, tamoxifen showed only a slight, if any, effect on the  $E_2$ -induced C-myc up-regulation in R-27 cells. By contrast, almost complete inhibition by ICI 182780 was observed on the  $E_2$ -induced up-regulation of C-myc in R-27 cells as well as MCF-7.

**Overexpression of ER- $\alpha$  in ER- $\alpha$ -negative cells down-regulates Cap43 expression.** We examined whether the  $E_2$ -induced down-regulation of Cap43 was specifically mediated through its interaction with ER- $\alpha$ . We introduced ER- $\alpha$  cDNA into the ER- $\alpha$ -negative lines, MDA-MB-231 and SK-BR-3, and established six cell lines, MDA/ER-1, MDA/ER-2, MDA/ER-3, SK-BR/ER-1, SK-BR/ER-2, and SK-BR/ER-3. We also isolated transfectants of the vector alone (MDA/Vec-1 and SK-BR/Vec-1). ER- $\alpha$  gene expression was observed in the cDNA transfectants from the SK-BR-3 and MDA-MB-231 lines (Fig. 6A and B). Three ER- $\alpha$ -expressing cell lines derived from SK-BR-3 and MDA-MB-231 showed a marked decrease in their expression of the Cap43 protein in comparison with their vector counterparts (Fig. 6C and D). Taken together, these data consistently indicated a close association between Cap43 gene expression and  $E_2$ -ER- $\alpha$  signaling in human breast cancer cells.

**Expression of Cap43 and ER- $\alpha$  in clinical samples of human breast cancer.** We next performed immunohistochemical analysis to examine whether the expression of Cap43 was associated with ER- $\alpha$  expression in tissue samples from breast

cancer patients. Ninety-six breast cancer patients were included in this study. The relationship between Cap43 expression and clinicopathologic findings is shown in Table 1. There was no significant correlation between the expression of Cap43 and age, tumor size, menopausal status, lymph node metastasis, EGFR expression, or HER-2 expression. However, because Cap43 expression was detected in 42% of grade 1 and 2 and 71% of grade 3 tumors, there was a significant correlation between Cap43 expression and tumor grade ( $P = 0.0387$ ; Table 1). Univariate analysis for 5-year postoperative overall survival done on these patients showed that there was no significant difference in Cap43 expression according to the postoperative prognosis ( $P = 0.345$ ).

Immunohistochemical analysis showed that breast cancers were variously positive and negative for the expression of Cap43 and ER- $\alpha$ . Figure 7 shows representative immunohistochemical data from two breast cancer patients. The tissue shown from case 1 with high expression of Cap43 and negligible expression of ER- $\alpha$  was therefore scored positive for the expression of Cap43 and negative for ER- $\alpha$  expression. By contrast, case 2 showed high expression of ER- $\alpha$  and negligible expression of Cap43, indicating that it was positive for ER- $\alpha$  and negative for Cap43. Positive expression of both Cap43 and ER- $\alpha$  was detected in 20 of 63 (31.7%) patients, and 43 of 63 (68.3%) patients were classified as being Cap43 negative and ER- $\alpha$  positive (Table 2). Cap43 expression was thus generally decreased in breast cancer cells from ER- $\alpha$  positive patients, and Cap43 expression was increased in breast cancer cells of ER- $\alpha$ -negative patients, indicating that the expression of Cap43 is inversely correlated with the expression of ER- $\alpha$  in breast cancer patients ( $P = 0.0374$ ). On the other hand, there was no relationship between PgR and Cap43 expression in breast cancer patients ( $P = 0.8405$ ; Table 2).

Table 1. Relationship between Cap43 expression and clinicopathologic variables in breast cancer ( $n = 96$ )

Factor	Total	Cap43 expression		P
		Negative, n (%)	Positive, n (%)	
Age		50.3 $\pm$ 12.1	51.2 $\pm$ 12.9	0.6629
Tumor size		3.7 $\pm$ 2.3	2.6 $\pm$ 1.3	0.1579
Menopausal status				
Pre	44	19 (43)	25 (57)	0.5432
Post	52	26 (50)	26 (50)	
Histologic grade				
1,2	55	32 (58)	23 (42)	0.0387
3	21	6 (29)	15 (71)	
Lymph node metastasis				
Absent	53	24 (45)	29 (55)	0.8375
Present	43	21 (49)	22 (51)	
EGFR				
Negative	73	38 (52)	35 (48)	0.0941
Positive	23	7 (30)	16 (70)	
HER-2				
Negative	62	29 (47)	33 (53)	>0.999
Positive	34	16 (47)	18 (53)	

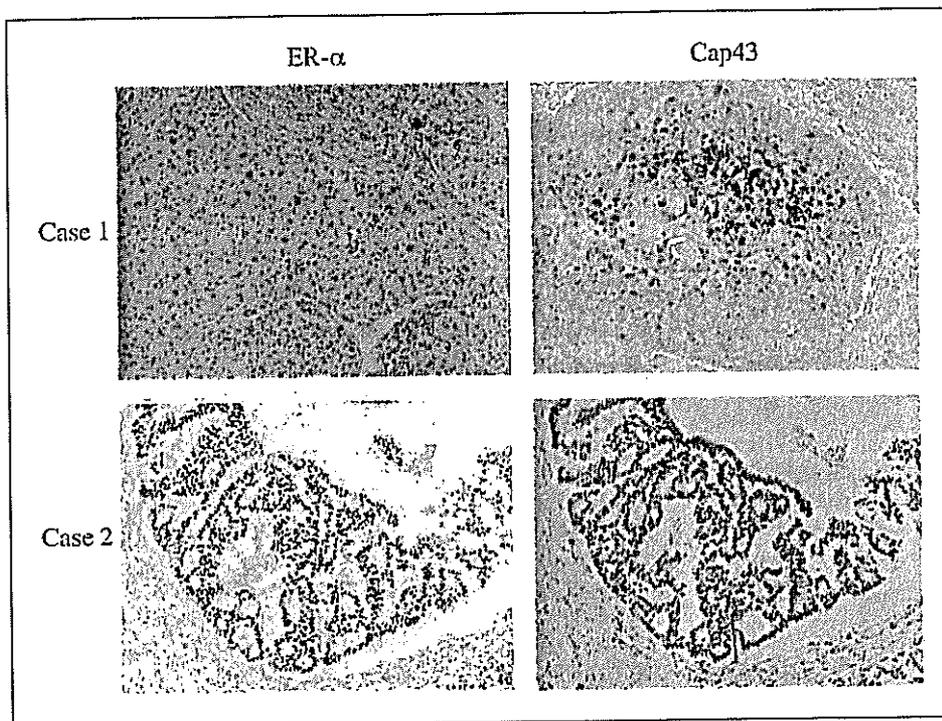


Fig. 7. Expression of Cap43 and ER- $\alpha$  in human breast cancer. Expression of Cap43 and ER- $\alpha$  in human breast cancer was analyzed by immunohistochemistry. Sections were analyzed for detection of ER- $\alpha$  (left) and the same site was also analyzed for Cap43 expression (right). Case 1 was evaluated as having high levels of Cap43 expression and not expressing ER- $\alpha$ . By contrast, case 2 showed high levels of ER- $\alpha$  expression and weak Cap43 expression. (Original magnification,  $\times 200$ ).

## Discussion

In our present study, we observed that Cap43 expression levels were inversely correlated with expression levels of ER- $\alpha$  in all seven human breast cell lines (Fig. 1). One tamoxifen-resistant line, R-27, which was derived from the MCF-7 line, however, showed expression of both Cap43 and ER- $\alpha$ . The addition of E<sub>2</sub> was found to markedly down-regulate the expression of the *Cap43* gene in ER- $\alpha$ -positive cell lines but not in ER- $\alpha$ -negative lines (Fig. 3A and B). Because the expression of a representative ER-responsive gene, *pS2*, could be modulated by E<sub>2</sub> only in ER- $\alpha$ -positive lines (Fig. 3C), we concluded that ER-dependent signaling operated in these ER- $\alpha$ -positive cell lines but not in the ER- $\alpha$ -negative lines. Furthermore, overexpression of ER- $\alpha$  in ER- $\alpha$ -negative cell lines induced down-regulation of both protein and mRNA levels of Cap43. Exposure to nickel, however, markedly increased the expression of the *Cap43* gene in both ER- $\alpha$ -positive and ER- $\alpha$ -negative lines (Fig. 4), suggesting that the E<sub>2</sub>-induced specific down-regulation of the *Cap43* gene depends on ER- $\alpha$ . Taken together, these studies indicate that the presence of the functional ER- $\alpha$  could be required for E<sub>2</sub>-induced down-regulation of Cap43.

We also showed that coadministration of tamoxifen or ICI 182780 abrogated the E<sub>2</sub>-induced down-regulation of the *Cap43* gene in ER- $\alpha$ -positive lines. Expression of the *Cap43* gene is thus modulated in response to E<sub>2</sub> or antiestrogen possibly through the ER- $\alpha$  expressed in human breast cancer cells. Tamoxifen at 10<sup>-6</sup> mol/L or ICI 182780 at 10<sup>-7</sup> mol/L almost completely abrogated the E<sub>2</sub>-induced down-regulation of *Cap43* gene in ER- $\alpha$ -positive breast cancer cell lines, MCF-7 and T47D. The abrogatory effect of tamoxifen seemed to be much less in a tamoxifen-resistant subline, R-27, compared

with the parental counterpart, MCF-7, in the presence of E<sub>2</sub> (10<sup>-8</sup> to 10<sup>-6</sup> mol/L). However, ICI 182780 could almost completely abrogate the E<sub>2</sub>-induced down-regulation of *Cap43* gene in R-27 cells. Moreover, the abrogatory effect of 10<sup>-6</sup> mol/L tamoxifen on expression of the E<sub>2</sub>-sensitive *pS2* gene also seemed to be much less than that of ICI 182780 at 10<sup>-7</sup> mol/L when R-27 cells were exposed to E<sub>2</sub> at 10<sup>-8</sup> mol/L (Fig. 3C). ICI 182780 showed  $\sim 10$ -fold higher antiestrogenic activity in the regulation of *Cap43* as well as *pS2* gene compared with tamoxifen, and ICI 182780 could thus overcome tamoxifen resistance in breast cancer cells, consistent with previous reports (26, 27). *Cap43* could be a molecular target for the functional hormone-dependent cell growth signal of breast cancers and also a target that is useful to determine the therapeutic efficacy of antiestrogenic anticancer agents.

Expression of *Cap43* gene is negatively regulated by *myc* gene (3). We examined the effect of E<sub>2</sub> on *C-myc* expression, and

Table 2. Relationship between *Cap43* and hormone receptor expression in clinical samples ( $n = 96$ )

	Total	Cap43 expression		P
		Positive, n (%)	Negative, n (%)	
ER- $\alpha$				
Positive	63	20/63 (31.7)	43/63 (68.3)	0.0374
Negative	33	21/33 (63.6)	12/33 (36.4)	
PgR				
Positive	50	26/50 (52.0)	24/50 (48.0)	0.8405
Negative	46	25/46 (54.3)	21/46 (45.7)	

E<sub>2</sub>-induced up-regulation of *C-myc* gene was observed only in ER- $\alpha$ -positive breast cancer cell lines. E<sub>2</sub> thus up-regulated *C-myc* and down-regulated Cap43 possibly through the E<sub>2</sub>-ER- $\alpha$  pathway. E<sub>2</sub>-induced down-regulation of *Cap43* gene might be mediated through up-regulation of *C-myc*. However, further study should be required to determine how *C-myc* is involved in the E<sub>2</sub>-induced down-regulation of *Cap43* gene. Oncogenes, tumor suppressor genes, and several physiologic stimuli are known to modulate expression of *Cap43* (see Introduction). Recent studies have shown that *Cap43* is a p53 target gene (16, 17). *Cap43* inhibited polyploidy in p53-negative cancer cell lines and increased the cell population at M phase when exposed to Taxol, a microtubule inhibitor, indicating that *Cap43* plays a role in the p53-dependent mitotic spindle checkpoint (17). Stein et al. have also reported that *Cap43* is necessary for p53-dependent apoptosis (16). Of the breast cancer cell lines used in this study, SK-BR-3, MDA-MB-231, and T47D have mutant p53 and MCF-7 has wild-type p53 (IARC TP53 Mutation Database, <http://www-p53.iarc.fr/>). Both ER- $\alpha$ -positive and ER- $\alpha$ -negative breast cancer cell lines with wild-type p53 showed a marked increase in *Cap43* expression when exposed to doxorubicin, an anticancer agent that mediates cytotoxicity through p53.<sup>6</sup> Nickel also promoted the increased expression of *Cap43* in all ER- $\alpha$ -positive and ER- $\alpha$ -negative lines (Fig. 4). The up-regulation of *Cap43* by nickel or doxorubicin occurred irrespective of the presence of p53. Tamoxifen- and ICI 182780-induced abrogation of the E<sub>2</sub>-induced down-regulation of *Cap43* might also occur irrespective of the presence of the p53 pathway but does depend on the presence of ER- $\alpha$ .

*Cap43* is a putative metastasis suppressor gene in human colon and prostate cancer, and its expression in these cancers is closely correlated with the prognosis of patients (18, 19). We also observed an inverse correlation between *Cap43* expression and the prognosis of patients with pancreatic cancers.<sup>7</sup> Immunohistochemical studies by Bandyopadhyay et al. (20) on tissue from 85 breast cancer patients have shown that patients positive for *Cap43* have a significantly more favorable prognosis than those with reduced expression of *Cap43*. The *Cap43* protein was detected in normal mammary gland cells in all 85 breast cancer patients; but its expression was significantly reduced in the tumor cells of 30% of patients (20). In our present study, immunohistochemical analysis of breast cancers showed an inverse correlation between *Cap43* expression and histologic grade or ER- $\alpha$  expression (Tables 1 and 2). However, there was no apparent correlation between *Cap43* expression and other clinical and pathologic features, including lymph

node metastasis and prognosis (Tables 1 and 2). At present, the reasons underlying discrepancies on the correlation between *Cap43* expression and prognosis in breast cancer patients are unclear. Such discrepancies might be due to differences in the background of patients and the methods and evaluation of immunohistochemical staining used. There are also conflicting studies regarding the effects of *Cap43* expression levels in colon cancer. *Cap43* has been reported to be down-regulated in colon cancers with low levels of metastasis (4, 18). By contrast, a study by Wang et al. reported that *Cap43* expression was well correlated with the progression of colon cancer (28). Further studies are required to understand the pathways by which *Cap43* protein could modulate malignant characteristics and mechanisms, including tumor progression in breast cancer.

The action of estrogen is mediated by nuclear-localizing ER through the regulation of target gene transcription (i.e., genomic signaling). On the other hand, recent studies presented another pathway of so-called nongenomic signaling that is mediated by activation of membrane-associated ER. This nongenomic signaling events by membrane-associated ER could be also involved in some physiologic functions of E<sub>2</sub> (29–33). The E<sub>2</sub> concentrations that cause the maximal effect on the down-regulation of *Cap43* in this study might be rather higher than physiologically most effective concentrations, suggesting that this E<sub>2</sub>-induced down-regulation of *Cap43* could be mediated through nongenomic signaling by membrane-associated ER. We, however, observed that most of ERs were localized in nucleus of the cells and we did not observe any apparent differences in the membrane localization of ER when the cells exposed to 10<sup>-12</sup> to 10<sup>-6</sup> mol/L E<sub>2</sub> by immunocytochemistry (data not shown). It is also known that nongenomic signaling by membrane-associated ER was not blocked by the pure antiestrogen ICI 182780 (29, 34, 35). Consistent with these findings, treatment with ICI 182780 effectively blocked the E<sub>2</sub>-induced down-regulation of *Cap43* in breast cancer cells (Fig. 3). Taken together, it seems unlikely that membrane-associated ER plays a critical role in the E<sub>2</sub>-induced down-regulation of *Cap43* gene in our present study. However, further study is required to understand how E<sub>2</sub>-dependent down-regulation of *Cap43* is associated with genomic signaling.

In conclusion, the expression of *Cap43*, a putative differentiation- and metastasis-related gene, was greatly modulated by E<sub>2</sub> and/or antiestrogen in ER- $\alpha$ -positive breast cancer cells. Therefore, decreasing the susceptibility of *Cap43* to antiestrogens would be a potential therapeutic strategy for the treatment of breast cancer.

## Acknowledgments

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<sup>6</sup>A. Fotovati, unpublished data.

<sup>7</sup>Y. Maruyama et al., unpublished data.

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